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-TESIS DOCTORAL-
Mención "Doctorado Internacional"

ESTUDIO MOLECULAR DEL CARCINOMA ESCAMOSO DE CABEZA Y CUELLO EN LA POBLACIÓN ESPAÑOLA

Javier Fernández Mateos

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Memoria presentada por D. Javier Fernández Mateos para optar al Grado de Doctor por la Universidad de Salamanca. Mención “Doctorado Internacional”.

Firmado: Javier Fernández Mateos

Salamanca, 8 de junio de 2017

El Prof. Dr. JUAN JESÚS CRUZ HERNÁNDEZ, Catedrático del Departamento de Medicina, Universidad de Salamanca.

El Prof. Dr. ROGELIO GONZÁLEZ SARMIENTO, Catedrático del Departamento de Medicina, Universidad de Salamanca.

CERTIFICAN:

Que el trabajo titulado "*Estudio molecular del carcinoma escamoso de cabeza y cuello en la población española*", que presenta el Licenciado en Biología y Bioquímica Don Javier Fernández Mateos, ha sido realizado bajo su dirección en el Departamento de Medicina y reúne, a su juicio, todos los requisitos necesarios para ser presentado ante el tribunal correspondiente, a fin de optar al Grado de Doctor por la Universidad de Salamanca.

Y para que así conste y a los efectos oportunos, se expide el presente certificado en Salamanca, a 8 de junio de 2017.

Dr. Juan Jesús Cruz Hernández

Dr. Rogelio González Sarmiento

PROCEDIMIENTO PARA LA PRESENTACIÓN DE LA TESIS DOCTORAL EN LA UNIVERSIDAD DE SALAMANCA EN EL FORMATO DE COMPENDIO DE ARTÍCULOS/PUBLICACIONES

(Comisión de Doctorado y Posgrado, 15 de febrero de 2013)

1.- Justificación

El capítulo II del Reglamento de Doctorado de la Universidad de Salamanca, sobre la elaboración y defensa de La Tesis Doctoral, en su artículo 14.1. establece que "*los estudios de doctorado concluyen con la elaboración y defensa de una tesis doctoral, que consistirá en un trabajo original de investigación, elaborado por el doctorando, en cualquier campo del conocimiento, siguiendo el formato determinado por la Comisión Académica del Programa de Doctorado, entre los posibles formatos establecidos por la Comisión de Doctorado.*"

Asimismo, el artículo 14.3 determina que "La redacción de la tesis doctoral se hará en castellano o en una de las lenguas habituales para la comunicación científica en su campo de conocimiento. Si la tesis doctoral está redactada en un idioma diferente al castellano, se acompañará de un documento, avalado por el Director de la misma, en el que consten el título, el índice, la introducción, un resumen significativo y las conclusiones de la tesis doctoral en castellano."

Las publicaciones científicas en revistas especializadas, del trabajo original e inédito realizado en una Tesis Doctoral, garantizan que el autor de la Tesis, personal investigador en formación de un programa de doctorado haya adquirido una de las competencias básicas definidas en el RD 99/2011, de 28 de enero, por el que se regulan las enseñanzas oficiales de doctorado, en concreto la capacidad de ***comunicación con la comunidad académica y científica y con la sociedad en general acerca de sus ámbitos de conocimiento en los modos e idiomas de uso habitual en su rama de conocimiento en la comunidad científica internacional.***

De acuerdo con ello, la Comisión de Doctorado y Posgrado establece como posible formato de presentación de Tesis Doctoral, la modalidad de Tesis por Compendio de Artículos/Publicaciones, publicados o aceptados en revistas especializadas y de prestigio, siempre que sean resultado de la actividad investigadora desarrollada por el doctorando.

A tal efecto, con el objetivo de garantizar la calidad y validez de dichas tesis, se establece a continuación el procedimiento a seguir, que comienza con las recomendaciones y requisitos mínimos de la estructura de este formato de tesis.

2.- Tramitación, número y tipo de publicaciones, autoría:

2.1. Dentro del Plan de Investigación del doctorando, bien en su origen o bien en su revisión anual, podrá indicar su intención de realizar su tesis en el formato de compendio de artículos/publicaciones. El informe correspondiente que el tutor y director del doctorando realizan anualmente sobre dicho Plan, así como la evaluación del mismo que realiza la Comisión Académica del Programa de Doctorado, se pronunciará sobre la autorización de dicho formato.

2.2.- En el momento de la solicitud de depósito de la Tesis por compendio de artículos/publicaciones los artículos/publicaciones aportadas por el doctorando deberán cumplir los supuestos siguientes:

a. Un mínimo de tres artículos o capítulos publicados o aceptados para su publicación, con posterioridad al inicio de los estudios de doctorado, en revistas científicas del ámbito de la especialidad del trabajo desarrollado en la tesis e indexadas en el *Science Citation Reports* (o equivalente según la CNEAI en los campos científicos en los que dicho criterio no sea aplicable). Excepcionalmente se podrá admitir un número inferior de publicaciones a las tres mencionadas cuando su relevancia y excelencia esté bien documentada.

b. El doctorando deberá ser primer autor de al menos dos de las aportaciones presentadas. En el caso de que no sea primer autor, el doctorando especificará cuál ha sido su aportación científica a la publicación lo que deberá estar certificado por el director de la tesis doctoral.

No se aceptarán como aportaciones válidas publicaciones de trabajos no relacionados con el proyecto de tesis doctoral, ni trabajos en forma de abstract, los recogidos en proceedings o en suplementos de revistas, los trabajos de revisión, las cartas, artículos de opinión, comentarios, introducciones, etc.

En el caso de que el doctorando no sea primer autor de al menos dos de las aportaciones presentadas o el número de aportaciones sea inferior a tres, se requiere un informe de valoración de la calidad de las aportaciones por parte de la Comisión Académica del Programa de Doctorado para aprobar la continuación de los trámites de la tesis doctoral.

3.- Documentación a aportar junto con la solicitud de depósito de Tesis Doctoral por Compendio de Artículos/Publicaciones:

Además de la documentación prevista en el artículo 17 del Reglamento de Doctorado de la Universidad de Salamanca en el caso de tesis doctoral por compendio de artículos, la solicitud de depósito deberá ir acompañada por los siguientes documentos

3.1.- Aceptación por escrito y con firma original de los coautores, a que el doctorando presente el trabajo y, declaración de que el doctorando es el autor principal de la investigación recogida en los artículos. No más de dos coautores que sean doctores, podrán formar parte de la propuesta de miembros para constituir el tribunal que juzgue la tesis.

3.2.- Renuncia expresa de los coautores no doctores a presentar los trabajos como parte de otra Tesis Doctoral

4.- Formato y estilo de la Tesis por Compendio de Publicaciones:

Las tesis que se presenten en la modalidad de Tesis Doctoral como compendio de publicaciones en la Universidad de Salamanca, deberán al menos incluir los siguientes apartados:

4.1.- Una página inicial especificando que la tesis corresponde a un compendio de trabajos previamente publicados o aceptados para publicación, el nombre y afiliación de los autores, la referencia completa de la revista o editorial, la carta de aceptación si no han sido publicados y el DOI si existiera de los artículos, libros o capítulos de libro que se incorporan a la tesis.

4.2.- A continuación se incluirá la autorización del director o codirectores para la presentación de la tesis en esta modalidad, el informe de la Comisión Académica del Programa de Doctorado y la autorización de la Comisión de Doctorado y Posgrado de la Universidad para su tramitación en dicha modalidad.

4.3.- Un apartado en castellano que refleje la coherencia y relación directa entre los artículos/publicaciones presentados. Dicho apartado incluirá al menos una introducción con los antecedentes del tema objeto de estudio, la hipótesis de trabajo y los objetivos así como las principales conclusiones. Cuando se considere oportuno podrá incluirse un anexo metodológico que complete descripción de la metodología indicada en los artículos

4.4.- Seguidamente, se incluirá una copia completa de las publicaciones originales que conformarán la Tesis Doctoral (artículos, capítulos de libro, libro o libros aceptados o publicados)

4.5.- En el supuesto de que los artículos que integran la tesis estén redactados en una lengua distinta al castellano, se incluirá para cada uno un resumen en castellano en el cual se especificarán: los objetivos de la investigación, la metodología utilizada, los resultados alcanzados, y las conclusiones finales.

4.6.- Si la Comisión Académica lo considera, la tesis contendrá un apéndice en el que se incluyan los principales índices de calidad de las publicaciones aportadas y cualquier otra documentación necesaria que permita acreditar los requisitos exigidos.

5.- Regulaciones particulares de los Programas de Doctorado:

La Comisión Académica de cada Programa de Doctorado podrá indicar requisitos adicionales para el formato de tesis por artículos/publicaciones presentadas en el programa en cuestión, estableciendo una regulación particular compatible con el presente procedimiento.

PROCEDIMIENTO PARA LA OBTENCION DE LA MENCIÓN DE "DOCTOR INTERNACIONAL" EN EL TITULO DE DOCTOR POR LA UNIVERSIDAD DE SALAMANCA

(Aprobado por la Comisión de Doctorado, en su sesión de fecha 10 de Noviembre de 2011)

En aplicación del Real Decreto 99/2011, de 28 de enero, por el que se regulan las enseñanzas oficiales de doctorado, y del Reglamento de Doctorado de la Universidad de Salamanca aprobado por el Consejo de Gobierno de 25 de octubre de 2011, la Universidad de Salamanca podrá otorgar la mención de *'Doctor Internacional'* a aquellos títulos de Doctor o Doctora que cumplan todos y cada uno de los siguientes REQUISITOS:

a) Que durante su etapa de formación necesaria para la obtención del título de doctor, el doctorando haya realizado una estancia mínima de 3 meses fuera de España en una institución de enseñanza superior o centro de investigación de prestigio, cursando estudios o realizando trabajos de investigación. La estancia y las actividades estarán avaladas por el director y autorizadas por la Comisión Académica y se habrán incorporado en el documento de actividades del doctorando.

b) Que parte de la tesis doctoral, al menos el resumen y las conclusiones se hayan redactado y presentado en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales en España. Esta norma no será de aplicación cuando las estancias, informes y expertos procedan de un país de habla hispana y la tesis esté redactada en castellano.

c) Que la tesis haya sido informada por un mínimo de 2 expertos doctores pertenecientes a alguna institución de educación superior o centro de investigación de prestigio, no español.

d) Que al menos un experto perteneciente a alguna institución de educación superior o centro de investigación no española, con el título de doctor, y distinto del responsable de la estancia mencionado en el punto primero, haya formado parte del tribunal evaluador de la tesis.

En consecuencia, para la TRAMITACION ADMINISTRATIVA de la mención de *'Doctor Internacional'* se establece el siguiente procedimiento:

Primero. El doctorando que quiera optar a la mención de *'Doctor Internacional'*, en el momento de solicitud de depósito de su tesis doctoral ante el órgano académico responsable del Programa de Doctorado, deberá presentar:

1. El ejemplar de la tesis doctoral redactada en parte, al menos el resumen y las conclusiones, en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta de las lenguas oficiales de España. Esta norma no será aplicable cuando las estancias, informes y expertos mencionados en el párrafo anterior procedan de un país de habla hispana y la tesis esté redactada en castellano.

2. La siguiente documentación adicional:

2.1. Modelo de solicitud de la mención *'Doctor Internacional'*.

2.2. Certificación de haber realizado, dentro de las actividades avaladas por su Director, autorizadas por la comisión Académica y reflejadas en el Documento de Actividades del Doctorando, una estancia mínima de 3 meses fuera de España, cursando estudios o realizando trabajos de investigación, en una institución de enseñanza superior o en un centro de investigación de prestigio.

2.3. Informes que avalen la tesis doctoral de al menos 2 expertos doctores pertenecientes a una institución de enseñanza superior o centro de investigación de prestigio no española

Segundo. El órgano responsable del Programa de Doctorado, una vez aceptado el depósito de la tesis doctoral, remitirá la documentación adicional señalada en el punto anterior al Presidente de la Comisión de Doctorado, que en el plazo máximo de quince días hábiles, y a la vista de los informes y documentos aportados, dictará una resolución favorable o no a que en el caso de la tesis sea evaluada con una calificación de "apto", el consiguiente título de doctor por la Universidad de Salamanca incluya la mención de *'Doctor Internacional'*.

Tercero. La Comisión de Doctorado remitirá su resolución al órgano responsable del Programa de Doctorado, para que sea incluida en la documentación que este órgano ponga a disposición de los miembros del tribunal de evaluación de la tesis doctoral

Cuarto. Tras el acto de defensa de la tesis, el Secretario del Tribunal encargado de evaluar la Tesis Doctoral, deberá certificar:

1. Si la redacción y presentación de la Tesis Doctoral fue realizada en parte, al menos el resumen y las conclusiones, en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta de las lenguas oficiales de España. Esta norma no será aplicable cuando las estancias, informes y expertos mencionados en el párrafo anterior procedan de un país de habla hispana y la tesis esté redactada en castellano.

2. Si el Tribunal ha sido constituido con al menos un experto con el título de doctor, perteneciente a alguna institución de enseñanza superior o centro de investigación de prestigio fuera de España, distinto del responsable de la estancia acreditada en la documentación adicional presentada para optar a la mención de *'Doctor Internacional'*.

Quinto. El Secretario del Tribunal remitirá los certificados señalados en el punto anterior, junto con el acta de calificación global de la tesis y el resto de informes del Tribunal sobre la misma, al Presidente de la Comisión de Doctorado, que a la vista de la documentación aportada dictará una resolución favorable o no a que el título de Doctor o Doctora por la Universidad de Salamanca incluya la mención de *'Doctor Internacional'*.

Sexto. La mención de *'Doctor Internacional'* se reflejará en el anverso del título de Doctor o Doctora por la Universidad de Salamanca.

Séptimo. La mención de *'Doctor Internacional'* no implicará en ningún caso medidas excepcionales en la financiación del procedimiento de lectura de la Tesis Doctoral.

El presente trabajo ha sido financiado por los siguientes organismos:

-El Instituto de Salud Carlos III mediante los proyectos PI11/00519, PI13/01741, PI14/00071, PI16/01920

-Gerencia Regional de Salud, Junta de Castilla y León, mediante los proyectos GRS 969/A/14, BIO/SA49/13, GRS 1385/A/16

El doctorando fue financiado mediante un contrato de Personal Investigador a cargo del Grupo Español de Tratamiento de Tumores de Cabeza y Cuello (TTCC), desde noviembre de 2011 hasta julio de 2013, cuando le fue concedido un contrato de Personal Investigador de Reciente Titulación Universitaria (PIRTU) de la Junta de Castilla y León (BOCYL 20 de mayo de 2013). Durante su estancia en el "Institute of Cancer Research", Londres (Reino Unido), el doctorando fue beneficiario de una ayuda de estancia de la "European Molecular Biology Organization" (*EMBO-Short term fellowship*).

Índice de contenidos

Glosario de abreviaturas

A

A: adenina
ADCC: citotoxicidad celular dependiente de anticuerpo
AJCC: *American Joint Committee on Cancer*
AKT/PKB: proteína quinasa B
APC: *adenomatous polyposis coli*
ATG: genes relacionados con la autofagia (del inglés *autophagy related genes*)

B

BAX: *Bcl-2-associated X protein*
BCL: B-cell lymphoma
BECN1: beclin1
BER: reparación por escisión de bases (del inglés *base excision repair*)

C

C: citosina
CCC: cáncer de cabeza y cuello
CCND1: ciclina D1
CDK: quinasa dependiente de ciclina
CDKN2A: inhibidor 2A de quinasa dependiente de ciclina o p16
CECC: carcinoma escamoso de cabeza y cuello
CSF1R: receptor del factor 1 estimulante de colonias
CTCAE: criterios de terminología común para eventos adversos
CYP: citocromo P450

D

DNA: ácido desoxiribonucleico (del inglés *deoxyribonucleic acid*)
DNMT: DNA metil-transferasas
DSB: reparación por rotura de doble cadena (del inglés *double-strand break repair*)

E

E: proteínas de fase temprana (del inglés *early*)
E6AP: ubiquitina ligasa E3A
EBV: virus de Epstein-Barr
ECOG: escala del grupo cooperativo de oncología (del inglés *Cooperative Oncology Group*)
EGF: factor del crecimiento epidérmico (del inglés *epidermal growth factor*)
EGFR: receptor del factor de crecimiento epidérmico (del inglés *epidermal growth factor receptor*)
ERCC: *excision repair cross-complementing*
ESCRT: complejos de clasificación endosomal requerido para el transporte (del inglés *endosomal sorting complexes required for transport*)

F

FBXW7: *F-box/WD repeat- containing protein 7*
FGFR: receptor del factor de crecimiento de fibroblastos
FU: 5-fluorouracilo

G

G: guanina
G0-1-2: fase de intervalo (del inglés *gap*), G0: quiescentes, G1: intervalo 1, G2: intervalo 2
GDP: guanosín difosfato
GTP: guanosín trifosfato
GST: *glutathione S-tranferase*

H

HDAC: histona deacetilasas
HPV: virus del papiloma humano (del inglés *Human Papillomavirus*)
hTERT: telomerasa transcriptasa inversa (del inglés *telomerase reverse transcriptase*)
H&E: hematoxilina y eosina

I

IgG: inmunoglobulina
IL: interleucina
L: proteínas de fase tardía (del inglés *late*)

L

LC3B: *microtubule-associated protein 1b-light chain 3*

M

M: fase de mitosis
mATG: genes relacionados con la autofagia en mamíferos
MDM2: *murine doble minute 2*
MGMT: metilguanidina-DNA metiltransferasa
miRNA: microARN
MTOR: gen de la diana de la rapamicina en mamíferos (del inglés *mammalian target of rapamycin*)
mTORC1: complejo 1 de la diana de la rapamicina en mamífieros

N

NCI: Instituto Nacional del Cáncer (del inglés *National Cancer Institute*)
NEFL2: *nuclear factor (erythroid-derive 2)-like 2* o Nrf2
NER: reparación por escisión de nucleótidos (del inglés *nucleotide excision repair*)
NFX1: factor de transcripción nuclear de unión a X-Box1
NGS: técnicas de secuenciación de nueva generación (del inglés *next generation sequencing*)
NKK: nitrosamina 4-(metilnitrosamino)-1-(3-piridil)-1-butanona
NOD2: *nucleotide binding oligomerization domain containing 2*

O

OIS: senescencia inducida por oncogén (del inglés *oncogen induced senescence*)
OR: razón de problabilidades (del inglés *odds ratio*)

P

PCR: reacción en cadena de la polimerasa (del inglés *polymerase chain reaction*)
PDGFR: receptor del factor de crecimiento derivado de plaquetas
PE: fosfatidiletanolamina
PF: cisplatino-5-fluorouracilo
PI3K: fosfatidilinositol-4,5-bisfosfato 3-quinasa
PIK3CA: subunidad catalítica alfa de la fosfatidilinositol-4,5-bisfosfato 3-quinasa
PKB/AKT: proteína quinasa B
PTEN: fosfatidilinositol-3,4,5-trifostato 3-fosfatasa

R

RARB-2: receptor del ácido retinoico B2
RAS: *Rat sarcoma*, H (*Harvey*), K (*Kirsten*), N (*neuroblastoma*)
RB: retinoblastoma
RFLP: polimorfismos de longitud de fragmentos de restricción (del ingés *restriction fragment length polymorphisms*)
ROS: especies reactivas de oxígeno (del inglés *reactive oxigene species*)

S

S: fase de síntesis
SEOM: Sociedad Española de Oncología Médica
SG: supervivencia global
SLP: supervivencia libre de progresión
SNP: polimorfismo de nucleótido simple (del inglés *single nucleotide polymorphism*)

T

T: timina
TNFα: factor de necrosis tumoral α
TNM: sistema de estadificación basado en tamaño-nódulos-metástasis
TP53: *tumor protein 53*
TPF: taxotere-cisplatino-5-fluorouracilo

U

UTR: región no traducida (del inglés *untranslated region*)

INTRODUCCIÓN

1. El cáncer de cabeza y cuello

El cáncer de cabeza y cuello (CCC) engloba a un conjunto de neoplasias localizadas en el tracto aerodigestivo superior. Incluye tumores localizados en el labio y cavidad oral, faringe (nasofaringe, orofaringe e hipofaringe), así como laringe, glándulas salivares y glándulas tiroides (Figura 1). Aproximadamente un 90% de estos tumores tiene una clasificación histopatológica de carcinoma escamoso de cabeza y cuello (CECC), originándose principalmente en la cavidad oral, orofaringe, hipofaringe o laringe[1,2].

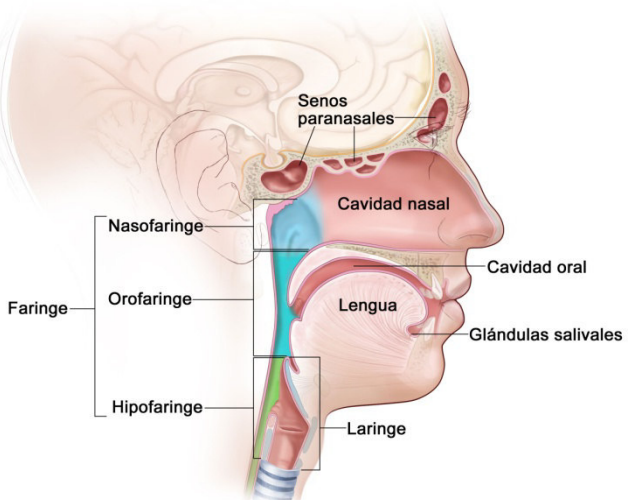


Figura 1. Regiones en el cáncer de cabeza y cuello. Obtenido de www.cancer.gov

A pesar de su origen común en la mucosa escamosa, estos tumores revelan un inesperado grado de heterogeneidad que complican su consideración como una única entidad[3].

Debido a la gran diversidad subyacente en estos tumores, en el presente trabajo nos centramos en el estudio de los carcinomas escamosos de cabeza y cuello de cavidad oral, faringe (excluyendo nasofaringe) y laringe, tumores con una misma clasificación histopatológica y una mayor incidencia.

2. Epidemiología

El carcinoma escamoso de cabeza y cuello es el sexto cáncer más común a nivel mundial, teniendo una incidencia de 600.000 nuevos pacientes diagnosticados por año, de los

cuales tan solo el 40-50% sobreviven a los cinco años[4,5]. En Europa se estima una incidencia de 130.000 nuevos casos por año[6].

En cuanto a España, los datos recogidos por GLOBOCAN[7], así como los datos calculados por la Sociedad Española de Oncología Médica (SEOM), estimaron una incidencia de CCC en la población española para el año 2015 de 16.296 casos (Figura 2), convirtiéndolo así en el sexto tumor más frecuente a nivel nacional, coincidiendo con la incidencia a nivel mundial[8,9].

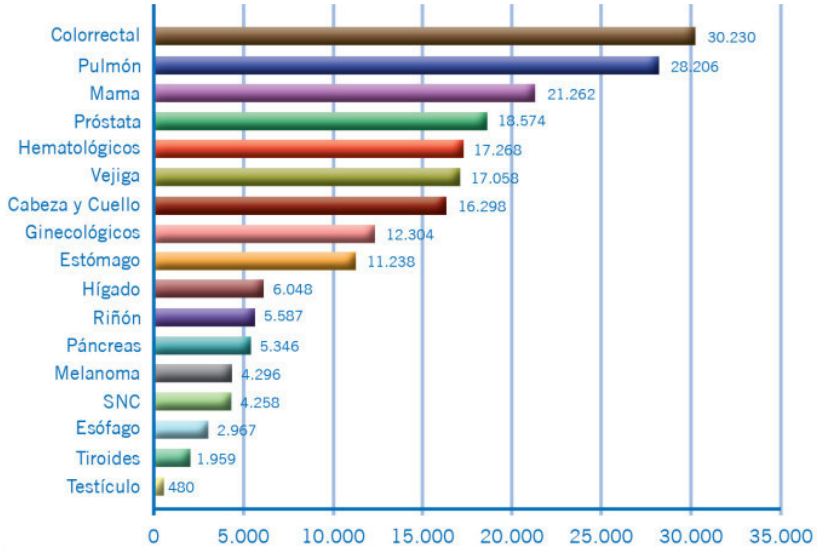


Figura 2. Incidencia del cáncer extrapolados para la población estimada para 2015 en España por el Instituto Nacional de Estadística (INE)[10].

Respecto a la mortalidad de CCC a nivel mundial, el número de fallecidos por esta enfermedad anualmente es de 333.400 casos[1]. Datos de la SEOM indican que este tumor causó 4.099 muertes en España en el año 2007[10].

Según su aparición por sexo, el CCC se presenta en mayor porcentaje en hombres, variando la proporción según el tipo tumoral, doblando las tasas para cáncer de cavidad oral y faringe, y triplicándolas en el cáncer laríngeo[1]. En España esta variación es más acusada, alcanzando un ratio 9:1 en detrimento al género masculino, debido a una mayor exposición al tabaco y alcohol, principales factores etiológicos que se comentarán más adelante. No obstante, debido a un cambio en el consumo de tabaco y alcohol, la incidencia por sexos en estos tumores se está igualando, disminuyendo su incidencia en hombres y aumentando en mujeres[11,12].

La edad media de aparición es aproximadamente 60 años. Sin embargo, la incidencia en adultos jóvenes (menores de 40 años) está en aumento[1] como consecuencia de la aparición de un mayor número de tumores de cabeza y cuello causados por la etiología del virus del papiloma humano (HPV) en países desarrollados[11]. Por localización, se ha observado que el tumor que aparece a edad más temprana es el carcinoma de cavidad oral u orofaringe, mientras que el carcinoma laríngeo es más frecuente en edades avanzadas[13].

Su incidencia varía según la distribución geográfica, siendo un tumor poco frecuente en Estados Unidos, mientras que en otras partes del mundo se trata de una de las enfermedades más comunes en hombres, como es el caso del sur y centro de Asia. Por otro lado, según la localización, en las regiones del sur y el este de Europa, el cáncer de laringe representa el 40% de todos los CECC diagnosticados[1], seguido del de orofaringe, cavidad oral y por último el de nasofaringe.

3. Factores de riesgo

El consumo de tabaco y alcohol se ha considerado como factor clásico asociados al desarrollo de CECC. Además, diversos estudios epidemiológicos han caracterizado otros agentes ambientales y genéticos importantes en la etiología de este tumor[14].

3.1 Tabaco

El consumo de tabaco es uno de los factores etiológicos más establecidos en el desarrollo tumoral[15]. La exposición al tabaco tiene un efecto carcinogénico dosis-dependiente asociado con la frecuencia y duración del consumo, en el cual la duración es más importante que la intensidad de la exposición[16].

El contenido en nitrosaminas, especialmente la nitrosamina 4-(metilnitrosamino)-1-(3-piridil)-1-butanona (NNK) e hidrocarburos aromáticos policíclicos, entre otros carcinógenos conocidos, parece iniciar un modelo particular de carcinogénesis inducida por el tabaco, considerándolo como el principal agente etiológico en estos tumores. El efecto carcinogénico del tabaco puede relacionarse con varios procesos biológicos, incluyendo la reparación del

DNA, la saturación de activación de vías de señalización, y un incremento en la activación de las enzimas detoxificadoras[17].

Numerosos estudios epidemiológicos han demostrado un mayor riesgo relativo en el rango de 3 a 12 veces mayor de padecer CECC en fumadores[1]. El efecto del tabaco se asocia principalmente al desarrollo de tumores de laringe, con un riesgo relativo diez veces mayor, seguido por los tumores de oro e hipofaringe con un riesgo relativo de cuatro veces mayor. Sin embargo, se correlaciona con una asociación menor en otras neoplasias como carcinomas de cavidad oral, nasofaríngeos o nasosinusales[16,18,19]. Todo ello sugiere que la laringe es el órgano más susceptible a los efectos carcinogénicos del tabaco y, aunque el mecanismo de aumento de susceptibilidad no está definido, una posible explicación podría ser que la laringe y faringe tienen una mayor exposición al humo del tabaco inhalado que la cavidad oral[19].

Teniendo en cuenta el tipo de tabaco consumido, se observó un mayor riesgo de desarrollar CECC en fumadores de cigarrillos (OR=3.46), seguido de puros (OR=2.54) y, por último, en pipa (OR=2.08). En todos los casos, ese riesgo se incrementa con la frecuencia, la duración del hábito tabáquico y su ratio acumulativo[20]. En cuanto al consumo de tabaco en otras formas, se ha visto una asociación menor en tabaco esnifado comparado con el tabaco de mascar (OR=1.71 y OR=1.20 respectivamente), teniendo un riesgo más elevado en tumores de cavidad oral[21]. Por último, la exposición involuntaria al humo del tabaco, como sería el caso de los fumadores pasivos, se asoció con un 60% de riesgo elevado en aquellos individuos expuestos durante más de quince años, siendo de nuevo mayor en tumores de laringe y faringe que de cavidad oral[22].

3.2 Alcohol

El consumo de alcohol es el segundo agente etiológico en importancia después del tabaco aunque, en este caso, el mayor efecto cancerígeno se produce al potenciar el efecto del tabaco[18]. De hecho, en ausencia del hábito tabáquico, la asociación entre el consumo de alcohol y el riesgo a desarrollar cáncer de cabeza y cuello es débil, estableciéndose una relación sólo en altas dosis[23].

El etanol actúa como iniciador carcinogénico y como promotor, ya que incrementa la permeabilidad de las células a otros carcinógenos ambientales, principalmente al humo del tabaco. Durante la metabolización, el etanol es oxidado en un primer paso a acetaldehído a

través de la actividad de la enzima alcohol deshidrogenasa y, en menor medida por las enzimas del complejo citocromo P450, incluyendo CYP2E1, especialmente en bebedores crónicos. El acetaldehído, clasificado como un posible carcinógeno humano (grupo 2B), es posteriormente metabolizado por la enzima aldehído deshidrogenasa a acetato[17]. El efecto potenciador de la ingesta de alcohol es dosis dependiente y está relacionado con el tiempo de exposición, estando más relacionado con tumores de cavidad oral y faringe, donde el acetaldehído derivado del metabolismo del alcohol parece ser el principal factor etiopatogénico[1].

Sin embargo, a pesar del efecto principal del acetaldehído, se ha sugerido que la ingesta de alcohol puede desencadenar otros mecanismos que expliquen la mayor incidencia en tumores de cavidad oral y faringe. Entre estos factores pueden encontrarse la producción de acetaldehído por la flora bacteriana de la cavidad oral, proceso que se ve incrementado en bebedores habituales, así como una pobre higiene bucal, que es frecuente en bebedores y fumadores y que produce un aumento en la liberación de citocinas proinflamatorias[17,24].

La interacción entre el consumo de tabaco y alcohol es la causa más frecuente del desarrollo de CECC. Al menos el 75% de estos tumores son atribuibles a la combinación de ambos carcinógenos[25]. Varios estudios confirman este efecto multiplicativo en el riesgo a padecer tumores de la cavidad oral y faringe y, en menor medida, en la laringe[26]. Los efectos de esta interacción es biológicamente plausible, puesto que el alcohol actúa como un solvente para los carcinógenos del tabaco, haciendo a la mucosa más permeable, aumentando las propiedades carcinogénicas en estas localizaciones con mayor tiempo de exposición[18]. Este efecto dual se asocia en mayor proporción a tumores de cabeza y cuello en hombres que en mujeres, ya que la combinación de tabaco y alcohol es más común en varones[25].

En resumen, la exposición crónica a carcinógenos del tabaco y alcohol da lugar a cambios moleculares en el tracto aerodigestivo, lo que puede dar lugar a lesiones premalignas, como leucoplasia y eritoplasia, que pueden desencadenar el posterior desarrollo de lesiones malignas como CECC u otras enfermedades secundarias[1].

3.3 Infecciones víricas

En los últimos años, se ha evidenciado la implicación de la etiología vírica en el desarrollo de CECC. Es el caso del virus de Epstein-Barr (EBV) en tumores de nasofaringe y del virus del papiloma humano, principalmente el subtipo 16 (HPV-16), en tumores de cavidad oral y orofaringe[1]. La incidencia de tumores causada por la infección de ambos agentes víricos, especialmente HPV, ha aumentado en los últimos años. Este aumento aparece particularmente en grupos de menor edad y es atribuible a las prácticas sexuales, principal modo de transmisión del virus[27]. Este aumento es más acusado en ciertos países, como Estados Unidos, donde el 60% de los tumores derivados de orofaringe son HPV-16 positivos[28], mientras que la proporción en población europea varía, comprendiendo entre el 90% de los casos en Suecia y menos del 20% en otras comunidades europeas con mayores ratios de consumo de tabaco y alcohol, como es el caso de España[28,29].

Sin embargo, el modelo de carcinogénesis desencadenado por la infección vírica identifica un subgrupo de tumores con desarrollo y respuesta ante tratamientos totalmente diferente, con mejor pronóstico[30,31], por lo que actualmente se clasifican como una entidad diferente a los causados por tabaco y alcohol [32,33].

3.4 Factores ocupacionales

Aunque la exposición ocupacional probablemente desempeñe un papel menor, se ha descrito la asociación de ciertos factores ambientales con un aumento en el riesgo de desarrollar tumores cérvico-faciales[1]. Existe una mayor incidencia de CECC en los trabajadores de la metalurgia, madera, industria textil y cuero, refinerías, construcción y agricultura. Todo ello se relaciona con una exposición continuada a numerosos agentes carcinogénicos, como pueden ser el asbesto, el polvo del cemento, los pesticidas o fertilizantes, solventes químicos, humos tóxicos, etc. Se ha observado que este riesgo aumenta con la duración de la exposición al tóxico y que la exposición a estos agentes se asocia principalmente con el desarrollo de tumores de laringe y faringe[34].

3.5 Factores dietéticos

Diversos estudios caso-control han puesto de manifiesto la importancia de la dieta en la epidemiología de este tumor. Dietas altas en grasas animales o carnes procesadas y bajas en consumo de frutas y verduras se asocian con un mayor riesgo al desarrollo de CECC[1].

El consumo de frutas y verduras protege frente al desarrollo de varios tipos de tumores[35], entre ellos el CECC[11,36,37]. Su efecto parece ser debido a las propiedades anticarcinogénicas y antioxidantes de numerosos compuestos presentes en frutas y verduras, como pueden ser vitaminas, carotenoides, fibra, ácido fólico, flavonoides, esteroles y ácido fenólico, que desempeñan un papel importante en diversos procesos, como la protección frente al estrés oxidativo y la reparación del DNA[38]. Este resultado se observa también con el consumo de suplementos vitamínicos o de minerales, donde la ingesta de vitamina C, E o calcio se ha asociado con un riesgo reducido de CECC[39].

Así mismo, el consumo de dietas ricas en carne roja o carnes procesadas han mostrado un aumento en el riesgo a desarrollar CECC, aunque esta asociación se ha establecido con mayor riesgo en el cáncer colorrectal, de esófago y pulmón. Este efecto parece estar relacionado con la presencia de radicales libres u otros carcinógenos producidos en los procesos de preparación o preservación de la carne que provoca, entre otras modificaciones estrés oxidativo[37]. En contraste, se ha observado una asociación inversa entre el consumo de carne blanca o pescado y el riesgo a desarrollar CECC, debido a que son alimentos bajos en grasas saturadas, hierro y al ser alimentos menos procesados, originan un número menor de carcinógenos[37].

3.6 Susceptibilidad genética

La aparición de agregaciones familiares de CECC sugiere la existencia de factores genéticos de predisposición[40]. Varios estudios caso-control han determinado esta susceptibilidad genética al desarrollo de CECC, incrementando el riesgo entre 2 y 8 veces para familiares de primer grado con antecedentes de CECC[1,41], independientemente de la localización[42,43]. Este riesgo es significativamente marcado en sujetos fumadores[40], por lo que la agregación familiar puede indicar tanto la existencia de factores genéticos heredables en el

riesgo a desarrollar CECC, como reflejar la tendencia familiar a un comportamiento de consumo de tabaco y alcohol similares[40,42].

Diversos estudios de asociación han puesto de manifiesto la existencia de polimorfismos (SNPs) en genes implicados en importantes cascadas de señalización como la metabolización de carcinógenos, la reparación del DNA y/o el control del ciclo celular, la apoptosis, etc., asociándolo con una influencia en el desarrollo y susceptibilidad genética en tumores[1,44–49], aunque los resultados no siempre son consistentes.

3.7 Otros factores de riesgo

El uso abusivo de colutorios con contenido antiséptico alcohólico parece incrementar el riesgo a padecer carcinoma escamoso de la cavidad oral debido a sus efectos perjudiciales sobre el epitelio oral en la metabolización del etanol al acetaldehído carcinogénico, aunque estas observaciones no están muy consensuadas[50]. A pesar de la falta de asociación, si se han visto *odds ratios* ligeramente elevados en aquellas personas con un uso prolongado de colutorios con contenido alcohólico[51].

El consumo de marihuana parece ser otro de los factores de riesgo establecidos en el desarrollo de CECC. El humo de la marihuana tiene hasta cuatro veces mayor contenido de alquitrán y un 50% más de concentración en benzopireno e hidrocarburos aromáticos que el tabaco. Sin embargo, tan sólo estudios aislados han encontrado asociación con un mayor riesgo de desarrollar estos tumores en consumidores de marihuana, con una clara relación dosis-dependiente[1]. Esto sugiere una gran heterogeneidad de los efectos de esta droga sobre la susceptibilidad al CECC[52].

Diversos estudios observacionales han sugerido la asociación entre la existencia de reflujo gastroesofágico con una mayor incidencia de tumores laringo-faríngeos, aunque de manera conflictiva[1]. Esto es debido a que el efecto del reflujo gastroesofágico es difícil de analizar por el papel del tabaco y alcohol como variables de confusión[53,54].

Otro factor importante que ha demostrado una fuerte relación con un mayor riesgo a desarrollar CECC es la radiación ionizante. Aunque la radiación ultravioleta se asocia fundamentalmente con tumores de labio, una exposición prolongada a irradiación gamma se relaciona con tumores de tiroides, sarcomas de cabeza y cuello y neoplasias de glándulas salivares, incluidos los tumores en senos paranasales[1].

Por último, otro factor etiológico descrito es la influencia del estatus social, especialmente para tumores de cavidad oral. El riesgo de cáncer tiene una relación inversa con el estatus socioeconómico[11] y el nivel educativo, independientemente del consumo de tabaco y alcohol[55]. De manera similar, el soporte familiar es un factor importante donde individuos sin apoyo familiar tienen un mayor riesgo de desarrollar CECC, asociado con una alimentación menos sana y mayor consumo de tabaco y alcohol.

4. Modelo de desarrollo tumoral

A pesar de que el 90% de los tumores de cabeza y cuello tienen un origen de carcinoma de células escamosas, éste es un grupo de tumores muy heterogéneo, distinguiéndose varias subclases según su perfil de expresión génica[56]. La clasificación principal de los CECC diferencian dos grupos: aquellos derivados por la infección de HPV y aquellos en los que el virus no interviene en la génesis del tumor. Ambos tipos de tumores se consideran entidades moleculares diferentes (Tabla 1).

Tabla 1. Diferencias entre las características biológicas y clínicas de CECC HPV negativo y positivo. Modificado de Leemans et al[57] y Marur et al[58].

Características	CECC HPV negativo	CECC HPV positivo
Incidencia	En descenso	En aumento
Etiología	Tabaco y alcohol	Sexo oral
Edad de aparición	>60 años	40-60 años
Estatus socioeconómico	Bajo-medio	Alto
Cancerización de campo	Si	Desconocido
Mutaciones en *TP53*	Frecuente	Poco frecuente
Localización mayoritaria	Ninguna	Orofaringe
Estadio	Avanzados	Tempranos
Pronóstico	Pobre	Favorable

El cáncer se produce a partir de la acumulación de cambios genéticos o epigenéticos en genes implicados en importantes cascadas de señalización, que desregulan y causan la adquisición de fenotipos tumorales. Estos fenotipos se caracterizan por un potencial replicativo ilimitado, un aumento de señales proliferativas, habilidad para evadir la apoptosis, y una inducción de la invasión, metástasis y angiogénesis[59]. Sin embargo, todos estos cambios pueden producirse de diversas maneras. Por ello, a continuación pasamos a definir los dos modelos principales de carcinogénesis en carcinomas escamosos de cabeza y cuello.

4.1 Carcinogénesis en tumores HPV negativos

La patogénesis del carcinoma escamoso de cavidad oral es el modelo mejor conocido dentro de los CECC debido a la fácil observación de las lesiones precursoras, frecuentemente diagnosticadas en estos tumores, como leucoplasias o eritroplasias orales. Estas lesiones producen un crecimiento de poblaciones clonales con acumulación de alteraciones genéticas y una progresión fenotípica a la neoplasia invasiva. Desde el inicio de los modelos de progresión tumoral se ha tenido en cuenta el proceso de cancerización de campo, muy frecuente en estos tumores[60]. Este término se define como la presencia de cambios genéticos en el epitelio de la mucosa que rodea al carcinoma al estar expuesto a los mismos carcinógenos, siendo una fuente importante de recurrencias locales y segundos tumores, eventos muy frecuentes en los CECC[57].

La carcinogénesis en estos tumores conlleva una serie de alteraciones genéticas bien descritas hasta el momento. El primer cambio observable en lesiones premalignas, como la displasia, es la pérdida de heterocigosidad en 9p21 (70-80%). En esta región se encuentra el *locus* génico de *CDKN2A*, que codifica dos proteínas importantes, p16 y $p14^{ARF}$, responsables de la regulación del ciclo celular en G1 y la degradación de p53 mediada por Mdm2. La telomerasa, implicada en el mantenimiento telomérico e inmortalización, se ha encontrado reactivada en el 90% de las lesiones premalignas de CECC. Además, se ha observado en etapas tempranas un aumento en la expresión del receptor del factor de crecimiento epidérmico (*EGFR*), presente en más del 90% de los casos de CECC[61,62]. Otro evento temprano común es la pérdida de la región cromosómica 3p, donde se encuentran varios genes supresores de tumores. La pérdida de heterocigosidad en 17p, así como mutaciones en *TP53* aparecen en más del 50% de los CECC aunque en estadios más avanzados.

La amplificación de la región 11q13 y la sobreexpresión de la ciclina D1 (*CCND1*) son eventos asociados al carcinoma *in situ*, permitiendo esta sobreexpresión la progresión del ciclo celular de G1 a S, lo que se relaciona con un comportamiento tumoral agresivo. Pacientes con progresión de carcinoma *in situ* a carcinomas escamosos invasivos muestran pérdidas adicionales de las regiones 4q, 8p, 11q o 17p (Figura 3)[57,61–63]. Los cambios epigenéticos son también un evento importante en la patogénesis del CECC, encontrando una metilación aberrante en ciertas islas CpG en *RARB-2*, *p15* y *MGMT*[64,65].

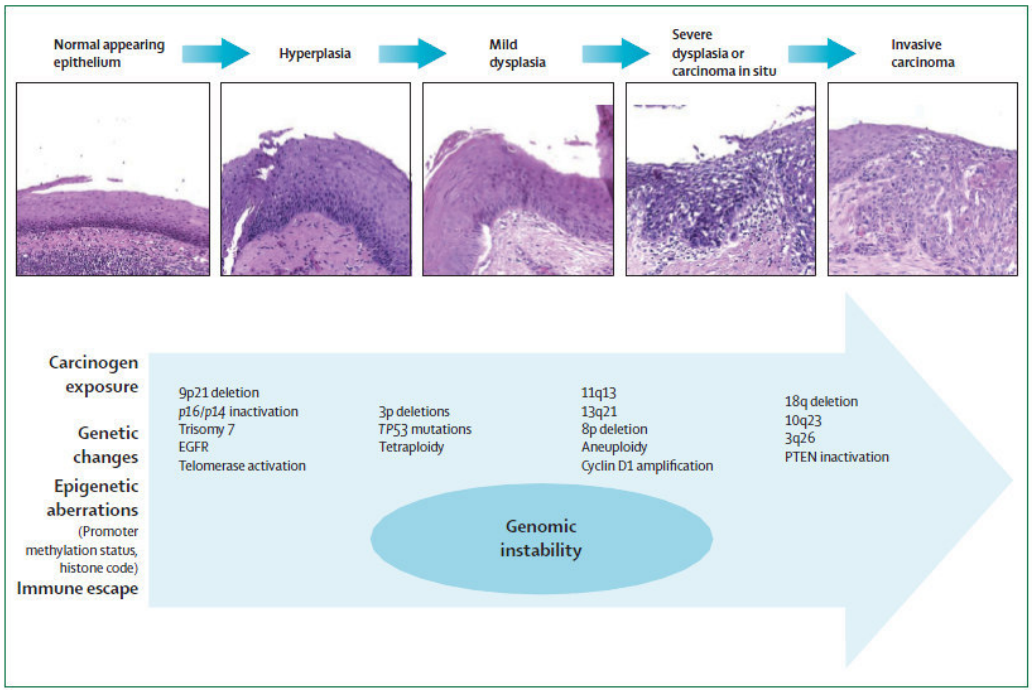


Figura 3. Representación de la progresión fenotípica y acumulación de alteraciones moleculares en la carcinogénesis del cáncer de cabeza y cuello. Obtenido de Argiris et al.[61].

4.2 Carcinogénesis producida por HPV

Los principales subtipos de virus del papiloma humano con alto riesgo oncogénico son HPV-16 y HPV-18. El genoma del virus del papiloma humano es un DNA circular de doble cadena de 8000 pares de bases que codifica diez proteínas. Este material genético contiene dos clústeres, divididos según su expresión en genes "tempranos", que codifican dos proteínas reguladoras (E1-E2) y tres oncoproteínas (E5-E6-E7), y genes "tardíos" (L1-L2), que codifican proteínas estructurales de la cápsida[66].

La proteína E5 juega un papel importante durante la infección temprana al unirse a los receptores EGFR, PDGFR y al receptor del factor 1 estimulante de colonias (CSF1R) promoviendo la proliferación celular. Sin embargo, durante la integración vírica en el genoma de la célula, la secuencia E5 suele deleccionarse, teniendo mayor importancia en la carcinogénesis y el mantenimiento del fenotipo maligno las proteínas E6 y E7[62,67] (Figura 4).

La proteína E7 interacciona con el complejo E3 ubiquitin ligasa cullin 2 e induce la degradación proteolítica de la proteína supresora de tumores retinoblastoma (pRb) y otras proteínas relacionadas. En ausencia de pRb, la familia de factores de transcripción E2F es liberada y activa la transcripción de los genes de fase S induciendo la proliferación celular que

también se produce por la acción de E7 sobre inhibidores de quinasas dependientes de ciclinas. De igual modo produce la inducción del remodelado y activación transcripcional de la cromatina, teniendo un papel crucial en la regulación epigenética de la transcripción génica[66].

La proteína E6 forma un complejo con una ubiquitina ligasa E3A (E6AP), ubiquitinando la proteína supresora de tumores p53, acelerando su proteólisis, desregulando los puntos de control G1/S y G2/M del ciclo celular tras daño al DNA y otros cambios de estrés celular, dando lugar a inestabilidad genómica[68].

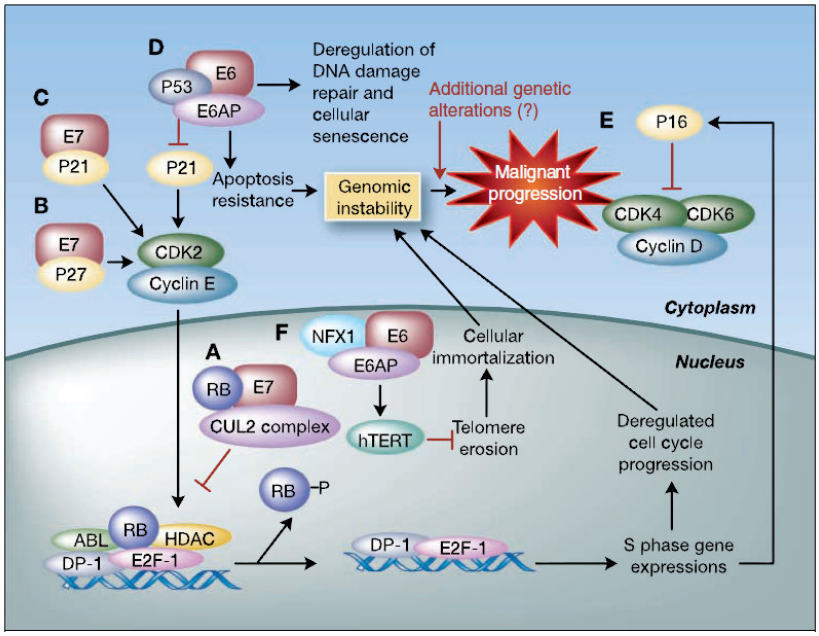


Figura 4. Diagrama de la carcinogénesis producida por las proteínas víricas E6 y E7, obtenido de Chung et al.[68]. A, ubiquitinación por el complejo E7 y la ubiquitin ligasa cullin 2 para la degradación de pRB; B, interacción entre E7 y p27Kip1 dando lugar a la inhibición de la parada del ciclo celular, contribuyendo a la carcinogénesis; D, ubiquitinación por E6 and la ubiquitin ligasa E6AP produciendo la degradación de p53; E, aumento en la expresión de $p16^{INK4A}$ debido a la ausencia de pRB; y F, degradación de NFX1, un represor transcripcional de hTERT, mediante la asociación con E6/E6AP dando lugar a la activación de hTERT y la inmortalización celular.

De acuerdo con las propiedades oncogénicas de E6, aproximadamente un 90% de los tumores producidos por el papilomavirus son *TP53 wild type*. Debido a la habilidad de E7 de dirigir la degradación de Rb, la expresión de esta proteína está también disminuida. La ausencia de mutaciones en *CDKN2A* y la sobreexpresión de $p16^{INK4A}$ se utiliza también como un marcador molecular de la carcinogénesis producida por el virus[69].

Diversos estudios de secuenciación de exomas en tumores HPV positivos corroboran un perfil molecular diferencial de esta etiología, acumulando un menor número de mutaciones. Esto es debido a que la carcinogénesis de tumores HPV positivos está modulada

por la actividad de las oncoproteínas virales E6/E7, necesitando un menor número de mutaciones para su transformación[66], mientras que en tumores sin infección vírica la carcinogénesis se produce por la adquisición de múltiples alteraciones en diferentes vías de señalización.

5. Caracterización genómica en carcinomas escamosos de cabeza y cuello

La introducción de técnicas de secuenciación masiva, más conocida como secuenciación de nueva generación (NGS), ha permitido analizar de una manera rápida y sencilla el genoma humano completo.

Desde la publicación de los dos primeros estudios de secuenciación masiva en cáncer de cabeza y cuello en 2011[70,71] se han definido los genes más frecuentemente mutados en este grupo de tumores. Uno de los estudios más importantes fue el proyecto "*The Cancer Genome Atlas*" (TCGA) donde se caracterizaron las alteraciones moleculares de más de 500 tumores entre los que se encontraban los carcinomas escamosos de cabeza y cuello. Este estudio incluyó 279 CECC describiendo las alteraciones somáticas más frecuentes en estas neoplasias[72]. A continuación se presenta un resumen de estos genes:

***TP53* (*Tumour protein p53*)**

El gen *TP53* se localiza en el brazo corto del cromosoma 17 (17p13.1). Conocido como el "guardián del genoma", la proteína codificada por este gen supresor de tumores tiene como función principal producir la parada del ciclo celular ante daño al DNA permitiendo su reparación. Si el daño no se puede reparar, p53 inicia como factor de transcripción la cascada de señalización de apoptosis y/o senescencia[73]. Estas funciones previenen que células dañadas proliferen y acumulen más errores, inhibiendo los eventos que llevan a la malignidad celular.

Se trata del gen más frecuentemente mutado en cáncer[74], siendo también uno de los más alterados en el CECC, variando su porcentaje según la serie estudiada desde el 50%[70] al 70%[75]. Estas mutaciones se detectan en estadios tempranos de la carcinogénesis inducida por tabaco e incluso están presentes en lesiones displásicas premalignas[69]. La presencia de alteraciones en el gen *TP53* en pacientes consumidores de tabaco y alcohol dobla su incidencia

en comparación con pacientes no fumadores[76]. Esto puede relacionarse con la asociación de una mayor incidencia de mutaciones en *TP53* en tumores HPV negativos[72,77], probablemente debido a que la pérdida de la función de p53 en tumores HPV positivos es a través de la oncoproteína viral E6, impidiendo la presión selectiva de ganancias de mutaciones en *TP53*[78].

Por último, la presencia de mutaciones en este gen se ha asociado con una respuesta clínica pobre y progresión de la enfermedad, disminuyendo la supervivencia[79].

***PIK3CA* (P*hosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha*)**

Las mutaciones en la vía de la familia fosfatidilinositol-3-quinasa (PI3K) son las más frecuentes en los tumores de cabeza y cuello, detectándose en torno a un 30% de los casos[80,81]. Dentro de esta familia cobra especial interés el gen *PIK3CA*, segundo más mutado en tumores de cabeza y cuello, con una media del 11%[76], predominando en ciertas localizaciones como la laríngea donde se encuentra mutado en casi un 17%[80]. Mutaciones en este gen se han encontrado principalmente en tumores HPV positivos, que presentan un menor porcentaje de mutaciones en *TP53*[78,82].

El gen *PIK3CA* se encuentra en el locus 3q26.32 y codifica la subunidad catalítica p110α de la clase 1A de la familia PI3K encargada de la conversión enzimática de fosfatidilinositol bifostato a trifosfato, activando las quinasas Akt/PKB. De este modo ejerce su función promoviendo el crecimiento y supervivencia celular y la reorganización del citoesqueleto[79]. Las mutaciones en este gen son aquellas que generan ganancia de función, aumentando la proliferación celular[73].

NOTCH1

Estudios de secuenciación masiva han definido a este gen como uno de los más frecuentemente mutados en CECC, encontrándose en aproximadamente el 15% de estos tumores[71].

El gen *NOTCH1* se localiza en la región cromosómica 9q34.3. La proteína NOTCH1 es un receptor transmembrana estructuralmente dividido en dos dominios: intracelular y extracelular. La ruptura del dominio intracelular y su translocación al núcleo como factor de transcripción hace que se activen cascadas de señalización cruciales para diferenciación

celular[79]. Aunque *NOTCH1* presenta mutaciones activadoras, la gran mayoría de mutaciones en CECC afectan al dominio de unión al ligando del factor de crecimiento epidérmico (EGF) y al dominio C-terminal produciendo proteínas truncadas y por lo tanto una pérdida de función[70,82], jugando un papel dual como oncogen o gen supresor de tumores según su mutación[83].

***CDKN2A* (*Cyclin dependent kinase inhibitor 2A*)**

El gen del inhibidor de la quinasa dependiente de ciclina 2A (*CDKN2A*) es un gen supresor de tumores localizado en el *locus* 9p21.3. Este gen está implicado en la regulación de la progresión del ciclo celular y se encuentra alterado en el 9% de los CECC ya sea por pérdida de heterocigosidad o deleciones, o mediante silenciamiento epigenético[79].

Su transcrito, p16, juega un papel crítico en la regulación del ciclo celular mediante la interacción con el supresor de tumores Rb, ya que inhibe a CDK4/6, impidiendo la fosforilación de Rb, estabilizando la unión de Rb y el factor de transcripción E2F e impidiendo la progresión a la fase S del ciclo celular[79,82]. Los niveles de expresión de esta proteína se usan como un marcador subrogado de tumores CECC HPV positivos, estando establecido que un 90% de estos tumores pierden la expresión de p16 debido a mutaciones, pérdidas de heterocigosidad o hipermetilación del promotor[78]. Aunque las alteraciones genéticas en *CDKN2A* son comunes en estadios tempranos, es poco probable que sean mutaciones *drivers* por sí mismas[79].

Familia de genes RAS

Mutaciones en los genes *RAS* están presentes en al menos un tercio de los cánceres humanos. Estas proteínas de la familia de GTPasas se localizan en la membrana plasmática, teniendo como función la activación de cascadas de señalización como Raf/MEK/ERK y/o PI3K, alternando entre el estado activo-GTP e inactivo-GDP. Esta familia incluye tres genes cuyas proteínas varían únicamente en su extremo C-terminal: *HRAS* (*Harvey*), *KRAS* (*Kirsten*) y *NRAS* (neuroblastoma)[84].

Aunque las mutaciones el gen *KRAS* son las más comunes encontradas a nivel global en tumores, *HRAS* es el gen más mutado de la familia en CECC, presente en un 8% de estos carcinomas[85]. Las mutaciones más frecuentes en estos genes causan una activación continua de la proteína dando lugar a un incremento en la proliferación y supervivencia celular[79,84].

***FBXW7* (*F-box and WD repeat domain containing protein 7*)**

Este gen localizado en 4q31.3 es miembro de la familia de proteínas F-box, un componente importante del complejo ubiquitina ligasa, codificando una ubiquitina ligasa E3, diana de un gran número de proteínas promotoras de crecimiento que se degradan por la vía proteasoma, incluyendo la ciclina E, MYC, NOTCH y MTOR[71].

Mutaciones en *FBXW7* se asocian con CECC HPV positivo[71,82]. Este gen supresor de tumores se ha encontrado mutado en múltiples tumores, puesto que su pérdida de función se ha correlacionado con inestabilidad genética y crecimiento tumoral, estando asociado con la activación de la señalización de NOTCH[86]. En CECC este gen se encuentra mutado entorno al 5%[80,85].

Otros genes implicados

Existe una gran variedad de genes implicados en el desarrollo de CECC entre los que se encuentra *EGFR*, *CCND1*, *PTEN*, *MET* y *FGFR2/3*[73,87]. Mientras que la alteración de la ciclina D1 (*CCND1*) suele ser por amplificación génica, evento que ocurre junto con amplificaciones en *EGFR*, las alteraciones más comunes en los genes *PTEN*, *MET* y *FGFR2/3* suelen ser mutaciones[78,88].

La sobreexpresión de EGFR está ampliamente descrita en CECC, demostrando una actividad incrementada en el 90% de los tumores en ausencia de mutaciones, debido a amplificación en su *locus*[79]. *PTEN* aparece mutado entorno al 10% de los casos[78] mientras que *MET* se ha encontrado mutado en un 4% en ciertas series de tumores de cavidad oral[86]. Por último *FGFR2/3* se presenta mutado en aproximadamente el 2% de estos tumores[88]. Sin embargo, el aumento de estudios de secuenciación está permitiendo la identificación de otros genes con menor penetrancia pero igualmente importantes en el desarrollo de CECC.

En resumen, los estudios realizados hasta el momento corroboran la heterogeneidad del espectro mutacional en los CECC. En general, comparando los tumores HPV positivo y negativo, se observa que los tumores HPV negativos presentan mayor rango de mutaciones que los positivos, asociándose los tumores HPV negativos con mutaciones en *TP53*, *CCND1*, *CDKN2A/B* y *PIK3CA* mientras que los positivos suelen presentar mutaciones en *PIK3CA*, *PTEN* y *FBXW7* en mayor porcentaje[80].

6. Diagnóstico

Los pacientes con tumores de cabeza y cuello en estadios tempranos presentan síntomas leves con nulos o mínimos hallazgos físicos, mientras que en estadios más avanzados las manifestaciones clínicas de este conjunto de tumores varía según su localización[89,90].

- Los pacientes con carcinomas escamosos en labio y cavidad oral presentan generalmente masas, excrecencias y úlceras que pueden causar dolor, disfagia, pérdida de peso y sangrado.

- Las neoplasias orofaríngeas tienen un curso clínico insidioso debutando con dolor de garganta, disfagia crónica u odinofagia, con persistencia al menos durante seis semanas, otalgia referida y trismus. De manera similar, el cáncer de hipofaringe se manifiesta en un curso avanzado de la enfermedad con disfagia, otalgia, disfonía y más de la mitad de los pacientes presentan adenopatías cervicales.

- Los síntomas en pacientes con tumores en la laringe varían según su sublocalización. Los tumores en la glotis se suelen diagnosticar en estadios tempranos y tienen un alto porcentaje de curación ya que estos pacientes presentan de manera precoz disfonía y tos persistente; mientras que en diagnósticos más avanzados las sintomatologías más presentes son: disnea, disfagia, odinofagia u otalgia refleja. Al contrario, los pacientes con tumores supraglóticos solo se identifican en enfermedad muy avanzada con adenopatías cervicales.

- Por último, los tumores en la cavidad nasal o senos paranasales se asocian con síntomas de sinusitis, rinorrea, obstrucción nasal unilateral y epistaxis. Los síntomas clásicos de tumores nasofaríngeos incluyen otitis, obstrucción nasa, epistaxis y parálisis de los nervios craneales.

Aproximadamente el 5% de los pacientes debutan con metástasis cervicales palpables como primera manifestación, alcanzando alrededor del 50% en el caso de tumores de nasofaringe e hipofaringe, donde constituyen de forma frecuente el primer signo de la enfermedad. Sin embargo, son raras las manifestaciones derivadas de la presencia de metástasis a distancia como primer signo o síntoma del CECC.

7. Factores relacionados con el tumor

A la hora de estimar la agresividad tumoral o su extensión se utiliza el grado histológico y la estadificación.

El grado histológico denota la semejanza del tejido tumoral con el tejido normal, tanto por la morfología celular, como por su organización. De este modo, los tumores se clasifican en[91]:

- **Grado 1**: bien diferenciado. Si las células del tumor así como su organización se asemejan al tejido normal. Se asocian con un crecimiento y extensión lentos. Se denomina también de bajo grado

-**Grado 2**: moderadamente diferenciado o grado intermedio.

-**Grado 3**: pobremente diferenciado.

-**Grado 4**: indiferenciado, células con morfología anormal y falta de estructura de tejido normal, asociado con un crecimiento y diseminación mayor que los grados inferiores. Los tumores con grado 3 y 4 pueden agruparse bajo el término de alto grado.

El sistema de estadificación aporta las características morfológicas del tumor así como su diseminación. El más reconocido es el sistema TNM dado por las iniciales de tumor, nódulo y metástasis, creado por el Comité Colectivo Americano del Cáncer (*American Joint Committee on Cancer*, AJCC)[91]. Éste agrupa a los tumores por:

-**T**: tamaño y extensión de la masa tumoral principal o primaria. Cuanto mayor es el número de la T, mayor es el tumor. Este sistema aporta según el tamaño un rango de T0, si no puede encontrarse el tumor primario, a T4, con un tumor mayor de 4cm de diámetro mayor y enfermedad local avanzada.

-**N**: extensión tumoral a los ganglios o nódulos linfáticos cercanos tanto por su número como por su ubicación y tamaño. N0 indica que no se ha extendido a los ganglios linfáticos, mientras que N3 determina la mayor extensión vía linfática.

-**M**: diseminación desde el tumor primario a otras partes del cuerpo o presencia de metástasis, siendo M0 ausencia de metástasis y M1 evidencia de metástasis a distancia. Aunque la incidencia de pacientes metastásicos se sitúa en torno al 10%, las localizaciones más comunes en estos tumores son pulmonares, óseas, hepáticas y en menor incidencia cerebrales.

La clasificación TNM agrupa los tumores en cinco estadios diferentes (0-IV) que a su vez están divididos en distintos subgrupos (Tabla 2).

Tabla 2. Clasificación por estadios en carcinomas escamosos de cabeza y cuello, siguiendo los criterios de la AJCC (excepto nasofaringe, tiroides y melanoma de cabeza y cuello)[91].

Estadio	T	N	M
Estadio 0 (in situ)	Tis	N0	M0
Estadio I	T1	N0	M0
Estadio II	T2	N0	M0
Estadio III	T3	N0	M0
	T1, T2, T3	N1	M0
Estadio IVA	T4a	N0, N1	M0
	T1, T2, T3, T4a	N2	M0
Estadio IVB	Cualquier T	N3	M0
	T4b	Cualquier N	M0
Estadio IVC	Cualquier T	Cualquier N	M1

8. Tratamiento

El manejo de los CECC ha evolucionado de forma muy importante en las últimas décadas, delimitando grupos de tumores por su diferente pronóstico y tratamiento. El abordaje de esta enfermedad tiene un tratamiento multidisciplinar según el estadio o la localización del tumor primario[92]. De esta manera, al considerar la terapeútica de los CECC nos referiremos a los tumores epidermoides de cavidad oral, orofaringe, laringe e hipofaringe, considerando aparte los tumores de fosas nasales, glándulas salivares y los localizados en nasofaringe.

Estadios tempranos (I y II)

Aproximadamente un tercio de los pacientes diagnosticados de CECC presentan una enfermedad en estadio temprano. Este grupo habitualmente se trata únicamente con cirugía y/o radiación, obteniendo, según su estadio I o II, un 90% y un 70% de curación respectivamente. Sin embargo, la elección depende de la localización, siendo la cirugía la opción preferida en tumores en la cavidad oral y laringe; mientras que en faringe, la radioterapia es la terapia de elección, demostrando igual resultado en comparación con la cirugía pero asociada con una menor morbilidad[61]. La intensidad habitual de radiación empleada en el tratamiento de CECC es de 60 a 70 Gy.

Estadios localmente avanzados (III y IVA/B)

Es el grupo más heterogéneo ya que incluye pacientes con enfermedad resecable o irresecable. Las principales opciones para el manejo de CECC localmente avanzado incluyen la cirugía, radioterapia, terapias biológicas o quimioterapia.

El mayor avance del tratamiento de los tumores en estos estadios ha sido la introducción del tratamiento concomitante de radioterapia y quimioterapia (quimiorradioterapia), obteniendo con la adicción de cisplatino mejores resultados que la radioterapia sola o la administración secuencial de ambos tratamientos, estableciéndose por tanto como tratamiento estándar[93]. Esta combinación tiene un mayor beneficio clínico cuando hay rotura extracapsular o márgenes quirúrgicos positivos. El beneficio de esta terapia resulta en un incremento en supervivencia del 8% a los cinco años, aunque esta concomitancia tiene un incremento de toxicidades agudas[61].

En pacientes irresecables, la quimiorradioterapia es el tratamiento estándar si la adicción de quimioterapia de inducción no está indicada por un estado basal pobre o comorbilidad de la enfermedad. En pacientes resecables, la cirugía normalmente se continúa con quimiorradioterapia. Sin embargo, en este apartado es donde se están introduciendo las grandes novedades con la adición de la quimioterapia de inducción, seguida de radioterapia para preservación de órgano, principalmente en laringe, mostrando resultados similares que la laringectomía[61].

La quimioterapia de inducción continúa siendo un tema de controversia[94,95]. Se introdujo con la combinación de cisplatino y 5-FU (PF) en CECC localmente avanzado,

mostrando unas altas tasas de respuestas y un posible aumento de supervivencia[96]. Posteriormente, la adición de un taxano (TPF) se convirtió en el régimen de elección más activo, mostrando mejores resultados que PF[97–100], aunque no mostró beneficios convincentes en supervivencia en comparación con datos de tratamiento de quimorradioterapia[101–103], excepto en el estudio de Paccagnella et al.[104]. No obstante, la quimioterapia de inducción debe ser una alternativa a la quimiorradioterapia para mejorar la preservación de órgano.

Cetuximab, un anticuerpo monoclonal quimérico dirigido contra el receptor del factor de crecimiento epidérmico (EGFR), es la primera terapia dirigida que muestra datos positivos en la supervivencia del CECC[105,106]. La combinación de este fármaco con la radioterapia (bioradioterapia) mostró un mejor control locorregional, supervivencia libre de progresión y supervivencia global, siendo una buena alternativa en aquellos pacientes que no toleran la quimioterapia[107–109]. El uso de bioradioterapia en pacientes con cáncer laríngeo estadio III-IVA que respondieron a TPF mejoró la preservación de órgano[110].

Enfermedad metastásica

El tratamiento para la enferemedad metastásica suele ser con intención paliativa para preservar la calidad de vida de los pacientes. Aunque pacientes con un buen estado basal pueden tratarse con poliquimioterapia, el uso más habitual es con monoquimioterapia debido a una condición basal pobre[92]. En muchas ocasiones se utiliza la combinación de fármacos utilizada para la enfermedad recurrente, que se define a continuación.

Enfermedad recurrente

Al menos el 50% de los pacientes localmente avanzados desarrollan recidivas locorregionales o a distancia, que suelen detectarse en los primeros dos años de tratamiento[61]. Las opciones terapéuticas para la enfermedad recurrente local o regional dependen de la localización, carga tumoral y el esquema terapéutico previo, pudiendo oscilar desde la cirugía radical, en aquellos pacientes con resecabilidad potencial de la recurrencia, a la reirrradiación sola o en combinación con quimioterapia. En casos donde la enfermedad no es susceptible de un tratamiento agresivo como la cirugía o radiación, la mono o poliquimioterapia es la única opción como tratamiento paliativo o aumento de supervivencia[92]. Entre estos agentes quimioterápicos predomina el uso del cisplatino, fluorouracilo o metrotexato, aunque el uso

de dianas terapéuticas contra EGFR como el cetuximab[111], o inhibidores tirosina-quinasa, gefitinib o erlotinib, están actualmente en estudio[61].

Debido a la caracterización genómica de los CECC por estudios de secuenciación masiva y la identificación de los genes más frecuentemente alterados, el uso de terapias dirigidas puede ser de gran importancia en el futuro del tratamiento de estos tumores[76].

9. Toxicidad al tratamiento

Como se ha comentado anteriormente, el abordaje del CECC es multidisciplinar. Debido a la intensificación de los tratamientos y al aumento de regímenes concomitantes, la toxicidad en pacientes con CECC ha aumentado[112]. La evaluación de la toxicidad ante el tratamiento se lleva a cabo mediante diferentes escalas, siendo la más utilizada la clasificación acorde con los Criterios Comunes de Toxicidad (CTCAE) del *National Cancer Institute* (NCI)[113]. Esta guía divide los eventos adversos en grados según su severidad siguiendo estos criterios:

- **Grado 1**: leve. Asintomático o con síntomas leves, únicamente observaciones clínicas o diagnósticas, sin indicación de intervención.
- **Grado 2**: moderado. Indicación de intervención no invasiva mínima o local, sin limitación para actividades básicas de la vida diaria.
- **Grado 3**: grave o de importancia médica, pero no potencialmente mortal. Indicada la hospitalización o prolongación de la hospitalización; existe limitación para las actividades básicas de la vida diaria.
- **Grado 4**: pone en peligro la vida del paciente. Indicada una intervención urgente.
- **Grado 5**: muerte relacionada con efectos adversos.

Los pacientes de CECC desarrollan complicaciones agudas y tardías como resultado específico de la localización de la enfermedad así como del tratamiento recibido.

Los efectos adversos más comunes en los pacientes con CECC son debidos al tratamiento con platino, principal agente quimioterápico que se utiliza en el tratamiento de estos tumores. Las toxicidades más comunes a la terapia con platino incluyen aquellas que causan efecto de daño celular, como náuseas y vómitos, mielosupresión (leucopenia, trombopenia y anemia) y un descenso en la respuesta a la infección (inmunosupresión). Otros efectos más específicos incluyen daño renal (nefrotoxicidad), neurotoxicidad y ototoxicidad[114].

La toxicidad más común asociada con la radioterapia es la mucositis, presente en más del 50% de los pacientes que reciben este tratamiento concomitante con quimioterapia[115], así como incremento en las secreciones, disfagia (ocasionalmente con aspiración), pérdida del gusto, disfonía y dermatitis. En cuanto a la toxicidad tardía se incluye la aparición de osteoradionecrosis, caries dental, fibrosis subcutánea, trismus, disfunción tiroidea, pérdida de audición, estenosis faríngea o esofágica y mielitis. La xerostomía inducida por la radiación es común en largos supervivientes, alcanzando un 60%[61,112].

Por último, el uso del anticuerpo monoclonal frente a EGFR, cetuximab, produce comúnmente toxicidad dérmica, ya sea por dermatitis, sequedad de piel, prurito y aparición de erupción o *rash* acneiforme[116,117]. Tanto el platino como el cetuximab concomitante a la radioterapia aumenta la incidencia de toxicidad severa (grado 3-4) comparado con el tratamiento único con radioterapia.

10. Autofagia

El término autofagia deriva del griego *auto* (uno mismo) y *phagos* (comer) y fue acuñado por primera vez por Christian de Duve basado en la observación de la degradación de estructuras intracelulares en lisosomas de rata. Recientemente, este mecanismo ha sido redescubierto caracterizándose su vía de señalización y su significación fisiológica. Estudios en levaduras han identificado la implicación de al menos 32 genes relacionados con la autofagia (ATG) y conservados en organismos superiores, enfatizando la importancia de este proceso autofágico en respuesta a la falta de nutrientes a lo largo de la filogenia[118].

Aunque el proceso de autofagia se caracteriza por una degradación proteolítica de componentes citosólicos vía lisosoma, se han definido tres tipos diferentes de autofagia: macro-autofagia, micro-autofagia y autofagia mediada por chaperonas. De modo resumido la macro-autofagia se caracteriza por un transporte de la carga citoplasmática al lisosoma a través de una vesícula intermediaria de doble membrana, denominada como autofagosoma, que se fusiona al lisosoma para formar el autofagolisosoma. Por el contrario, en la micro-autofagia los componentes citosólicos se incluyen directamente en la membrana del lisosoma por invaginación. Por último, en la autofagia mediada por chaperonas, las proteínas diana se translocan a lo largo de la membrana del lisosoma en un complejo con proteínas chaperonas siendo reconocidas y degradadas[118,119]. Debido a la importancia de la macro-autofagia y su

papel en la enfermedad, a continuación se define únicamente el papel de la misma bajo el término general de autofagia.

10.1 Bases moleculares de la autofagia

Como se indicó en el apartado anterior, hasta la fecha se han identificado al menos 32 genes implicados en el proceso de autofagia. Las proteínas codificadas por estos genes se clasifican en seis grupos diferentes según la función que tengan en el desarrollo del proceso autofágico[119] (Figura 5):

1. Inducción de la autofagia: complejo quinasa ULK1 (ULK1-mAtg13-FIP200-Atg101)

Ante condiciones de estrés, como la falta de nutrientes, la autofagia se inicia mediante la activación de mTORC1, que desempeña un papel central en esta vía de señalización[120]. De modo similar como ocurre en levaduras, donde se necesita la existencia de un pre-autofagosoma compuesto por Atg1-Atg17, Atg29-Atg31, en mamíferos el complejo quinasa ULK1 (unc-51-like kinase 1, también conocido como RB1CC1) formado por ULK1 (Atg1), mAtg13 (Atg13), FIP200 (Atg17) y Atg101 es esencial para el inicio de la vía. Así, en condiciones de falta de nutrientes, mTORC1 se disocia del complejo quinasa ULK1 dando lugar a la fragmentación del complejo en sus componentes desfosforilados, como mAtg13 y FIP200, dirigiéndose al sitio de ensamblaje del fagóforo e induciendo la autofagia[121].

2. Reciclaje de la membrana: mAtg9 y VMP1

Aunque el origen de las vacuolas autofágicas continúa en discusión, una de las principales hipótesis es la formación "*de novo*", donde la proteína Atg9 actuaría como reservorio[119]. Las proteínas mAtg9 (Atg9) y VMP1 son dos proteínas transmembrana requeridas para la autofagia. Aunque la función de mAtg9 continúa en estudio, potencialmente contribuye a la entrega de la membrana emergente al autofagosoma en formación[122].

3. Nucleación de la vesícula: complejo fosfatidilinositol 3-quinasa clase III (PIK3), formado por Vps34 (PI3K clase III)-Beclin1 (Atg6) –p150 (Vps15) -mAtg14

Este complejo formado por la PI3K clase III tiene un papel esencial en la nucleación de la membrana en mamíferos. Como primer paso de la iniciación en la formación del autofagosoma se requiere la proteína Beclin1 dando lugar a diferentes respuestas autofágicas, debido a la existencia de numerosos reguladores positivos y negativos que interaccionan con esta proteína[119]. Uno de los reguladores más importantes es la proteína antiapoptótica Bcl-2, que inhibe la autofagia al secuestrar Beclin1 bajo condiciones de abundancia de nutrientes, mientras que en ausencia de los mismos se disocia para inducir la vía. Tras esta liberación Beclin1 se une con Vps34, activando a esta última para que fosforile el fosfatidilinositol a fosfatidilinositol 3-fosfato, reclutando las proteínas al fagóforo para el crecimiento de la membrana[123,124].

4. Inicio del proceso de expansión: complejo fosfatidilinositol 3-fosfato (PIP3)-Atg2-Atg18 (WIPI1/2 en mamíferos)

Efectores del complejo fostatidilinositol PI3K clase III, como la proteína DFCP1 (también conocida como ZFYVE1) y proteínas con repeticiones WD, interactúan con proteínas fosfatidilinositol, entre las que se encuentra el complejo ATG2-WIPI. Estas proteínas parecen estar implicadas en la expansión de la membrana del autofagosoma al funcionar como transportador para abastecer la membrana emergente[125].

5. Expansión de la membrana del autofagosoma

La expansión de la membrana del autofagosoma se lleva a cabo por dos sistemas de conjugación basados en proteínas de tipo ubiquitina.

a. **Sistema de conjugación Atg12-Atg5-Atg16L1**. Atg7 actúa como una ubiquitina E1 activando Atg12 uniendo a su extremo carboxiterminal un residuo de glicina. Atg12 es luego transferido a la ubiquitina E2 Atg10, potenciando el enlace covalente de la lisina 130 de Atg12 a Atg5. Este complejo se conjuga en pares con dímeros de Atg16L formando un complejo Atg5-Atg12-Atg16L que se asocia con el fagóforo en extensión[126,127].

b. **Sistema de conjugación LC3.** También nombrado expansión de la membrana mediante la conjugación de LC3 (Atg8 en bacterias) con fosfotidiletanolamina (LC3-PE). En el sistema de conjugación de LC3 en mamíferos, esta proteína primero se procesa mediante la cisteína proteasa Atg4 exponiendo un residuo de glicina en el C-terminal, siendo la forma citosólica o LC3-I. La misma enzima Atg7 participa en la activación de LC3 y lo transfiere a Atg3. LC3 es finalmente conjugado a la diana lipídica fosfatidiletanolamina, formando LC3-II, anclada en la membrana del autofagosoma dirigiendo la elongación de la misma[124]. Esta conversión de LC3-I a LC3-II se considera un marcador de inducción de autofagia[128,129]. Así mismo, la proteína p62 (SQSTM1) puede ser un marcador del proceso autofágico, debido a su unión a LC3 y la degradación preferencial vía autofagia[130].

6. Cierre del autofagosoma, fusión del autofagolisosoma y degradación del contenido

Una vez se ha formado el autofagosoma éste se fusiona con un lisosoma para formar el autofagolisosoma (o autolisosoma) y completar la cascada de autofagia. En células de mamífero, la maduración del autofagosoma es un paso previo a la fusión entre los autofagosomas y lisosomas. Cuando la formación del autofagosoma está completa, la proteína LC3 adherida a la membrana exterior es escindida de la fosfatidiletanolamina por Atg4 y devuelta al citosol[124].

De manera similar a levaduras, la actividad de las GTPasas monoméricas como Rab22 y Rab24 participan en la maduración del autofagosoma y los ortólogos de los miembros de la familia SNARE y la proteína NSF pueden estar implicados en la maduración de las vesículas autofágicas. Estudios recientes han identificados otros reguladores como UVRAG, Tubicon, presenilina-1, VCP y las proteínas del complejo sintaxina-5 SNARE. ESCRT, complejo de clasificación endosomal requerido para el transporte de proteínas ubiquitinadas en cuerpos multivesiculares (entre las que se encuentra las proteínas LAMP) adquiere también un papel importante en la fusión autofagosoma-lisosoma. Aparte de su implicación en la fusión, ESCRT es así mismo requerido para el cierre del fagógoro y la biogénesis del lisosoma[119].

Tras la fusión, la degradación del contenido es dependiente de una serie de hidrolasas ácidas, incluidas las catepsinas B y D (homólogos de proteinasa A en levaduras). Las moléculas degradadas, particularmente los aminoácidos son transportados al citosol para la síntesis

proteica y el mantenimiento de las funciones celulares bajo condiciones de falta de nutrientes[124].

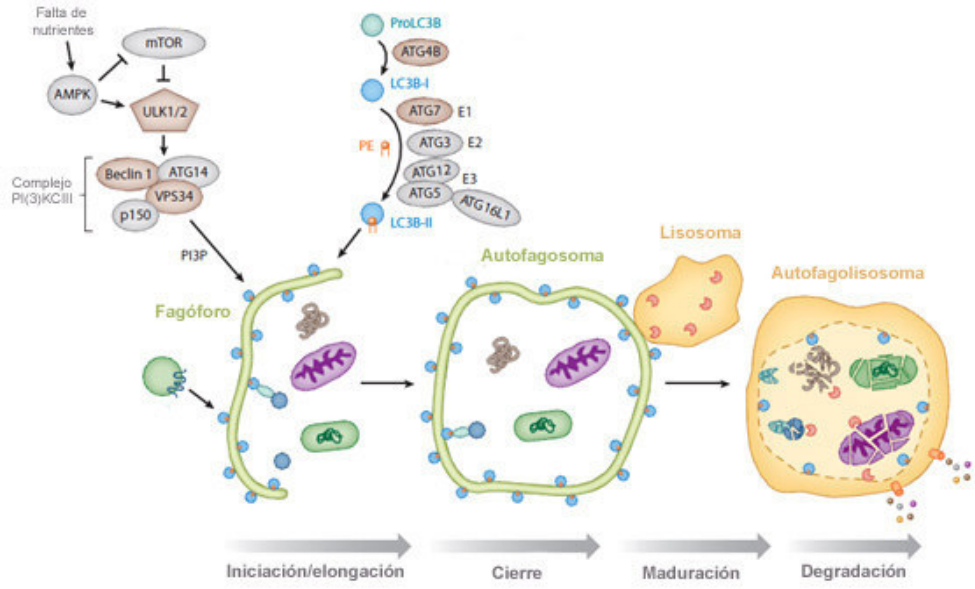


Figura 5. Mecanismo molecular resumido del proceso autofágico[131]. En la parte inferior se observan las diferentes etapas del proceso autofágico. Las moléculas u orgánulos a degradar se engloban en el fagóforo de doble membrana que se cierra formando el autofagosoma. Posteriormente este se une al lisosoma, formando el autofagolisosoma, donde la carga es degradada por las enzimas lisosomales y los productos de degradación son reciclados al citosol por medio de transportadores.

10.2 Autofagia y cáncer

La homeostasis celular requiere un balance constante entre procesos de biosíntesis y catabolismo. Las células eucariotas utilizan dos mecanismos diferenciales para degradaciones a larga escala, el proteasoma y la autofagia, teniendo ambas un papel crucial en el mantenimiento de la fisiología celular. La autofagia se induce por un cambio en las condiciones ambientales, principalmente ante la falta de nutrientes. Sin embargo, este proceso está también implicado en importantes procesos como desarrollo, diferenciación y remodelado tisular de organismos, y su alteración se asocia con ciertas enfermedades humanas, entre ellas el cáncer[132]. La autofagia tiene un efecto dual actuando tanto en la promoción como en la prevención al cáncer durante la progresión tumoral[133,134].

10.2.1 Autofagia como supresor tumoral

El papel de la autofagia como un supresor de tumores se estableció inicialmente por estudios genéticos donde el *locus* de Beclin1 (*BECN1*) se encontró alterado en un alto porcentaje de tumores de mama, ovario y próstata, asociado con una baja expresión de la proteína[135]. Estudios *in vitro* demostraron que la expresión ectópica de Beclin1 reducía la proliferación, disminuyendo su potencial cancerígeno. Además de alteraciones genéticas en *BECN1*, se identificaron deleciones de *ATG5* así como de otros importantes reguladores de la autofagia, con un incremento de susceptibilidad al desarrollo de ciertos tumores[135–137].

Por otro lado, se ha demostrado que la autofagia basal está implicada en la restricción del crecimiento celular e inhibición de la proliferación ante estrés oncogénico. Este efecto como supresor tumoral se ha reportado en distintos estudios, asociándose con un papel protector ante el estrés genotóxico manteniendo la integridad del genoma. Estos estudios sugirieron que defectos en la autofagia aumentan el daño al DNA, causando un incremento de mutaciones en células que sobreviven al estrés metabólico, favoreciendo la tumorogénesis[138,139]. Además, ante situaciones de estrés metabólico, la autofagia limita la necrosis tumoral previniendo de la infiltración leucocitaria del tumor primario, asociada con una inmunidad pro-tumoral que favorece el desarrollo de la neoplasia[135].

Finalmente, la autofagia es requerida para el establecimiento de la "senescencia inducida por oncogén" (OIS), aportando un mecanismo alternativo de la supresión tumoral mediada por la autofagia. La OIS causa una parada del ciclo celular limitando la proliferación de células dañadas con señales hiperproliferativas aberrantes de oncogenes, siendo una barrera al desarrollo tumoral[140].

10.2.2 Autofagia como promotor tumoral

Aunque la autofagia puede actuar como supresor de tumorogénesis en etapas iniciales a través de muchos mecanismos, diversos estudios han demostrado que este mecanismo puede, a su vez, favorecer el crecimiento tumoral de múltiples maneras, al igual que promover resistencia a gran variedad de terapias[131]. La disminución de la expresión o deleción de genes autofágicos ha mostrado una reducción en la supervivencia celular y tumorogénesis en líneas tumorales, estableciendo un papel funcional en la promoción tumoral[137,141].

La reducción en el proceso autofágico promueve la formación tumoral, a pesar de que un mínimo nivel de autofagia parece contribuir a la adaptación y supervivencia de células tumorales en respuesta a estrés. Puesto que las células tumorales tienen una alta demanda de nutrientes y oxígeno para facilitar su alto ratio proliferativo, los tumores normalmente se encuentran en estrés metabólico e hipoxia, especialmente en tumores sólidos pobremente vascularizados. Por ello, se encuentran niveles elevados de autofagia en células en el interior del tumor, promoviendo la supervivencia ante falta de nutrientes reciclando sus componentes intracelulares, protegiéndolos de la apoptosis o necrosis[135].

El efecto tumorigénico más importante de la autofagia se da en estadios tardíos de la progresión tumoral, como la diseminación y metástasis, ya que, por ejemplo, la separación de la matriz extracelular induce autofagia en células epiteliales, protegiéndolas de la muerte por anoikis (apoptosis inducida por la pérdida de anclaje de la célula a la matriz extracelular) y favoreciendo su propagación[142].

Finalmente, la autofagia actúa como un mecanismo importante de supervivencia celular durante terapias antitumorales ya que retira las macromoléculas u orgánulos dañados permitiendo la supervivencia de las células transformadas[133]. Esta vía se ha visto incrementada en células en respuesta a la quimioterapia y radiación, observando como la inhibición de la autofagia sensibiliza a las células tumorales ante un amplio espectro de tratamientos[135]. Por todo ello el uso de fármacos inhibidores de autofagia puede ser una nueva vía terapéutica efectiva para tumores.

10.2.3 Autofagia en tumores de cabeza y cuello

Debido a las diversos mecanismos modulados por la autofagia, esta vía tiene una implicación importante en el desarrollo de carcinoma escamoso de cabeza y cuello a diferentes niveles[143].

Genética

A pesar de que los tumores englobados bajo el término de cabeza y cuello son altamente heterogéneos, datos de secuenciación genómica han demostrado aberraciones somáticas comunes en vías implicadas en la autofagia, como pueden ser mutaciones en *PIK3CA*

(produciendo activación de la vía PI3K/Akt/mTOR promoviendo autofagia) o sobreexpresión de proteínas como EGFR, muy común en estos tumores[144].

Aunque la autofagia ha sido estudiada en todas las localizaciones del CECC, la mayoría de estudios se ciñen a carcinomas escamosos de la cavidad oral[144,145]. Estos estudios han revelado un alto nivel de p62 citoplasmático, sugiriendo una autofagia inactiva, mostrando una correlación con una menor supervivencia. Así mismo, se ha demostrado una reactivación de la autofagia en estadios avanzados de la enfermedad, al observarse altos niveles de LC3B-II relacionados con una supervivencia global reducida. Estos resultados apoyan la paradoja de que desregulación de la autofagia en estadios tempranos promueve la tumorogénesis mientras que su reactivación en estadios tardíos facilita la supervivencia[146].

Tabaco y alcohol

La exposición al tabaco y al alcohol da lugar a una desregulación en varias vías metabólicas entre ellas aquellas implicadas en inflamación, metabolización de especies reactivas de oxígeno (ROS) y autofagia. El consumo de tabaco y alcohol produce estrés oxidativo y una inflamación crónica favoreciendo la presencia de mutaciones oncogénicas e induciendo la autofagia[143].

Infección microbiana

Además de su efecto en cáncer, la autofagia juega un papel importante como un mecanismo de defensa celular en la infección por microorganismos.

Un alto porcentaje de personas desarrolla periodontitis severa o moderada, un importante factor en el ambiente tumoral del carcinoma de cavidad oral. *Porphyromonas gingivalis*, la bacteria que produce esta enfermedad, induce tanto la autofagia como una interrupción en el ciclo celular de las células tumorales, dando lugar a una proliferación tumoral reducida tras su infección. Además se ha visto que la infección por esta bacteria aumenta los niveles de especies reactivas de oxígeno (ROS), siendo la autofagia de estas moléculas la base molecular por la que *P. gingivalis* induce alteración de la proliferación celular y la regulación del ciclo en células tumorales[145].

Debido al aumento en la etiología vírica del CECC, diversos estudios han relacionado la infección vírica con el proceso de autofagia. En los procesos de infección, el HPV activa la vía PI3K/Akt/mTOR inhibiendo la vía autofágica previniendo el mecanismo de limpieza viral[147]. De modo similar, se ha visto que las oncoproteínas víricas E6/E7 modulan la autofagia, determinando el balance entre ambas la inducción de esta vía en células infectadas[143]. Por el contrario el virus de Epstein-Bar, una vez introducido en el huésped, utiliza la maquinaria autofágica como producción de partículas víricas[148]. Por todo ello, a pesar del papel claro de la autofagia en la infección vírica y patogénesis, la relación entre ambos procesos en CECC no está clara, dando lugar a un interesante y novedoso tema de investigación[144].

Tratamiento

El uso de fármacos quimioterápicos y/o radioterapia ha demostrado inducir la autofagia, dirigiéndose bien a muerte o supervivencia celular mediada por esta vía debido a su efecto dual durante el proceso de tumorogénesis[143].

La radioterapia causa un aumento en el estrés celular y una autofagia mantenida en estos tumores. La radiorresistencia en células tumorales se ha relacionado con una sobreexpresión de Bcl-2 asociada a la inhibición de la apoptosis, inhibiendo la autofagia dependiente de beclin-1 y manteniendo niveles de autofagia basales para la supervivencia celular[149]. Puesto que las células cancerígenas radiorresistentes emplean la inhibición de la autofagia como una estrategia de supervivencia celular, el uso de inductores de este proceso puede radiosensibilizar estas células de nuevo[150].

De modo similar a la radioterapia, los agentes quimioterápicos inducen un aumento de la autofagia que en colaboración con la apoptosis, producen la inducción de la muerte celular[143]. Actualmente, la combinación de inhibidores autofágicos con drogas citotóxicas es uno de los esquemas de tratamiento más atractivo en la terapia contra el cáncer. En estudios *in vitro* en CECC, de modo similar a otros tipos celulares, la combinación de un inhibidor de autofagia, como la cloroquina y el cisplatino, demostraron una apoptosis incrementada, sugiriendo que la inhibición de la autofagia puede ser una estrategia adyuvante útil para pacientes que reciben quimioterapia[144]. Sin embargo, esta vía de señalización puede actuar durante la quimioterapia tanto como un mecanismo de supervivencia como de muerte celular autofágica[151]. Además, el uso de ciertos fármacos utilizados en el tratamiento de CECC como cetuximab, inhibidor de EGFR, está ligado a una inducción en la vía autofágica[145].

En conclusión, el uso de moduladores de autofagia está emergiendo como una posible diana terapéutica contra el cáncer, predominando entre ellos el CECC. Puesto que este proceso implica a diversos genes, las vías de modulación de la autofagia son variadas, siendo el uso de inhibidores de PI3K/Akt/mTOR una de las vías que más opciones tiene para el tratamiento dirigido[152]. La modulación específica del proceso, fundamentalmente con el fármaco cloroquina[153]; al igual que el uso de modificadores epigenéticos que regulen la expresión de los genes implicados en este mecanismo[154] son unas de las opciones más comunes.

El uso de la autofagia como nueva diana terapéutica puede ampliar las oportunidades de administración de fármacos que son muy necesarias en CECC, donde la supervivencia a los 5 años continúa por debajo del 50%. Sin embargo, la resistencia al tratamiento requiere definir estrategias óptimas para modular la autofagia como una ventaja terapéutica[143].

HIPÓTESIS Y OBJETIVOS

Los CECC son tumores heterogéneos con etiopatogenia compleja en la que se encuentran involucrados factores ambientales, víricos y genéticos. El consumo de tabaco y alcohol son los carcinógenos relacionados de forma más directa, aunque la infección vírica, principalmente por el virus HPV, define un subgrupo de tumores con desarrollo y pronóstico diferenciado. Estos factores varían según su distribución geográfica, teniendo datos escasos en la población española.

La mayoría de los CECC se diagnostican en estadios avanzados de la enfermedad y a pesar de que el tratamiento combinado ha conseguido un aumento en la conservación del órgano, apenas se ha observado efecto en la supervivencia. El conocimiento del perfil genético en CECC permitiría definir su implicación en la susceptibilidad y evolución de la enfermedad así como la influencia en la respuesta y toxicidad al tratamiento. Esto permitiría definir subtipos pronósticos y factores moleculares predictivos de respuesta y/o toxicidad que establecerían la mejor estrategia terapéutica en cada paciente. De modo similar, la implicación de otras vías de señalización en el desarrollo de CECC podría definir nuevas dianas terapéuticas para este conjunto de tumores.

Ante estos antecedentes, nuestra hipótesis es que los CECC representan un conjunto de cánceres con diferencias epidemiológicas y moleculares dependiendo de su localización.

En el presente trabajo nos planteamos como objetivo general profundizar en el conocimiento de las alteraciones genéticas del CECC, así como evaluar el impacto de éstas en el comportamiento clínico y biológico de la enfermedad. Esto permitirá la búsqueda de biomarcadores y terapias alternativas que mejoren las escasas respuestas de estos tumores. Para ello, se propusieron los siguientes objetivos:

1. Definir las características epidemiológicas del CECC en la población española, así como la distribución de los factores de riesgo según las localizaciones más frecuentes: cavidad oral, faringe (oro e hipofaringe) y laringe.

2. Realizar un estudio de caracterización de variantes alélicas en genes candidatos de vías relacionadas con la carcinogénesis en relación con la susceptibilidad, respuesta al tratamiento y toxicidad en una cohorte representativa de la población española de CECC.

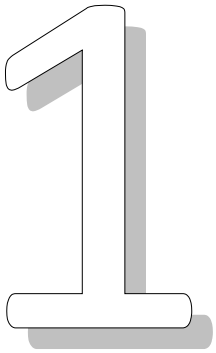
3. Analizar el estado mutacional en un panel de genes frecuentemente mutados en tumores y determinar si existe un patrón de mutaciones específico en la población de CECC, tanto a nivel global como diferenciando según el estado de infección por el virus del papiloma humano (HPV), relacionándolo con respuesta al tratamiento y la supervivencia para evaluar su posible papel pronóstico.

4. Analizar el efecto en la modulación del proceso autofágico de los fármacos panobinostat, cloroquina, metformina, paclitaxel y decitabina sobre la expresión proteica, viabilidad y ciclo celular en líneas tumorales comerciales y establecidas de las localizaciones más frecuentes del carcinoma escamoso de cabeza y cuello para determinar nuevas estrategias terapéuticas en el tratamiento de este tumor.

MATERIALES, MÉTODOS Y RESULTADOS

En esta sección de la memoria se describen los pacientes y los materiales y métodos empleados, así como los resultados obtenidos en relación con cada uno de los objetivos planteados mediante la inclusión de los artículos científicos originales redactados y publicados o sometidos a publicación como consecuencia del trabajo realizado. Cada uno de los artículos referidos está precedido de un breve resumen en castellano que facilita una revisión rápida de la información contenida en los mismos.

Epidemiological characteristics of a Spanish cohort of patients diagnosed with squamous cell carcinoma of head and neck: distribution of risk factors by tumor location



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Artículo 1: "Epidemiological characteristics of a Spanish cohort of patients diagnosed with squamous cell carcinoma of head and neck: distribution of risk factors by tumor location"

El carcinoma escamoso de cabeza y cuello es una enfermedad altamente heterogénea que engloba un alto número de tumores del área cérvico-facial. Los factores de riesgo clásicamente asociados con la enfermedad son el uso del tabaco y alcohol, aunque hay otros factores que tienen importancia en la etiología de este tumor. Estos factores varían según la población, existiendo datos escasos en la población española.

Con estos antecedentes, se planteó un estudio multicéntrico observacional y retrospectivo de CECC en la población española para determinar las características epidemiológicas de estos tumores así como la distribución de los factores de riesgo según la localización tumoral. Tras tres años de reclutamiento, se incluyeron 459 pacientes (75 carcinomas de cavidad oral, 167 carcinomas oro/hipofaríngeos y 217 carcinomas de la laringe).

La mayoría de pacientes fueron varones (88.4%), grandes fumadores y bebedores, diagnosticados en estadios avanzados de la enfermedad, con una media de 59 años. La distribución según la localización tumoral en varones fue similar a la incidencia global. Sin embargo, nuestro estudió mostró una proporción particularmente baja de mujeres con tumores en la laringe, predominando la presencia de tumores de oro/hipofaringe y cavidad oral ($p<0.01$). Estas localizaciones están asociadas a un mayor consumo de alcohol, observando en la muestra de mujeres un exceso de incidencia aún ajustado por la cantidad de alcohol consumido. Esto puede explicarse debido a una menor especificación del consumo de alcohol al no estar aceptado socialmente o al mayor efecto carcinogénico del alcohol en el sexo femenino.

La mayoría de los pacientes con carcinomas de cavidad oral o laringe provinieron de áreas urbanas, mientras que los tumores faríngeos fueron más comunes en áreas rurales, bien por la mayor exposición al tabaco en áreas rurales o por la infección vírica, debido a la diferencia en prácticas sexuales entre el modo de vida rural o urbana.

Respecto al nivel educacional, un alto porcentaje de nuestra muestra tuvo un bajo nivel (primaria o menor). Por otra parte, un tercio de los pacientes tuvieron historia familiar de cáncer (47% de ellos con CECC), sugiriendo un alto riesgo de desarrollar CECC en pacientes con historia familiar de la enfermedad, particularmente en aquellos con un familiar de primer grado. En cuanto a sus manifestaciones clínicas, los pacientes con cáncer laríngeo presentaron

disfonía, mientras que los tumores de faringe debutaron con masa en el cuello y en la cavidad oral lo más frecuente fueron las lesiones ulceradas presentes durante un tiempo prolongado. La pérdida de peso apareció en un tercio de los pacientes.

Observando los factores de riesgo clásicos, la mayoría de los pacientes incluidos en el estudio eran grandes fumadores con un inicio temprano, predominando el consumo de cigarrillos de tabaco negro, revelando el análisis bivariado una asociación entre aquellos pacientes que fuman más de 20 paquetes/año y una mayor proporción de tumores laríngeos que faríngeos. De modo similar, los sujetos eran grandes bebedores variando su consumo según la localización tumoral, asociándose un mayor consumo de alcohol en tumores de la faringe que de la laringe ($p<0.01$).

Aunque el estudio tiene ciertas limitaciones debido a su diseño retrospectivo, es el primer estudio de estas características en población española, describiendo las variables epidemiológicas de un amplio grupo de CECC en nuestro territorio.

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RESEARCH ARTICLE

Epidemiological characteristics of a Spanish cohort of patients diagnosed with squamous cell carcinoma of head and neck: distribution of risk factors by tumor location

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Abstract
Purpose Head and neck cancer is a highly heterogeneous disease comprising a large number of tumors located in the cervicofacial area. This study aimed to determine the epidemiological characteristics of squamous-cell carcinomas of the head and neck in the Spanish population, and the distribution of risk factors based on tumor locations.
Methods/patients A cohort of 459 patients (75 oral cavity, 167 oro-/hypopharyngeal and 217 laryngeal cancers) recruited in 19 hospitals participating in the Spanish head and neck cancer cooperative group were included over 3 years (2012–2014). Epidemiological parameters and risk factors were obtained from a self-administered questionnaire, and tumor characteristics were obtained from clinical records. Multivariate multinomial logistic regression was used to assess factors associated with tumor location.
Results Most patients were males (88.4 %), smokers (95 %) and drinkers (76.5 %). Relative to laryngeal cancer, pharyngeal cancer and oral cancer were more common in women than men (OR 3.58, $p = 0.003$ and 4.33,

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$p = 0.001$, respectively); pharyngeal cancer was more associated with rural environment (OR 1.81, $p = 0.007$) and weekly alcohol intake (10–140 g: OR 2.53, $p = 0.012$; 141–280 g: OR 2.47, $p = 0.023$; >280 g: OR 3.20, $p = 0.001$) and less associated with pack-years of smoking (21–40 packs: OR 0.46, $p = 0.045$; 41–70 packs: OR 0.43, $p = 0.023$; ≥71 packs: OR 3.20, $p = 0.015$).

Conclusions The distribution of these tumors differs between the sexes, with a higher proportion of oral cavity and pharyngeal tumors in women than in men. Oro-/hypopharyngeal cancers were more strongly associated with rural areas and with alcohol consumption, although less strongly associated with smoking than laryngeal tumors.

Keywords Head and neck cancer · Squamous cell carcinoma · Epidemiological characteristics · Spain · Risk factors

Introduction

Head and neck cancer (HNC) includes a large number of tumors located in different anatomical regions of the cervicofacial area, which, taken together, represent one of the most common cancers in the developed world [1]. Most of them are squamous cell carcinomas diagnosed in locally advanced stages, the oral cavity, pharynx and larynx being the most frequent locations [2].

Clinical presentation of HNC varies significantly depending on the location of the primary tumor and the extent of the disease. Thus, the first sign of the disease could be a palpable mass or, alternatively, location-specific symptoms such as dysphonia in laryngeal cancer or dysphagia in oropharyngeal cancer [3].

Major geographical differences in the incidence and location of the primary tumor in HNC are a consequence of the diverse patterns of tobacco and alcohol consumption in the different regions, as well as genetic variation between populations [4, 5].

Several epidemiological studies have shown an association between tobacco and alcohol consumption and the risk of HNC. Alcohol potentiates the effects of tobacco, a very common risk factor for these tumors [6, 7]. Other studies have shown a protective effect of fruit and vegetable intake [8, 9]. Social factors such as low socioeconomic status and low levels of education also seem to be related to disease development [10] and they could be related to the exposures described above.

There is evidence that two viruses are involved in the development of HNC: the Epstein-Barr virus (EBV) and human papillomavirus (HPV). Other infectious agents may also act as risk factors, but confirmatory studies are needed [11].

Other conditions, such as gastroesophageal reflux, have been associated with laryngeal and pharyngeal cancer regardless of age, gender, or tobacco and alcohol consumption [12].

In general, occupational exposures play a limited role in HNC, but an increased risk of sinonasal tumors has been observed among metal, wood, textile and leather workers, as well as people working in refineries [11].

The exposure to radiation for diagnostic purposes has been linked to salivary gland cancer [13], as has the therapeutic irradiation of these areas with the development of sarcoma [14].

Besides environmental factors, several association studies have suggested that genetic predisposition may also play a role in these diseases, since there is a higher risk in those subjects with family history of HNC [15, 16].

The aim of this study was to determine the epidemiological characteristics of squamous-cell carcinomas of the head and neck (SCCHN) in Spain using a series of attended cases, as well as the distribution of risk factors based on the most frequent tumor locations (oral cavity, oro/hypopharynx and larynx). To our knowledge, this is the first study of its type conducted in Spain.

Patients and methods

Design and study population

The present work is an observational study, carried out in 19 hospitals from different areas of Spain (four each in Catalonia and Madrid, two each in Castile and Leon, the Balearic Islands, Aragon and the Valencian Community, and one each in Galicia, Castile-La Mancha and Andalusia) belonging to the Spanish Head and Neck Cancer Cooperative Group (TTCC). Cases with a histologically confirmed diagnosis of SCCHN in oral cavity, oro-/hypopharynx or larynx, treated in these hospitals were invited to participate. Nasopharyngeal tumors were not included in the study due to the different risk factors involved (namely, a greater involvement of the EBV). Patients whose primary tumor affected several locations ($n = 1$) and those for which there was no information about location ($n = 11$) were excluded from the final analysis. A total of 459 patients (75 oral cavity cancer-OC, 167 oro/hypopharyngeal cancer-OHPC, 217 laryngeal cancer-LC) over a period of 3 years (2012–2014) were included. Written informed consent was

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obtained from all study subjects and the protocol was approved by the Institutional Review Board of each hospital.

Variable information

At baseline, all participants completed a self-administered questionnaire, which included sociodemographic data, personal and familiar backgrounds, symptoms present at the onset of the disease, and the risk factors associated with HNC, including detailed information about alcohol consumption and smoking habits.

The variables included in the study are defined as follows:

- Age refers to the age at diagnosis.
- Gender: male or female.
- Area of origin: patients were divided into two groups according to the size of their area of habitual residence: "urban area" included cities with more than 100,000 inhabitants; "rural area" comprised all others.
- Family support: patients were considered to have family support if they did not live alone.
- Educational level: data were divided into two groups: at most primary education, and more than primary education.
- Family history of cancer refers to the existence of one or more family members of patients with any type of cancer.
- Family history of HNC refers to the existence of one or more family members of patients with an HNC.
- Degree of relationship: we considered patients' first-degree relatives (parents, siblings and children), and other relatives (uncles, aunts, nephews, nieces, cousins, grandparents and grandchildren).
- Clinical presentation refers to the signs and symptoms at the time of diagnosis.
- Time with symptoms is defined as the time from symptom onset to first specialist consultation.
- Weight loss refers to the amount of weight (kg) lost before the diagnosis of the disease.
- Gastroesophageal reflux was judged to occur if the patient had feelings of heartburn after meals or at bedtime.
- Tobacco consumption: we collected the following information: age of onset, type of tobacco smoked, and whether the patient usually found themselves in environments with smoke. The calculation of the package-years was performed using the following formula: (number of cigarettes a day × years smoking/20 cigarettes per pack).
- Alcohol consumption: we collected information about the specific consumption of wine, beer and other alcoholic drinks. We then calculated the amount of ethanol (in grams) consumed with reference to the Standard Drink Unit (SDU) in Spain, which is equivalent to consumption of a single wine or beer, or a half measure of spirits. One SDU corresponds to 10 g of pure ethanol [17].
- Anatomical regions (oral cavity, pharynx: oro- and hypopharynx, larynx), stage and grade: these data were recorded according to the definitions of the 7th edition of the Manual for Cancer Staging [18]. Medical charts were retrospectively reviewed to collect the data.

The different items were checked for missing values or inconsistent data, and problems with the information were resolved appropriately.

Statistical analysis

In descriptive analysis, categorical variables were expressed as frequencies or percentages and the differences between pairs of groups were tested with Chi square contingency tables. A P value <0.05 was taken to indicate statistical significance.

Multivariate multinomial logistic regression was used to estimate ORs and 95 % confidence intervals with respect to tumor location. Laryngeal tumors were used as the reference group. The final model included those variables that proved to be statistically significant in bivariate analyses and well-established risk factors (tobacco and alcohol consumption).

Statistical analyses were performed using the Stata version 12.0 (Stata Corp, College Station, TX, USA).

Results

The characteristics of the population with HNSCC included in this study (n = 459) are summarized in Table 1. Median age at diagnosis was 59 years (range 37.5–87.3 years) and the distribution of patients by age groups was similar across all tumor locations. Most patients were men. However, the proportion of women was particularly low in the case of laryngeal cancer. The majority of patients with OCC or LC lived in areas with more than 100,000 inhabitants. Most patients had family support and tended to have a lower educational level (primary or no education), with no significant differences among specific locations. One hundred sixty-six patients had a family history of cancer, 47 % of them with head and neck cancer. Of the family members with head and neck cancer, 62.8 % were a first-degree relative of the patient. The majority of patients diagnosed with laryngeal cancer presented clinically with dysphonia. In the case of pharyngeal cancer the most frequent sign was the presence of a

Table 1 Characteristics of cases: overall and by subtypes of squamous head and neck cancer

Head and neck cancer cases	Overall		Subtypes						P^b
			OCC[a]		OHPC[a]		LC[a]		
Variables and potential confounders	(*N* = 459)	%	(*N* = 75)	%	(*N* = 167)	%	(*N* = 217)	%	
Age at diagnosis									
<50	69	15.03	15	20.00	29	17.37	25	11.52	0.277
50–65	270	58.82	42	56.00	99	59.28	129	59.45	
>65	116	25.27	18	24.00	37	22.16	61	28.11	
Missing	4	0.87	0	0	2	1.20	2	0.92	
Gender									
Male	406	88.45	58	77.33	144	86.23	204	94.01	<0.001*
Female	53	11.55	17	22.67	23	13.77	13	5.99	
Area									
≤100,000 inhabitants	176	38.34	20	26.67	82	49.10	74	34.10	<0.001*
>100,000 inhabitants	268	58.39	49	65.33	79	47.31	140	64.52	
Missing	15	3.27	6	8.00	6	3.59	3	1.38	
Familiar support									
No	73	15.90	12	16.00	29	17.37	32	14.75	0.728
Yes	384	83.66	63	84.00	137	82.04	184	84.79	
Missing	2	0.44	0	0	1	0.60	1	0.46	
Level of education									
Primary or lower	296	64.49	43	57.33	112	67.07	141	64.98	0.336
Higher than primary	163	35.51	32	42.67	55	32.93	76	35.02	
Family history of cancer									
Yes	166	36.17	29	38.67	58	34.73	79	36.41	0.871
No	291	63.40	46	61.33	107	64.07	138	63.59	
Missing	2	0.44	0	0	2	1.20	0	0	
Family history of head-neck cancer									
Yes	78	16.99	13	17.33	31	18.56	34	15.67	0.723
No	379	82.57	62	82.67	134	80.24	183	84.33	
Missing	2	0.44	0	0	2	1.20	0	0	
Relative with head-neck cancer									
No	379	82.57	62	82.67	134	80.24	183	84.33	0.720
First-degree	49	10.68	10	13.33	20	11.98	19	8.76	
Second-degree	26	5.66	3	4.00	9	5.39	14	6.45	
Other	3	0.65	0	0	2	1.20	1	0.46	
Missing	2	0.44	0	0	2	1.20	0	0	
Symptoms									
Dysphonia	141	30.72	1	1.33	18	10.78	122	56.22	<0.001*
Dysphagia	38	8.28	6	8.00	22	13.17	10	4.61	
Bulk	62	13.51	9	12.00	35	20.96	18	8.29	
Ulcer	58	12.64	40	53.33	16	9.58	2	0.92	
Pain	66	14.38	15	20.00	33	19.76	18	8.29	
Other	27	5.88	1	1.33	12	7.19	14	6.45	
Various	51	11.11	2	2.67	24	14.37	25	11.52	
Missing	16	3.49	1	1.33	7	4.19	8	3.69	
Time with symptoms									
≤2 months	225	49.02	41	54.67	84	50.30	100	46.08	0.313
>2 months	223	48.58	32	42.67	77	46.11	114	52.53	
Missing	11	2.40	2	2.67	6	3.59	3	1.38	

Table 1 continued

Head and neck cancer cases	Overall		Subtypes						P^b
			OCC[a]		OHPC[a]		LC[a]		
Variables and potential confounders	(*N* = 459)	%	(*N* = 75)	%	(*N* = 167)	%	(*N* = 217)	%	
Weight loss									
Yes	141	30.72	24	32.00	60	35.93	57	26.27	0.107
No	312	67.97	50	66.67	104	62.28	158	72.81	
Missing	6	1.31	1	1.33	3	1.80	2	0.92	
Gastroesophageal reflux									
Yes	160	34.86	24	32.00	57	34.13	79	36.41	0.738
No	292	63.62	51	68.00	106	63.47	135	62.21	
Missing	7	1.53	0	0	4	2.40	3	1.38	

* Statistically significant results

[a] *OCC* oral cavity cancer; *OHPC* oro-/hypopharyngeal cancer; *LC* laryngeal cancer. [b] *P* values were calculated excluding missing values

mass in the neck, followed by pain. Finally, the presence of ulcerated lesions was the most frequent sign in the case of the oral cavity neoplasm. About half of the patients had symptoms for more than 2 months before diagnosis and 30.7 % suffered weight loss. Thirty-five percent of these patients had symptoms suggestive of gastroesophageal reflux, there being no significant differences for the different tumor locations.

Most patients were heavy smokers (Table 2) and many of them had begun smoking at an early age (10–15 years). Black tobacco or a mixture of black and blonde tobacco were the forms most frequently consumed. Some patients (14.4 %) smoked a pipe and/or cigars in addition to cigarettes and are included in the "others" category in Table 2. Most patients were heavy drinkers (Table 2) and had been drinking for an average of 30 years. The grams of ethanol consumed weekly differed significantly between tumor locations, with higher consumption being noted in patients diagnosed with pharyngeal and laryngeal tumors.

Tumor characteristics are listed in Table 3. Most of them were diagnosed in locally advanced stages. Tumors of the pharynx had a larger locoregional extension (stage IVa–b) and were more undifferentiated (histological grade 3) at the time of diagnosis.

Taking laryngeal tumors as the reference group, Table 4 shows the association of sociodemographic characteristics and tobacco and alcohol consumption, the main well-established risk factors, with specific locations. A higher proportion of women were noted in the oro-/hypopharyngeal and oral cavity tumor groups than in the laryngeal cancer group. OHPCs were more strongly associated with rural areas and with alcohol consumption, although less strongly associated with smoking. There were no statistically significant differences in these variables in OCCs with respect to LCs.

Discussion

Head and neck cancer is a very heterogeneous disease that comprises a large number of tumors with clinically and etiologically different characteristics. This study shows the epidemiological characteristics of a Spanish cohort of patients diagnosed with squamous cell carcinoma of oral cavity, pharynx (excluding nasopharynx) and larynx, overall and by tumor location.

Most of the patients were diagnosed at advanced stages and were men aged 50–65 years. There was a higher proportion of women in the group of patients with oral cavity and pharyngeal cancer than with laryngeal cancer. These data are consistent with previously published findings [2, 19]. The lower incidence of HNSCC in women compared with men is explained by differences in the patterns of tobacco and alcohol consumption. In recent decades the number of cases in women has been increasing due to a change in these patterns [19]. Nevertheless, it is not known why women develop oral cavity and pharyngeal cancer more frequently than laryngeal cancer. The two cancers are more frequently linked to alcohol consumption than is laryngeal cancer because of the direct exposure of these locations to alcohol beverages. However, we found a statistically significant excess of women even after adjusting for alcohol consumption. A possible explanation could be that women under-reported their consumption of alcohol, since it is considered less socially acceptable for women to drink. Moreover, women's possible lower consumption of alcohol would have been offset by the well-known stronger carcinogenic effects of alcohol on women than on men [20].

Considering the area of origin of the patients treated at the different hospitals revealed statistically significant differences with respect to primary tumor location, whereby a higher percentage of patients with pharyngeal

Table 2 Tobacco and alcohol consumption: overall and by subtypes of squamous head and neck cancer

Head and neck cancer cases	Overall		Subtypes						P^a
			OCC		OHPC		LC		
Variables and potential confounders	(*N* = 459)	%	(*N* = 75)	%	(*N* = 167)	%	(*N* = 217)	%	
Starting smoking age									
Never smokers	22	4.79	7	9.33	8	4.79	7	3.23	0.078
10–15 years old	227	49.46	28	37.33	80	47.90	119	54.84	
15–20 years old	160	34.86	29	38.67	64	38.32	67	30.88	
>20 years old	43	9.37	9	12.00	12	7.19	22	10.14	
Missing	7	1.53	2	2.67	3	1.80	2	0.92	
Active smokers									
Yes	436	94.99	68	90.67	159	95.21	209	96.31	0.104
No	22	4.79	7	9.33	8	4.79	7	3.23	
Missing	1	0.22	0	0	0	0	1	0.46	
Kind of tobacco									
Never smokers	22	4.79	7	9.33	8	4.79	7	3.23	0.085
Black	176	38.34	25	33.33	56	33.53	95	43.78	
Blond	94	20.48	21	28.00	34	20.36	39	17.97	
Mix	93	20.26	15	20.00	38	22.75	40	18.43	
Others	66	14.38	6	8.00	28	16.77	32	14.75	
Missing	8	1.74	1	1.33	3	1.80	4	1.84	
Pack-years of smoking									
≤20	84	18.30	19	25.33	38	22.75	27	12.44	0.120
21–40	72	15.69	11	14.67	26	15.57	35	16.13	
41–70	139	30.28	21	28.00	46	27.54	72	33.18	
≥71	150	32.68	22	29.33	51	30.54	77	35.48	
Missing	14	3.05	2	2.67	6	3.59	6	2.76	
Environmental smoke									
Yes	335	72.98	50	66.67	121	72.46	164	75.58	0.336
No	117	25.49	24	32.00	42	25.15	51	23.50	
Missing	7	1.53	1	1.33	4	2.40	2	0.92	
Alcohol consumers									
Yes	351	76.47	49	65.33	138	82.63	164	75.58	0.005*
No	104	22.66	26	34.67	26	15.57	52	23.96	
Missing	4	0.87	0	0	3	1.80	1	0.46	
Grams ethanol per week									
<10	104	22.66	25	33.33	26	15.57	53	24.42	0.025*
10–140	98	21.35	18	24.00	35	20.96	45	20.74	
141–280	80	17.43	16	21.33	26	15.57	38	17.51	
>280	164	35.73	16	21.33	69	41.32	79	36.41	
Missing	13	2.83	0	0	11	6.59	2	0.92	

OCC oral cavity cancer, *OHPC* oro-/hypopharyngeal cancer, *LC* laryngeal cancer

* Statistically significant results

[a] *P* values were calculated excluding missing values

cancer were from rural areas (<100,000 inhabitants). In the multivariate analysis, the area of origin remained independently significant, irrespective of cigarette and alcohol consumption. This could be due to different patterns of tobacco consumption, whereby there was greater exposure of the pharynx than the larynx to tobacco smoke in patients from areas with fewer than 100,000 inhabitants. Moreover, considering that a high percentage of oropharyngeal tumors (57–72 %, depending on the context) [21] is caused by HPV infection and that this infection is associated with an

Table 3 Tumor characteristics: overall and by subtypes of squamous head and neck cancer

Head and neck cancer cases	Overall		Subtypes						P^a
			OCC		OHPC		LC		
Variables and potential confounders	(*N* = 459)	%	(*N* = 75)	%	(*N* = 167)	%	(*N* = 217)	%	
Stage									
Ca in situ	3	0.65	0	0	1	0.60	2	0.92	<0.001*
I	24	5.23	9	12.00	2	1.20	13	5.99	
II	33	7.19	8	10.67	4	2.40	21	9.68	
III	96	20.92	10	13.33	20	11.98	66	30.41	
IV	258	56.21	43	57.33	131	78.44	84	38.71	
Metastases	9	1.96	1	1.33	4	2.40	4	1.84	
Missing	36	7.84	4	5.33	5	2.99	27	12.44	
Grade									
1	73	15.90	25	33.33	16	9.58	32	14.75	0.001*
2	160	34.86	27	36.00	48	28.74	85	39.17	
3	60	13.07	8	10.67	29	17.37	23	10.60	
Unknown	166	36.17	15	20.00	74	44.31	77	35.48	

OCC oral cavity cancer, *OHPC* oro-/hypopharyngeal cancer, *LC* laryngeal cancer

* Statistically significant results

[a] *P* value were calculated excluding missing values

Table 4 Multivariable multinomial logistic regression model, taking laryngeal tumors as reference, assessing factors associated with location of HNSCC

Variables	LC (*N* = 217)		OHPC (*N* = 167)		OR	95 % CI[c]	P^b	OCC (*N* = 75)		OR	95 % CI	P^c
Gender												
Male	204	94.01 %	144	86.23 %	Reference			58	77.33 %		Reference	
Female	13	5.99 %	23	13.77 %	3.58	1.55–8.27	0.003*	17	22.67 %	4.33	1.78–10.50	0.001*
Area[e]												
>100,000 inh.	140	64.52 %	79	47.31 %	Reference			49	65.33 %		Reference	
≤100,000 inh.	74	34.10 %	82	49.10 %	1.81	1.17–2.81	0.007*	20	26.67 %	0.80	0.44–1.47	0.482
Pack-years of smoking[f]												
≤20	27	12.44 %	38	22.75 %		Reference		19	25.33 %		Reference	
21–40	35	16.13 %	26	15.57 %	0.46	0.21–0.98	0.045*	11	14.67 %	0.56	0.22–1.44	0.228
41–70	72	33.18 %	46	27.54 %	0.43	0.22–0.84	0.013*	21	28.00 %	0.52	0.23–1.19	0.125
≥71	77	35.48 %	51	30.54 %	0.43	0.22–0.85	0.015*	22	29.33 %	0.58	0.25–1.34	0.202
Grams ethanol per week[f]												
<10	53	24.42 %	26	15.57 %	Reference			25	33.33 %		Reference	
10–140	45	20.74 %	35	20.96 %	2.53	1.22–5.24	0.012*	18	24.00 %	1.27	0.57–2.84	0.554
141–280	38	17.51 %	26	15.57 %	2.47	1.13–5.38	0.023*	16	21.33 %	1.51	0.64–3.55	0.341
>280	79	36.41 %	69	41.32 %	3.20	1.61–6.37	0.001*	16	21.33 %	0.81	0.36–1.86	0.626

LC laryngeal cancer, *OHPC* oro-/hypopharyngeal cancer, *OCC* oral cavity cancer, *OR* odds ratio, *CI* confidence interval

* Statistically significant results

[a] *P* values were calculated by multinomial logistic regression using multivariable analysis

[b] Missing values are described in Table 1

[c] Missing values are described in Table 2

earlier sexual debut and more sexual partners [22], it could also be due to differences in sexual practices between rural and urban populations.

The bivariate analysis revealed no statistically significant differences in cigarette consumption by tumor location. However, in the multivariate analysis we observed a significantly higher proportion of LCs than OHPCs in those patients who had smoked more than 20 pack-years.

In the case of alcohol we identified a higher risk of developing pharyngeal than laryngeal cancer in patients with high alcohol consumption. The development of pharyngeal and oral cavity cancers has been associated with alcohol consumption [20]. Our study did not find a higher risk of developing oral compared with laryngeal cancer among those who consumed alcohol. This may be due to a lack of statistical power, since this is the least-represented location among our sample of patients. Moreover, there was a notable percentage of women diagnosed with oral cavity cancer amongst whom, as discussed above, alcohol consumption may have been underestimated.

A high percentage of patients had family support regardless of tumor location. Family support is a common feature in Mediterranean countries. In Spain, the percentage of men over 65 who live alone is small (22.7 %), their partner being the care provider should this be necessary [23].

Head and neck cancer has been associated with low levels of education in previous studies [10]. Most of the patients in our study had a low educational level (64.5 % of participants had no education or only primary school education).

In most of the cases in our study with a family history of HNC, the affected family member was a first-degree relative (62.8 %). This figure is consistent with those of previous studies, suggesting that there is a higher risk of developing HNC in subjects with a family history of the disease, particularly in those with a first-degree relative with HNC [15, 16].

The clinical appearance of the disease matches the habitually discussed signs and symptoms [3]. Thus, the most common signs of laryngeal, oral cavity and pharyngeal cancers are, respectively, dysphonia, ulcers, and a mass in the neck.

We recognize that our study is limited by the retrospective nature of the design, which makes it susceptible to bias. Moreover, most of the information was obtained via self-administered questionnaires. Thus, in the case of surveys, even though they are accepted as being the best available method of learning about patterns of alcohol consumption [24], they are well known to underestimate a drinkers' risks, either due to recall bias and/or deliberate underreporting. Finally, we had no information about HPV infection, which is an important risk factor for some of these tumors.

In this study we describe the epidemiological characteristics of a group of Spanish patients diagnosed with head and neck cancer. We found a higher proportion of women with oral cavity and pharynx than with laryngeal cancer that could not be ascribed to the gender-based differences in consumption of tobacco and alcohol. We also noted a higher risk of developing pharyngeal cancer in patients who consumed alcohol beverages in great quantities, as has been reported previously by other groups. We demonstrated a higher risk of pharyngeal cancer in areas with fewer than 100,000 inhabitants regardless of cigarette and alcohol consumption consistent with differences between urban and rural areas with respect to tobacco consumption and/or sexual habits. In this context, it would be useful to develop prevention programs in the urban setting and extend them to rural areas.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All procedures performed in this study were in accordance with the ethical standards of the institutional research committees and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Identification of polymorphisms associated with head and neck squamous cell carcinoma susceptibility in the Spanish population

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Artículo 2: "Identification of polymorphisms associated with head and neck squamous cell carcinoma susceptibility in the Spanish population"

A pesar del efecto de los factores de riesgo ambientales, la presencia de factores genéticos de predisposición desempeña también un papel importante en el desarrollo de CECC. La carcinogénesis del tracto aerodigestivo implica la desregulación de múltiples vías de señalización y variantes en los genes que regulan estas vías dan lugar a diferencias en la susceptibilidad a desarrollar ciertos tumores, entre ellos el CECC. Sin embargo, estos resultados son contradictorios debido a sesgos en el diseño de los estudios en cuanto a heterogeneidad de la población a estudio o en los factores de riesgo.

No todos los individuos expuestos a los carcinógenos clásicos desarrollan CECC, por lo que la identificación de variantes genéticas en vías de señalización que regulan la carcinogénesis podría definir el riesgo diferencial a desarrollar la enfermedad. Por ello, se planteó un estudio multicéntrico en el cual se seleccionaron 296 pacientes con CECC (126 laringes, 100 faringes y 70 cánceres orales) y se compararon con una población control totalmente pareada en sexo, consumo de tabaco y alcohol y edad. Se estudiaron un total de 29 polimorfismos en genes implicados en reparación de DNA, inflamación, apoptosis o metabolismo de carcinógenos previamente asociados con riesgo a desarrollar CECC, así como variaciones en la respuesta y/o toxicidad en el tratamiento. EL DNA se extrajo de sangre periférica mediante el método de fenol-cloroformo. La discriminación alélica se realizó mediante sondas TaqMan® o PCR y posterior digestión con enzimas específicas (RFLP).

El análisis descriptivo mostró una población control totalmente pareada en factores de riesgo y edad, excepto en el grupo de cáncer laríngeo donde la edad fue usada como factor de ajuste. Teniendo en cuenta la distribución entre localizaciones observamos diferencias estadísticamente significativas en la distribución de varios SNPs.

En cáncer laríngeo, los genotipos con el alelo menos común de los polimorfismos *ERCC1* rs11615 y *ERCC2* rs13181 fueron asociados con un menor riesgo a desarrollar este tumor. Ambos SNPs se localizan en genes que forman parte de la vía de reparación por escisión de nucleótidos (NER) y se han relacionado con una menor expresión génica y una menor reparación del DNA, así como una mejor respuesta y supervivencia tras el tratamiento con platino. La diferencia de nuestro resultado podría estar en la naturaleza retrospectiva del estudio, donde la mayoría de pacientes incluidos tendrían el alelo común asociado a una mayor reparación del DNA, proceso altamente activo en pacientes fumadores y bebedores.

La inflamación es otra de las vías esenciales en la carcinogénesis. Ésta es mediada por las citocinas, donde destacan IL-2 y la proinflamatoria IL-6. Las variantes rs1800975 en *IL6* y rs2069762 en *IL2* se ha asociado con una mayor expresión génica, previamente asociadas con un aumento en la inflamación. Nuestro estudio mostró como la variante en *IL6* produjo un riesgo incrementado de desarrollar carcinoma de faringe y cavidad oral, mientras que *IL2* rs2069762 disminuyó la susceptibilidad a cáncer oral, estando esta citocina más implicada en la autoinmunidad que en la inflamación protumoral.

Mdm2 es un regulador negativo de la proteína supresora de tumores p53, y su SNP rs2279744 en la zona promotora atenúa esta inhibición. Nuestros datos confirman el efecto visto en otros tumores donde el alelo variante se asocia con un riesgo incrementado a desarrollar cáncer laríngeo, debido a una menor actividad supresora mediada por p53.

El polimorfismo Pro72Arg rs1042522 en *TP53* es uno de los más estudiados, relacionándose con un incremento al desarrollo de tumores, aunque sus resultados son debatidos. Este SNP es esencial en el aumento de la respuesta apoptótica. Nuestros resultados mostraron un riesgo menor a desarrollar tumores de faringe y laringe en portadores del alelo antiapoptótico Pro72 y, aunque estos resultados son contrarios al aumento de riesgo establecido en otros trabajos, podría deberse tanto al desequilibrio en su distribución (HWE $p<0.05$), como en la mayor supervivencia del alelo Pro previamente descrita, aumentando el número de individuos en nuestra muestra retrospectiva.

En nuestra serie, el polimorfismo rs2279115, localizado en la zona promotora del gen anti-apoptótico *BCL2* aparece asociado con un mayor riesgo de desarrollar cáncer oral, confirmando resultados previos en CECC. Este SNP en el promotor P2 produce un aumento en la unión a proteínas nucleares disminuyendo la actividad del promotor activador P1 y, como consecuencia, una disminución de la apoptosis.

Por último, dos polimorfismos en el gen *NFE2L2* se asociaron con menor riesgo a desarrollar cáncer laríngeo y faríngeo. *NFE2L2* rs1303586 se asoció con menor riesgo al desarrollo de ambos tumores, mientras que rs2706110 se relacionó con menor riesgo únicamente en carcinoma de laringe. Aunque hasta el momento no se han presentado análisis funcionales de estos SNPs, nuestra hipótesis es que incrementarían la inducción de los genes antioxidativos ante estrés, disminuyendo así el riesgo en CECC.

Por lo tanto, este estudio muestra la asociación entre diferentes polimorfismos en genes implicados en reparación de DNA, inflamación, oxidación y apoptosis con un riesgo

diferencial a desarrollar CECC. Así mismo, las diferencias encontradas en este estudio según la localización tumoral ponen de manifiesto la heterogeneidad subyacente en estos cánceres.

Identification of polymorphisms associated with head and neck squamous cell carcinoma susceptibility in the Spanish population

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Despite tobacco, alcohol and viral infection are the most common etiopathogenic factors, genetic predisposition is also an important cause. Not all individuals expose to the environmental carcinogens develop the illness so, the identification of polymorphism could define those variant associated with HNSCC susceptibility. In a multicentre case-control study 126 laryngeal, 100 pharyngeal and 70 oral cavity HPV- tumours with paired controls in sex, age and tobacco and alcohol consumption were included. A gene candidate association study of 29 selected SNPs in genes implied in important carcinogenic pathways was perfomed by TaqMan probes or RFLP assay in germinal DNA. The results showed that nine SNPs in genes implicated in DNA repair (*ERCC1* rs11615 and *ERCC2* rs13181), inflammation (*IL2* rs2069762 and *IL6* rs1800795), oxidation (*NFE2L2* rs13035806 and rs2706110) and apoptosis (*TP53* rs1042522, *MDM2* rs2279744 and *BCL2* rs2279115) were associated with HNSCC susceptibility in a Spanish population. The fact that these results change according to tumour location, confirm the diversity of the tumours grouped under the term of HNSCC.

Keywords: Head and neck squamous cell carcinoma, polymorphism, SNP, association study, susceptibility

Introduction

Head and neck cancer (HNC) includes a set of diverse neoplasms located in the lips, oral cavity, pharynx, larynx, salivary glands and thyroid glands among others[1]. Approximately 600.000 new cases are diagnosed per year, being the sixth cancer type on incidence worldwide. Treatment of early stages includes surgery and/or radiotherapy, while locally advanced tumours are also treated with chemotherapy and biological therapies[2]. Only the 40-50% reach five-years survival rate[3] causing and annual death of 271.000 patients[4,5]. Regarding its histological type, not all the HNC are the same, prevailing the squamous cell carcinomas[1].

Head and neck squamous cell carcinoma (HNSCC) development is a multifactorial process associated with a variety of risk factors. At least 75% HNSCC are attributable to the combination of cigarette smoking and alcohol drinking, the most classic carcinogens[6]. Diverse epidemiological studies have also revealed the existence of other environmental and genetic related factors. Similar to other tumours, viral aetiology has shown an implication in the development of HNSCC predominating Epstein-Barr virus (EBV) infection in nasopharynx and human papillomavirus (HPV), mainly subtype 16, in oral cavity and oropharynx tumours[6]. The carcinogenesis procedure triggered by viral infection define a different entity to that caused by tobacco and alcohol[8] allowing the classification of HNSCC in two main prognostic and therapeutic groups, in which HPV negative tumours are associated with an aggressive course and worst prognosis than HPV positive ones[9]. Despite the defined role of environmental factors there is also an evidence of familial aggregation and increased cancer risks amongst HNSCC relatives[9], suggesting the existence of genetic predisposition factors[10].

Not all individuals exposed to these carcinogens will suffer the disease so the identification of genetic variants in important signalling pathways could help to define tumour susceptibility as well as differences in response and toxicity to treatment. HNSCC carcinogenesis involves different pathways: carcinogen metabolism, DNA repair, cell cycle, immunity and inflammation[11–13]. Single Nucleotide Polymorphism (SNP) is the most abundant form of genetic variation, becoming an ideal genetic susceptibility marker[1].

Previously, diverse studies have evaluated the relationship between genetic variants and susceptibility to HNSCC[14–17]. However, the results were inconsistent because they were conducted in different populations with heterogeneity in the study design regarding risk factors, race or ethnicity.

In this context, we present the first association study of polymorphisms in genes involve in the main cancer pathways and susceptibility to develop HNSCC within a Spanish population whose control group was totally paired in most important risk factors (tobacco and alcohol consumption). Due to the equality in the risk factors between both groups, the importance lies on the genotype, assuming the result into a greater extent to the SNPs.

Material and methods

Study population

TTCC-2010-05 was an observational multicentre study conducted in 19 Spanish centres, all of them belonging to the Spanish Group of Head and Neck Cancer Treatment (TTCC) coordinated by the Medical Oncology Department of the University Hospital of Salamanca between January 2012 and December 2014. Epidemiological details have been previously described[18].

The inclusion criteria were histologically confirmed HPV negative HNSCC patients from larynx, oro/hypopharynx and oral cavity carcinomas. They were recruited in Oncology, Radiotherapy and Otorhinolaryngology departments. Controls were hospitalized patients without tumour history and paired by age, sex, smoking and alcoholism habit with the HNSCC cases. They were captured in Pneumology, Radiotherapy, Otorhinolaryngology and Internal Medicine departments. Only Spanish population were permitted, avoiding ethnicity bias.

Taking into account incidence in Spain, 10% of possible lost and duration of the study, initial calculations of recruitment were of 440 patients in each group. A total of 459 patients and 259 controls were included.

In this study the variables were polymorphisms in oncogenes, tumour suppressor genes, genes implicated in DNA reparation, inflammation, carcinogen metabolism and apoptosis, together with some risk factors collected in the socio-demographic (6 questions) and the data informed by patients (19 questions) questionnaires. The information of both questionnaires were collected by auto-application, being supervised by the members of the research team with the objective of correct filled. Clinicopathologic data as well as response and specific toxicity to treatment were collected by oncologists in the case report form questionnaire (CRF).

The study was approved by the University Hospital of Salamanca and the local ethics committees. All participants were previously informed and signed the provided written informed consent designed for this project. All data were treated with the security measures established in compliance with the Protection of Personal Data Organic Law 15/1999, 13th December, and safe-keeping by the University Hospital of Salamanca in its specific hospital server.

Selection of polymorphism

Candidate SNPs selection was done according to at least two of the following criteria: >5% allele frequency in Caucasian/European population, previously defined association with HNSCC susceptibility, earlier related different response or toxicity to chemotherapy or radiotherapy. At the initial stages of the project design, a huge search was performed in available databases using keywords as SNPs, susceptibility, HNSCC, response, toxicity, selecting only those with statistically significant results in other populations[12,14–17,19]. SNPs with some published evidence of functionality were preferred selected (Table 1).

DNA isolation and genotyping

DNA was extracted from leukocytes of peripheral blood by phenol-chloroform method. Genotyping was performed using the TaqMan® Allelic Discrimination Assay (Applied Biosystems, Foster, CA) in those SNPs where the probes are available. 40 ng/µl of DNA sample were added to 6.25 µl of Taqman® Universal PCR Master Mix and it was combined with specific forward and reverse primers, and allele-specific VIC (allele 1) and FAM (allele 2) labelled probes. The assay was performed in a 96 well plate and the detection was measured in the Step One Plus Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Foster, CA). Negative and positive controls were always added. 5% of random samples were re-genotyping to ensure the reproducibility.

In those candidate SNPs where TaqMan® probes were not available, genotyping was analyzed by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). Specific oligonucleotides were designed to amplify the polymorphic sequences and digestion was made by the specific restriction enzymes. The PCR products were run on 3% Syber-safe stained agarose gel and visualized under UV light.

Statistical analysis

The statistical analysis to associate the relation between the different clinical and molecular variables was analyzed by cross tabs and the X^2 test of Pearson. The Odds ratio (OR) and 95% confidence intervals were calculated by logistic regression analysis. Quantitative variable distribution was analyzed by ANOVA test in those examples where the sample followed a parametric distribution ($p>0.05$ in Levene´s test); while in those with a non-parametric distribution; a Mann Whitney U test was applied. Hardy-Weinberg equilibrium (HWE) was tested in control population by X^2 test. It was considered the existence of statistically significant differences where the two sided P-value was <0.05. Only TP53 rs1042522 and APEX rs1130409 were in disequilibrium ($p_{HWE}<0.05$).

Because of the lower inclusion of matched controls, the statistical analysis was realized matching the group by the Propensity Score method (PS). This potent matching technique allowed us to equate groups in a cohort study through a logistic regression introducing the confounders as predictive variables[20]. Groups were matched according to: packs per year consumed (PPY): no smokers, <20PPY and >20PPY, standard unit of alcohol per week (SDU/week): <14SDU/week and >14SDU/week and sex. Quantitative age was not included in the PS and it was introduced in the logistic regression as adjustment variable only in laryngeal cancer where the age between both groups was statistically significant different.

These analyses were performed with the statistical software SPSS v.21.0 (IBM-SPSS Inc., Chicago).

Results

After the application of the Propensity Score method 126 larynx, 100 pharynx and 70 oral cavity squamous cell carcinomas were totally paired with the control group. Descriptive study of the analysis by locations did not show any statistically significant differences between sex, age, tobacco and alcohol intake respect the control group (Table 2). Only age between laryngeal tumour and control was significant ($p<0.05$) so this variable was included in the logistic regression as an adjustment variable.

The laryngeal cancer susceptibility study showed a different distribution in *TP53*, *MDM2*, *ERCC1*, *ERCC2*, *IL6* and *NRF2* selected SNPs (Supplementary table 1). Less common genotypes in *ERCC1* rs11615 (p=0.011, OR=0.288 (0.110-0.751) in recessive model) and *ERCC2* rs13181 (p=0.046, OR=0.375 (0.143-0.982) in codominant model) were associated with lower risk to suffer laryngeal cancer. This also happened with *TP53* rs1042522 in which its mutant allele C was associated with a decrease risk to develop laryngeal cancer (p=0.002, OR=0.286 (0.119-0.607) in recessive model). Variant allele in *MDM2* rs2279744, p=0.029 OR=2.413 (1.094-5.323) in recessive model, and *IL6* rs1800795 p=0.002 OR=2.394 (1.376-4.163) in dominant model, were related with a higher risk to suffer this tumour. Lastly, rs1303586 variant genotypes GA+AA and CT+TT in rs2706110, both in the *NRF2* gene, were associated with a lower risk to suffer this carcinoma, p=0.035 (OR=0.478 (0.240-0.949)) and p=0.518 (OR=0.518 (0.299-0.900)) respectively (Table 3).

Pharyngeal squamous carcinoma showed an association between CC genotype in *TP53* rs1042522 and a lower probability to suffer this illness, p=0.001, OR=0.124 (0.035-0.476) in recessive model. Also, an association between *NRF2* rs2706110 less common allele gentoypes CC+CT and lower risk to develop pharyngeal carcinoma was also found, p=0.043, OR=0.552 (0.311-0.982) (Supplementary table 2 and table 4).

Finally, the analysis in oral cavity squamous carcinoma showed significant differences in the distribution of the polymorphism in inflammatory and apoptotic genes (Supplementary table 3). Inflammatory gene *IL2* rs2069762 variant was associated with a lower risk of oral cavity cancer (GG p=0.039, OR=0.300 (0.096-0.940)) while the variants CG+GG in the pro-inflammatory *IL6* rs1800795 were related with increased oral cavity susceptibility, p=0.018, OR=2.265 (1.148-4.467). CA+AA genotypes in *BCL2* rs2279115 were related with a higher risk to develop from oral carcinoma p=0.010, OR=2.753 (1.273-5.952) in dominant model (Table 5).

Discussion

Not all individuals exposed to the most classic carcinogens (tobacco and alcohol) develop HNSCC. Several susceptibility studies have identified SNPs in carcinogenesis-related pathways but their results are controversial due to the design and the risk factors misunderstanding. In this multicentre case-control study we examined the association between some polymorphisms and HNSCC susceptibility in a Spanish cohort with a control group totally paired by its risk factors considering their genetic background on its own.

Analysis of laryngeal squamous cell carcinoma showed an association with lower susceptibility risk in the *ERCC1* rs11615 and *ERCC2* rs13181 SNPs. These genes are part of the nucleotide excision repair (NER) pathway, implicated in the repair of adducts produced by the tobacco, DNA oxidative damage, alkylating agents damage and thymine dimers[21]. *ERCC1* Asn118Asn (rs11615; c.354G>A) and *ERCC2* Lys751Gln (rs13181; c.2251A>G) variants have been related with an increased risk to develop cancer in a huge variety of populations[22], among them in HNSCC cancer[23,24]. These polymorphisms were linked to a minor mRNA level or with a deficient DNA repair capacity respectively[25,26]. rs11615 and rs13181 variant genotypes have been also associated to a better response and longer survival for higher damage accumulation in patients treated with platinum[27], triggering cell death. A possible explanation of these results in our series could be the retrospective nature of our study, where those mutated allele carriers with less reparation and more aggressive illness would have been died along the follow-up and most of longest survivors with common allele would have been included in our series. This distribution is confirmed in our data.

Inflammation has been considered an important factor in the pathogenesis of human cancer[28,29], with a special interest in the context of oral cancer[30]. Inflammation is mediated by cytokines, being IL-2 and pro-inflammatory IL-6 two of the most important ones. SNPs in interleukin genes can influence in the magnitude of the inflammatory response, contributing into a different reaction[31]. rs1800795 -174C variant in the promoter of the *IL6* gene is related with lower level of serum proteins while -174G corresponds with a higher expression, increasing the inflammatory response[32]. Cytokine IL-2 is important in the activated T-lymphocytes proliferation and the phagocytes activation to destroy the uptaken material (Th1). The SNP -330G>T (rs2069762) in the gene promoter has been identified with a differentiating protein production in healthy subject, in which G allele produces a rise in the *IL2* gene expression, whereas GT/TT carriers have a decrease *IL2* expression skewing the balance Th1/Th2 towards Th2[33]. GT/TT genotypes were related with a certain susceptibility to suffer from diverse illnesses such as multiple sclerosis and minor risk to develop gastric cancer[33]. Our result with *IL6* variant can be related with the carcinogenesis induced by inflammatory process[29] in which G allele in *IL6* rs1800975 was associated with a higher gene expression and largest inflammatory response, increasing laryngeal and oral tumour susceptibility. However, GG genotype in *IL2* rs2069762 was associated with lower oral cavity carcinoma risk, in contrast with reported associations[34]. This result could be explained by the main role of IL-2 in self-reactive cells elimination[35], decreasing the antitumour efficacy by immune system.

Mdm2 is an important negative regulator of the tumour suppressor protein p53. Both form a complex in which Mdm2 attenuates p53 through proteasomal degradation by ubiquitinylation, while p53 induces *MDM2* transcription in response to genotoxic stress[36]. SNP rs2279744 309T>G, is located in the promoter P2 and increase *MDM2* expression by improving the binding affinity with Sp1 transcription factor, attenuating TP53 suppressor pathway[37]. Several correlations between *MDM2* rs2279744 and higher risk of HNSCC have been reported[38]. Our data confirm these results, showing a higher risk to suffer from laryngeal cancer in those patients with the GG genotype, related with an increase *MDM2* expression and lower p53 tumor suppressing activity.

It was found an association between the SNP in *TP53* rs1042522 and a higher susceptibility to develop pharyngeal and laryngeal cancer. *TP53* is a tumour suppressor gene and it is one of the main regulators of the genome integrity, cell cycle and apoptosis mechanism[39]. The polymorphism c.215C>G in the exon 4 of *TP53* produces a change of proline by arginine in the codon 72 (Pro72Arg) of the protein, being this domain essential in the apoptotic response and the carcinogenesis inhibition. In this sense, the arginine allele in the codon 72 is an apoptotic inductor more powerful than the proline one[40,41]. Lot of studies have related the Pro72Arg polymorphism with an increased risk to develop gastric, oesophageal, bladder cancer[42,43], although its role is controversial. In HNSCC, it has been only associated in isolated studies[17,44]. Though our results showed the contrary relationship, the explanation of this fact is difficult to define because SNP distribution was in disequilibrium (p_{HWE}>0.05). One plausible explanation could be for the influence of this SNP in survival, in which Pro72 was associated with longer survival after life-threatening diseases[40], tendency also shown in our series in laryngeal cancer (data not shown). So, our retrospective protocol would have selected those longest survivors, increasing the number of CC (Pro72) variant.

In addition to previous results, we found a statistically significant result in the polymorphism of the anti-apoptotic gene *BCL2* and oral cavity cancer. *BCL2*-938C>A (rs2279115) polymorphism is found in the gene promoter P2, which acts as a negative regulator element, decreasing the activity of the promoter P1[45]. C allele is related with a rise in the P2 promoter activity joining to nuclear proteins. The presence of C allele reduces the activity of P1 and the Bcl-2 protein expression increasing apoptosis. Our results showed similar results to that reported on breast cancer and acute myeloid leukaemia[46] where the presence of the A allele (CA+AA) increased susceptibility to these tumours due to an anti-apoptotic effect, being related with a higher Bcl-2 protein expression. This effect was also reported by Chen et al where the rs2279115 polymorphism was associated with an increase risk to HNSCC development[47].

NFE2L2 codifies for a transcription factor family (Nrf2) that induce many antioxidative genes under oxidative stress. SNPs in this gene have been associate to cancer risk[48]. In our sample, *NFE2L2* rs2706110 and rs1303586 less common allele genotypes were associated with lower risk to develop laryngeal cancer while in pharyngeal cancer only rs1303586 was found. This effect was different from that reported on a higher risk to suffer from breast cancer, related with AA genotype[49]. Functional analyses of these SNPs have not yet been described but our hypothesis is that these changes could increase antioxidative genes induction under stress, produced at high level in HNSCC by tobacco and alcohol consumption. Besides, the fact that Nrf2 and inflammation pathways are related[50], could enhance our results.

Overall, this study has shown the association between some polymorphisms in DNA repair, inflammation, antioxidative and apoptosis genes with different susceptibility to develop HNSCC. The characteristics of the control group favoured that this results were caused by the genetic background, avoiding confounder variables. In the same way, the differences found in this association study according to the location corroborate the heterogeneity in these tumours included under the same term of head and neck squamous cell carcinoma. The statistical power of this study is limited due to the moderate number of analyzed patients so; studies in larger groups should be done and would be necessary to confirm these results.

Interest conflicts

None of the authors who appear in this study have a potential interest conflict related with the manuscript.

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Table 1. SNPs selected in the study.

FUNCTION	GENE	RS	ID	Change
Oncogenes and tumor supressor genes	*TP53*	1042522	C_2403545_10	Pro72Arg
	MDM2	2279744	PCR-RFLP	Thr309Gly
	KRAS-LC6	61764370	PCR-RFLP	Intronic
	EGFR	2227983	C_16170352_20	Lys521Arg
Base excision repair (BER)	*XRCC1*	25487	C_622564_10	Gln399Arg
		1799782	C_11463404_10	Arg194Trp
	APEX	1130409	C_8921503_10	Asp148Glu
Nucleotide excision repair (NER)	*ERCC2(XPD)*	13181	C_3145033_10	Lys751Gln
	ERCC1	11615	C_2532959_10	Asn118Asn
	XPC	2228000	C_16018061_10	Ala499Val
Double-strand break repair genes	*XRCC3*	861539	C_8901525_10	Thr241Met
		1799794	C_2983904_10	c.-316A>G
	KU70	2267437	C_15872242_20	c.-731C>G
Inflammatory genes	*IL1*	16944	C_1839943_10	c.-598T>C
	IL2	2069762	C_15859930_10	c.-385T>G
	IL6	1800795	C_1839697_20	c.-237C>G
	IL10	1800872	C_1747363_10	c.-627A>C
	TNFA	361525	C_2215707_10	c.-418G>A
Apoptotic genes	*NOD2*	2066844	C_11717468_20	Arg702Trp
		2066845	C_11717466_20	Arg908Gly
	BAX	4645878	C_27848291_10	Intronic
	BCL2	2279115	C_3044428_30	Intronic
Carcinogen Metabolism genes	*CYP3A5*	776746	C_26201809_30	Intronic
	GSTP1	1695	C_3237198_20	Ile105Val
	GSTT1	N/A	PCR	Null/present
	GSTM1	N/A	PCR	Null/present
	NFE2L2 (NRF2)	13035806	C_11745134_10	3'-region
		2706110	C_11745133_10	3'-region
	KEAP1	1048290	C_9323035_10	Leu471Leu

Table 2. Descriptive case-control study.

	LARYNX N=126		CONTROL N=126		P-value	PHARYNX N=100		CONTROL N=100		P-value	ORAL CAVITY N=70		CONTROL N=70		P-value
Characteristics	N	%	N	%		N	%	N	%		N	%	N	%	
Age (years)	63.02±8.566		56.30±12.803		**0.000**	59.96±8.41		59.52±10.044		0.742	60.92±10.008		62.24±8.88		0.412
Sex															
Female	13	10.3	13	10.3	1.000	20	20.0	22	22.0	0.728	16	22.9	17	22.9	1.000
Male	113	89.7	113	89.7		80	80.0	78	78.0		54	77.1	54	77.1	
Tobacco smoking															
Never	7	5.5	7	5.5		7	7.0	8	8.0		7	10.0	7	10.0	
<20 PPY	20	15.9	22	17.5	0.944	22	22.0	23	23.0	0.943	12	17.1	12	17.1	1.000
>20 PPY	99	78.6	97	77.0		71	71.0	69	69.0		51	72.9	51	72.9	
Missing	0	0	0	0		0	0	0	0		0	0	0	0	
Alcohol drinking															
Never	53	42.1	51	40.5		26	26.0	27	27.0		23	32.9	23	32.9	
<14 SDU/week	28	22.2	31	24.6	0.904	30	30.0	30	30.0	0.985	19	27.1	19	27.1	1.000
>14 SDU/week	45	35.7	44	34.9		44	44.0	43	43.0		28	40.0	28	40.0	
Missing	0	0	0	0		0	0	0	0		0	0	0	0	

P-values related to controls. Statistically significant results in bold.

Table 3. Statistically significant SNPs in laryngeal cancer.

	Genotype	Larynx		Control		P-value*	OR (CI 95%)
SNPs		**N**	**%**	**N**	**%**		
***TP53* rs1042522**	GG	61	48.4	62	49.2	Ref.	1.00
	GC	54	42.9	37	29.4	0.165	1.505 (0.846-2.677)
	CC	11	8.7	27	21.4	**0.008**	**0.319 (0.136-0.745)**
Recessive	GG+GC	115	91.3	99	78.6	Ref.	1.00
	CC	11	8.7	27	21.4	**0.002**	**0.268 (0.119-0.607)**
Dominant	GG	61	48.4	62	49.2	Ref.	1.00
	GC+CC	65	51.6	64	50.8	0.596	0.986 (0.587-1.654)
***MDM2* rs2279744**	TT	44	34.9	62	49.2	Ref.	1.00
	TG	57	45.2	53	42.1	0.279	1.364 (0.778-2.392)
	GG	25	19.8	11	8.7	**0.015**	**2.826 (1.219-6.552)**
Recessive	TT+TG	101	80.2	115	91.3	Ref.	1.00
	GG	25	19.8	11	8.7	**0.029**	2.413 (1.094-5.323)
Dominant	TT	44	34.9	62	49.2	Ref.	1.00
	TG+GG	82	65.1	64	50.8	0.075	1.616 (0.953-2.742)
***ERCC1* rs11615**	TT	53	42.1	45	35.7	Ref.	1.00
	TC	67	53.2	58	46.0	0.872	0.956 (0.550-1.661)
	CC	6	4.8	23	18.3	**0.013**	**0.281 (0.103-0.768)**
Recessive	TT+TC	120	95.2	103	81.7	Ref.	1.00
	CC	6	4.8	23	18.3	**0.011**	**0.288 (0.110-0.751)**
Dominant	TT	53	42.1	45	35.7	Ref.	1.00
	TC+CC	73	57.9	81	64.3	0.354	0.778 (0.457-1.324)
***ERCC2* rs13181**	TT	72	57.1	52	41.3	Ref.	1.00
	TG	46	36.5	58	46.0	0.247	0.720 (0.413-1.255)
	GG	8	6.3	16	12.7	**0.046**	**0.375 (0.143-0.982)**
Recessive	TT+TG	118	93.7	110	87.3	Ref.	1.00
	GG	8	6.3	16	12.7	0.079	0.433 (0.170-1.102)
Dominant	TT	72	57.1	52	41.3	Ref.	1.00
	TG+GG	54	42.9	74	58.7	0.093	0.638 (0.377-1.078)
***IL6* rs1800795**	CC	43	34.1	62	50.8	Ref.	1.00
	CG	64	50.8	46	37.7	**0.003**	**2.471 (1.372-4.452)**
	GG	19	15.1	14	11.5	0.070	2.164 (0.938-4.991)
Recessive	CC+CG	107	84.9	108	88.5	Ref.	1.00
	GG	19	15.1	14	11.5	0.444	1.351 (0.625-2.921)
Dominant	CC	43	34.1	62	50.8	Ref.	1.00
	CG+GG	83	65.9	60	49.2	**0.002**	**2.394 (1.376-4.163)**
***NRF2* rs13035806**	GG	109	87.2	95	76.0	Ref.	1.00
	GA	14	11.2	29	23.2	**0.019**	**0.424 (0.207-0.869)**
	AA	2	1.6	1	0.8	0.520	2.235 (0.193-25.903)
Recessive	GG+GA	123	98.4	124	99.2	Ref.	1.00
	AA	2	1.6	1	0.8	0.444	2.600 (0.225-30.064)
Dominant	GG	109	87.2	95	76.0	Ref.	1.00
	GA+AA	16	12.8	30	24.0	**0.035**	**0.478 (0.240-0.949)**
***NRF2* rs2706110**	CC	92	73.6	72	57.1	Ref.	1.00
	CT	24	19.2	47	37.3	**0.005**	**0.425 (0.233-0.775)**
	TT	9	7.2	7	5.6	0.732	1.207 (0.411-3.541)
Recessive	CC+CT	116	92.8	119	94.4	Ref.	1.00
	TT	9	7.2	7	5.6	0.403	1.574 (0.544-4.560)
Dominant	CC	92	73.6	72	57.1	Ref.	1.00
	CT+TT	33	26.4	54	42.9	**0.020**	**0.518 (0.299-0.900)**

**P-values adjusted by age. Statistically significant results in bold.*

Table 4. Statistically significant SNPs in pharyngeal cancer.

	Genotype	Pharynx		Control		P-value	OR (CI 95%)
SNPs		**N**	**%**	**N**	**%**		
TP53 **rs1042522**	GG	53	53.0	47	47.0	Ref.	1.00
	GC	44	44.0	33	33.0	0.583	1.182 (0.650-2.151)
	CC	3	3.0	20	20.0	**0.002**	**0.133 (0.037-0.476)**
Recessive	GG+GC	97	97.0	80	80.0	Ref.	1.00
	CC	3	3.0	20	20.0	**0.001**	**0.124 (0.035-0.431)**
Dominant	GG	53	53.0	47	47.0	Ref.	1.00
	GC+CC	47	47.0	53	53.0	0.396	0.786 (0.451-1.370)
NRF2 **rs2706110**	CC	68	68.0	54	54.0	Ref.	1.00
	CT	25	25.0	41	41.0	**0.020**	**0.484 (0.262-0.893)**
	TT	7	7.0	5	5.0	0.863	1.112 (0.334-3.698)
Recessive	CC+CT	93	93.0	95	95.0	Ref.	1.00
	TT	7	7.0	5	5.0	0.553	1.430 (0.438-4.667)
Dominant	CC	68	68.0	54	54.0	Ref.	1.00
	CT+TT	32	32.0	46	46.0	**0.043**	**0.552 (0.311-0.982)**

Statistically significant results in bold.

Table 5. Statistically significant SNPs in oral cavity carcinoma.

SNPs	Genotype	Oral cavity		Control		P-value	OR (CI 95%)
		N	%	N	%		
***IL2* rs2069762**	TT	43	61.4	31	44.3	/	1.00
	TG	22	31.4	27	38.6	0.152	0.587 (0.284-1.217)
	GG	5	7.1	12	17.1	**0.039**	**0.300 (0.096-0.940)**
Recessive	TT+TG	65	92.9	58	82.9	/	1.00
	GG	5	7.1	12	17.1	0.078	0.372 (0.124-1.119)
Dominant	TT	43	61.4	31	44.3	/	1.00
	TG+GG	27	38.6	39	55.7	**0.043**	**0.499 (0.254-0.979)**
***IL6* rs1800795**	CC	25	35.7	39	55.7	/	1.00
	CG	33	47.1	23	32.9	**0.031**	**2.238 (1.077-4.653)**
	GG	12	17.1	8	11.4	0.104	2.340 (0.839-6.528)
Recessive	CC+CG	58	82.9	62	88.6	/	1.00
	GG	12	17.1	8	11.4	0.337	1.603 (0.612-4.203)
Dominant	CC	25	35.7	39	55.7	/	1.00
	CG+GG	45	64.3	31	44.3	**0.018**	**2.265 (1.148-4.467)**
***BCL2* rs2279115**	CC	13	18.6	27	38.6	/	1.00
	CA	43	61.4	30	42.9	**0.008**	**2.977 (1.325-6.688)**
	AA	14	20.0	13	18.6	0.116	2.237 (0.820-6.103)
Recessive	CC+CA	56	80.0	57	81.4	/	1.00
	AA	14	20.0	13	18.6	0.830	1.096 (0.473-2.540)
Dominant	CC	13	18.6	27	38.6	/	1.00
	CA+AA	57	81.4	43	61.4	**0.010**	**2.753 (1.273-5.952)**

Statistically significant results in bold.

Supplementary table 1. Distribution between selected SNPs in different candidate genes in laryngeal tumours (Ca) and controls (Co)*.

			Homozygotes common alleles		Heterozygotes				Homozygotes rarer allele			
FUNCTION	**GENE**	**RS**	**Ca**	**Co**	**Ca**	**Co**	**P-value**	**OR (95% CI)**	**Ca**	**Co**	**P-Value**	**OR (95% CI)**
Oncogenes y tumor suppressor genes	*p53*	1042522	61	62	54	37	0.165	1.505 (0.846-2.677)	11	27	**0.008**	0.319 (0.136-0.745)
	MDM2	2279744	44	65	57	53	0.279	1.364 (0.778-2.392)	25	11	**0.015**	2.826 (1.219-6.552)
	KRAS-LC6	rs61764370	97	87	27	33	0.382	0.762 (0.414-1.401)	2	6	0.247	0.367 (0.068-1.998)
	EGFR	2227983	78	72	41	44	0.514	0.830 (0.474-1.453)	7	10	0.283	0.536 (0.197-1.608)
Base excision repair (BER)	*XRCC1*	25487	58	57	50	55	0.794	0.929 (0.533-1.617)	18	14	0.724	1.160 (0.509-2.646)
		1799782	106	109	20	14	0.371	1.416 (0.663-3.012)	0	2	0.999	0.000 (0.000-)
	APEX	1130409	38	42	65	49	0.308	1.366 (0.750-2.490)	23	35	0.546	0.803 (0.394-1.637)
Nucleotide excision repair (NER)	*ERCC2=XPD*	13181	72	52	46	58	0.247	0.720 (0.413-1.255)	8	16	**0.046**	0.375 (0.143-0.982)
	ERCC1	11615	53	45	67	58	0.872	0.956 (0.550-1.661)	6	23	**0.013**	0.281 (0.103-0.768)
	XPC	2228000	62	61	57	52	0.530	1.190 (0.692-2.046)	7	13	0.384	0.636 (0.230-1.763)
Double-strand break repair genes	*XRCC3*	861539	49	47	59	58	0.721	0.902 (0.512-1.590)	18	21	0.504	0.766 (0.352-1.671)
		1799794	78	78	43	40	0.593	1.166 (0.664-2.047)	5	8	0.789	0.849 (0.255-2.823)
	KU70	2267437	39	44	65	58	0.995	0.998 (0.550-1.811)	22	24	0.780	0.898 (0.422-1.909)
Inflammatory genes	*IL1*	16944	56	58	58	56	0.849	1.054 (0.612-1.817)	12	12	0.930	0.959 (0.382-2.408)
	IL2	2069762	62	67	56	45	0.307	1.333 (0.768-2.311)	8	14	0.132	0.472 (0.178-1.253)
	IL6	1800795	43	62	64	46	**0.003**	2.471 (1.372-4.452)	19	14	0.070	2.164 (0.938-4.991)
	IL10	1800872	75	71	45	47	0.557	0.849 (0.491-1.467)	6	8	0.540	0.694 (0.216-2.229)
	TNFA	361525	104	103	19	21	0.694	0.866 (0.422-1.777)	3	2	0.632	1.565 (0.250-9.780)
Apoptotic genes	*NOD2*	2066844	110	109	16	17	0.876	0.940 (0.433-2.043)	0	0	-	-
		2066845	112	119	4	7	0.277	0.485 (0.131-1.789)	0	0	-	-
	BAX	4645878	92	93	31	31	0.806	1.078 (0.592-1.964)	2	2	0.986	1.017 (0.146-7.065)
	BCL2	2279115	37	40	66	55	0.124	1.614 (0.878-2.969)	23	31	0.896	1.050 (0.501-2.203)
Carcinogen Metabolism genes	*CYP3A5*	rs776746	116	110	8	14	0.246	0.573 (0.223-1.468)	1	1	0.914	1.167 (0.072-19.029)
	GSTP1	1695	65	62	54	51	0.805	1.071 (0.622-1.842)	12	6	0.254	0.535 (0.183-1.568)
	GSTT1	N/A	110	109	15	17	0.483	1.322 (0.606-2.882)	-	-	-	-
	GSTM1	N/A	66	59	59	67	0.337	1.289 (0.767-2.168)	-	-	-	-
	NFE2L2 (NRF2)	13035806	109	95	14	29	**0.019**	0.424 (0.207-0.869)	2	1	0.520	2.235 (0.193-25.903
		2706110	92	72	24	47	**0.005**	0.425 (0.233-0.775)	9	7	0.732	1.207(0.411-3.541)
	KEAP1	1048290	50	53	58	57	0.768	1.008 (0.622-1.900)	17	16	0.541	1.290 (0.569-2.925)

**P-values adjusted by age and related to controls. Statistically significant results in bold.*

Supplementary table 2. Distribution between selected SNPs in different candidate genes in pharyngeal tumours (Ca) and controls (Co).

			Homozygotes common alleles		Heterozygotes				Homozygotes rarer allele			
FUNCTION	**GENE**	**RS**	**Ca**	**Co**	**Ca**	**Co**	**P-value**	**OR (95% CI)**	**Ca**	**Co**	**P-Value**	**OR (95% CI)**
Oncogenes y tumor suppressor genes	*p53*	1042522	53	47	44	33	0.583	1.182 (0.650-2.151)	3	20	**0.002**	0.133 (0.037-0.476)
	MDM2	2279744	49	49	37	42	0.675	0.881 (0.487-1.595)	14	9	0.350	1.556 (0.616-3.928)
	KRAS-LC6	rs61764370	68	69	30	27	0.704	1.127 (0.607-2.093)	2	4	0.442	0.507 (0.090-2.862)
	EGFR	2227983	58	54	36	37	0.743	0.906 (0.502-1.634)	6	9	0.394	0.621 (0.207-1.860)
Base excision repair (BER)	*XRCC1*	25487	40	44	47	44	0.594	1.175 (0.649-2.127)	13	12	0.701	1.192 (0.487-2.913)
		1799782	90	87	10	12	0.634	0.806 (0.3311.961)	0	1	1.000	0.000 (0.000-)
	APEX	1130409	29	35	45	39	0.320	1.393 (0.725-2.675)	26	26	0.615	1.207 (0.580-2.513)
Nucleotide excision repair (NER)	*ERCC2=XPD*	13181	48	49	36	40	0.782	0.919 (0.504-1.676)	16	11	0.370	1.485 (0.625-3.526)
	ERCC1	11615	39	36	48	51	0.646	0.869 (0.477-1.584)	13	13	0.860	0.923 (0.378-2.253)
	XPC	2228000	48	54	46	38	0.296	1.362 (0.763-2.431)	6	8	0.768	0.844 (0.273-2.606)
Double-strand break repair genes	*XRCC3*	861539	36	35	43	49	0.616	0.853 (0.459-1.586)	21	16	0.550	1.276 (0.574-2.839)
		1799794	56	59	36	38	0.722	1.112 (0.620-1.995)	5	6	0.711	1.264 (0.365-4.377)
	KU70	2267437	39	33	47	49	0.504	0.812 (0.440-1.497)	14	18	0.328	0.658 (0.285-1.522)
Inflammatory genes	*IL1*	16944	47	52	43	39	0.506	1.220 (0.679-2.192)	10	9	0.681	1.229 (0.460-3.286)
	IL2	2069762	46	52	41	37	0.459	1.253 (0.690-2.273)	13	11	0.526	1.336 (0.546-3.272)
	IL6	1800795	46	48	45	35	0.336	1.342 (0.737-2.442)	9	13	0.498	0.722 (0.282-1.852)
	IL10	1800872	60	57	37	38	0.792	0.925 (0.518-1.652)	3	5	0.456	0.570 (0.130-2.495)
	TNFA	361525	83	83	17	15	0.746	1.133 (0.531-2.419)	0	2	0.999	0.000 (0.000-)
Apoptotic genes	*NOD2*	2066844	82	86	17	14	0.538	1.274 (0.590-2.749)	1	0	1.000	1694278518 (000-)
		2066845	96	95	4	5	0.734	0.792 (0.206-3.039)	0	0	-	-
	BAX	4645878	83	74	15	23	0.141	0.581 (0.282-1.197)	2	3	0.575	0.594 (0.097-3.655)
	BCL2	2279115	30	33	46	42	0.573	1.205 (0.630-2.302)	24	25	0.886	1.056 (0.500-2.229)
Carcinogen Metabolism genes	*CYP3A5*	rs776746	89	89	10	10	1.000	1.000 (0.397-2.520)	0	0	-	-
	GSTP1	1695	49	40	41	46	0.293	0.728 (0.402-1.317)	7	12	0.155	0.476 (0.171-1.322)
	GSTT1	N/A	84	86	16	14	0.692	0.855 (0.393-1.860)	-	-	-	-
	GSTM1	N/A	46	49	54	51	0.671	0.887 (0.509-1.545)	-	-	-	-
	NFE2L2 (NRF2)	13035806	78	75	18	25	0.292	0.692 (0.349-1.372)	3	0	0.999	-
		2706110	68	54	25	41	**0.020**	0.484 (2.262-0.893)	7	5	0.863	1.112 (0.334-3.698)
	KEAP1	1048290	42	44	49	46	0.713	1.116 (0.623-2.000)	9	10	0.908	0.943 (0.349-2.550)

P-values related to controls. Statistically significant results in bold.

Supplementary table 3. Distribution between selected SNPs in different candidate genes in tumours from oral cavity (Ca) and controls (Co).

			Homozygotes common alleles		Heterozygotes				Homozygotes rarer allele			
FUNCTION	**GENE**	**RS**	**Ca**	**Co**	**Ca**	**Co**	**P-value**	**OR (95% CI)**	**Ca**	**Co**	**P-Value**	**OR (95% CI)**
Oncogenes y tumor suppressor genes	*p53*	1042522	32	38	30	21	0.156	1.696 (0.818-3.518)	8	11	0.779	0.864 (0.310-2.407)
	MDM2	2279744	30	30	28	34	0.593	0.824 (0.404-1.678)	12	6	0.218	2.000 (0.664-6.026)
	KRAS-LC6	rs61764370	40	49	26	18	0.126	1.769 (0.851-3.678)	4	3	0.536	1.633 (0.345-7.727)
	EGFR	2227983	41	35	25	30	0.338	0.711 (0.354-1.428)	4	5	0.591	0.683 (0.170-2.742)
Base excision repair (BER)	*XRCC1*	25487	31	31	30	30	1.000	1.000 (0.492-2.034)	9	9	1.000	1.000 (0.350-2.856)
		1799782	62	62	8	8	1.000	1.000 (0.353-2.833)	0	0	-	-
	APEX	1130409	18	25	39	26	0.066	2.083 (0.952-4.559)	13	19	0.914	0.950 (0.375-2.408)
Nucleotide excision repair (NER)	*ERCC2=XPD*	13181	24	33	36	27	0.101	1.833 (0.888-3.785)	10	10	0.541	1.371 (0.495-3.821)
	ERCC1	11615	19	22	43	36	0.401	1.383 (0.649-2.948)	8	12	0.640	0.772 (0.261-2.284)
	XPC	2228000	36	37	27	26	0.857	1.067 (0.526-2.165)	7	7	0.963	1.028 (0.327-3.226)
Double-strand break repair genes	*XRCC3*	861539	27	32	29	28	0.582	1.228 (0.592-2.546)	14	10	0.301	1.659 (0.636-4.332)
		1799794	39	42	26	23	0.587	1.217 (0.598-2.477)	5	5	0.912	1.077 (0.289-4.007)
	KU70	2267437	19	22	35	34	0.657	1.192 (0.550-2.585)	16	14	0.561	1.323 (0.515-3.401)
Inflammatory genes	*IL1*	16944	27	37	36	27	0.093	1.827 (0.904-3.693)	7	6	0.443	1.599 (0.483-5.297)
	IL2	2069762	43	31	22	27	0.152	0.587 (0.284-1.217)	5	12	**0.039**	0.300 (0.096-0.940)
	IL6	1800795	25	39	33	23	**0.031**	2.238 (1.077-4.653)	12	8	0.104	2.340 (0.839-6.528)
	IL10	1800872	29	37	37	29	0.165	1.628 (0.819-3.237)	4	4	0.745	1.276 (0.294-5.542)
	TNFA	361525	61	60	8	9	0.796	0.874 (0.316-2.417)	1	1	0.991	0.984 (0.060-16.088)
Apoptotic genes	*NOD2*	2066844	62	63	8	7	0.785	1.161 (0.397-3.397)	0	0	-	-
		2066845	68	67	2	3	0.651	0.657 (0.106-4.057)	0	0	-	-
	BAX	4645878	51	51	19	18	0.888	1.056 (0.497-2.240)	0	1	1.000	0.000 (0.000-)
	BCL2	2279115	13	27	43	30	**0.008**	2.977 (1.325-6.688)	14	13	0.116	2.237 (0.820-6.103)
Carcinogen Metabolism genes	*CYP3A5*	rs776746	61	63	9	6	0.432	1.549 (0.520-4.614)	0	0	-	-
	GSTP1	1695	38	33	29	28	0.766	0.899 (0.448-1.808)	3	8	0.118	0.326 (0.080-1.329)
	GSTT1	N/A	8	11	62	59	0.461	1.445 (0.543-3.842)	-	-	-	-
	GSTM1	N/A	42	34	28	36	0.176	0.630 (0.322-1.230)	-	-	-	-
	NFE2L2 (NRF2)	13035806	56	54	12	16	0.448	0.723 (0.313-1.670)	2	0	0.999	-
		2706110	45	39	22	29	0.241	0.657 (0.326-1.325)	3	2	0.780	1.300 (0.206-8.184)
	KEAP1	1048290	24	27	39	36	0.586	1.219 (0.598-2.485)	7	7	0.845	1.125 (0.345-3.673)

P-values related to controls. Statistically significant results in bold.

Analysis of autophagy gene polymorphisms in Spanish patients with head and neck squamous cell carcinoma

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Artículo 3: "Analysis of autophagy gene polymorphisms in Spanish patients with head and neck squamous cell carcinoma"

La carcinogénesis del tracto aéreo-digestivo implica una alteración en el metabolismo de carcinógenos, una modificación de la reparación del DNA, la disrupción del ciclo celular y la desregulación de las vías implicadas en inmunidad, inflamación y degradación de componentes celulares. Variantes alélicas en genes implicados en estas vías pueden tener un papel importante en la susceptibilidad al desarrollo de CECC.

La autofagia, proceso catabólico de degradación de componentes celulares bajo estrés o deprivación de nutrientes, tiene un papel dual en la tumorogénesis, estando involucrada en el desarrollo del CECC. Para estudiar la importancia de la susceptibilidad al desarrollo de las diferentes localizaciones de CECC en la población española, se llevó a cabo un estudio de asociación de SNPs en los genes de autofagia implicados en la formación del autofagosoma. Se seleccionaron los polimorfismos *ATG2B* rs3759601, *ATG5* rs2245214, *ATG10* rs1864183 y *ATG16L1* rs2241880. Este estudio se realizó teniendo en cuenta los factores de riesgo más comunes en estos tumores, como son el tabaco y el alcohol, así como factores de confusión como sexo y edad, teniendo en cuenta únicamente el efecto de los SNPs.

450 pacientes diagnosticados de CECC (213 carcinomas laríngeos, 165 faríngeos y 72 de cavidad oral) con confirmación histológica negativa para el HPV fueron incluidos en el estudio. El DNA fue extraído de sangre periférica, realizando el genotipado mediante sondas TaqMan®. El análisis estadístico se realizó en dos fases, por un lado el ajuste multivariante con factores de riesgo de toda la población a estudio según su localización tumoral, y en segundo lugar el pareamiento de las muestras y controles por sus factores de riesgo mediante el método de *Propensity Score*.

El análisis de los resultados por ambos métodos mostró una asociación estadísticamente significativa entre los genotipos con el alelo menos común (CT+TT) en *ATG10* rs1864183 y un riesgo elevado de desarrollar cáncer laríngeo. Este SNP en el exón 4 produce un cambio en la proteína que causa la desregulación de la formación del autofagosoma, con menor autofagia y una acumulación del daño al DNA, aumentando el riesgo a desarrollar este tumor.

En cuanto al carcinoma escamoso de faringe, portadores del genotipo menos común GG en el SNP *ATG2B* rs3759601 presentaron un riesgo incrementado a desarrollar este tumor,

tanto en el modelo codominante como en el recesivo. La proteína ATG2B es esencial en la formación del fagosoma y mutaciones en este gen se han asociado con un riesgo incrementado en varios tumores. El cambio producido por este SNP podría producir una autofagia disminuida y un mayor riesgo a desarrollar cáncer de faringe.

Por último, la distribución del polimorfismo *ATG16L1* rs2241880 mostró asociación entre los pacientes portadores del genotipo menos común CC con un mayor riesgo a desarrollar cáncer oral. ATG16L1 es un adaptador central para la formación y elongación del autofagosoma. Varios estudios han relacionado este polimorfismo con un aumento en los procesos de inflamación y una menor autofagia, asociándolo de esta manera con nuestro resultado.

Este es el primer estudio que analiza estos SNPs en genes autofágicos y la susceptibilidad a desarrollar CECC en una población control totalmente pareada por sus factores de riesgo. Los resultados ponen de manifiesto la importancia de la vía autofágica en el desarrollo de CECC así como la heterogeneidad subyacente en estos tumores.

Title page

Analysis of autophagy gene polymorphisms in Spanish patients with head and neck squamous cell carcinoma

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth cancer on incidence worldwide. Tobacco and alcohol consumption are the most classical risk factors associated with its development. Autophagy process has a dual effect both in tumorigenesis and tumour suppressing activity. To investigate the importance of this pathway in HNSCC susceptibility, a risk factor matched case-control association study was performed with four candidate polymorphisms in autophagy genes (*ATG2B, ATG5, ATG10, ATG16L1*). We found an association between the variant in ATG10 rs1864183 and a higher susceptibility to develop laryngeal cancer, *ATG2B* rs3759601 and pharyngeal cancer and *ATG16L1* rs2241880 and oral carcinoma. *ATG5* rs2245214 SNP is not associated with any location. Overall, our results indicate the importance of the autophagy pathway in the susceptibility of head and neck squamous cell carcinoma and demonstrate the heterogeneity between its locations encompassed under a single terminology.

Introduction

Head and neck cancer (HNC) groups a set of different tumours located in the upper aero-digestive via. It includes tumours located in the lips, oral cavity, pharynx (nasopharynx, oropharynx and hypopharynx) as well as larynx, salivary glands and thyroid glands among others[1]. It is the sixth cancer type on incidence worldwide. Approximately 600.000 new cases are diagnosed each year and only 40-50% reach the five-years survival rate[2] causing an annual death of 271.000 patients[3,4]. Not all HNC present similar histology, prevailing in 90% of cases the squamous cell carcinomas which initiate in the mucosa[1].

Classic factors associated to the development of head and neck squamous cell carcinoma (HNSCC) are tobacco and alcohol consumption. At least 75% HNSCC are attributable to the combination of both carcinogens[5,6]. Moreover, different epidemiological studies have revealed the existence of other related factors, both environmental and genetic. In the last years the viral aetiology has been implicated in the development of HNSCC. This is the case of Epstein-Barr virus (EBV) in nasopharynx and the human papillomavirus (HPV), mainly subtype 16, in oral cavity and oropharynx tumours[7]. However, the carcinogenesis procedure triggered by viral infection identifies a totally different entity than the one produced by tobacco and alcohol[8,9]. On the other hand, the observation of familial aggregations in HNSCC suggests the existence of genetic predisposition factors. Lots of case-control studies have determined this genetic susceptibility, increasing the risk between 2-4 times for first grade HNSCC patients family[10].

Aero digestive tract carcinogenesis involves altered carcinogen metabolism, a modified DNA repair, cell cycle disruption and deregulation of pathways implicated in immunity, inflammation and cellular components degradation[11]. Allelic variants of genes implicated in essential cellular pathways play a very important role in tumour development as well as in treatment response. Polymorphism is defined as that mutation or variant which is found in at less 1% of the general population. Single Nucleotide Polymorphism (SNP) is the most abundant form of genetic variation[11].

Macro-autophagy is the catabolic process of damaged organelles or protein recycling under nutrient starvation or stress. It starts with the double-membrane autophagosome formation and finish with a fusion with the lysosomes to form the autophagolysosome which contains hydrolases for the degradation of the contents. This autophagosome complex is synthesised by autophagy-related genes (*ATG*)[12]. Autophagy takes part into both the initiation and prevention of cancer, and its function can be altered during tumor progression[13]. Although autophagy has a suppressing tumour activity, it is also involved in tumorigenesis by inhibiting cellular death and increasing drug resistance. It participates in important pathways connected to carcinogenesis as well as immune response, inflammation and genome stability[14]. However the precise mechanisms that involve autophagy in cancer are not yet defined[15]. In HNSCC, autophagy mechanisms are still unknown and they can symbolize an important area for future research[16].

To achieve our aim a candidate gene analysis was performed to study SNPs in autophagy genes: *ATG2B, ATG5, ATG10, ATG16L1* (Table 1) that could be associated to the risk to suffer HNSCC in a Spanish population. This association study was performed with a control group, selecting a cohort of subjects matched in gender, age and the two most important

environmental factors involved in the development of HNSCC, tobacco and alcohol consumption, avoiding confounder variables and considering genetic background on its own.

Results

A total of 450 patients distributed in 213 cases of larynx carcinoma, 165 of pharynx carcinoma and 72 of oral cavity were included in the study. The descriptive study of the global analysis by location showed some statistical differences between sex, age, tobacco and alcohol intake (Table 2). For this reason, SNPs analysis was calculated with an adjustment for these variables in the different locations.

The global study of susceptibility in laryngeal cancer (Table 3) showed an association between the heterozygote genotype of *ATG2B* rs3759601 and a lower risk to develop laryngeal squamous cell carcinoma, p=0.049 OR=0.607 (0.369-0.999). Moreover, although not statistically significant a tendency in ATG10 rs1864183 was found. The heterozygous genotype has a close relationship with an increased risk to develop laryngeal cancer (p=0.059, OR=1.648) (Table 3).

Analysis in pharyngeal squamous cell carcinoma shows that carriers of GG genotype in the SNP *ATG2B* rs3759601 have an increased risk to develop this tumour, both in the codominant and the recessive model, p=0.013 OR=2.493 (1.212-5.129) (Table 3). No other associations were found in the rest of SNPs between cases and controls.

ATG16L1 rs2241880 is unequally distributed in oral cavity cancer (Table 3). Patients with the less common allele C have higher risk to suffer from oral cavity cancer in our sample, p=0.017 in recessive model, OR=2.214 (1.150-4.263).

Due to the great significant differences in all the variables between groups, a second analysis was proposed by the Propensity Score method (PS). After its application we have totally paired 126 larynx, 100 pharynx and 70 oral cavity tumours according to sex, packs of tobacco per year (PPY) and standard drink units per week (SDU/week) with their specific control groups (Table 4). This method allowed us to corroborate the previous analysis avoiding the possible confounder variables. Quantitative age was also included as an adjustment variable in the logistic regression analysis of the laryngeal susceptibility study due to the significant differences between groups in the ANOVA test (p-value< 0.05) (Table 4). Because of pharyngeal and oral cavity carcinomas are paired by age, adjustment by quantitative age was not necessary (Table 4).

Once again, *ATG2B* rs3759601 heterozygote genotype was associated with a lower risk to develop laryngeal cancer p=0.028 OR=0.535 (0.307-0.935) (Table 5). Although not statistically significant in the previous analysis (p=0.059), we found a similar result in *ATG10* rs1864183 and a higher risk to develop laryngeal cancer in patients carrying the T allele, p=0.026 OR=1.888 (1.708-3.308) in the dominant model.

PS method corroborates the result in the previous analyses founding an association between *ATG2B* rs3759601 G allele and a higher risk to suffer from pharynx cancer (p=0.035, OR=2.721 (1.075-6.887)) (Table 6).

Finally, *ATG16L1* rs2241880 CC genotypes still being inversely associated with a higher risk to develop oral carcinoma after the PS application, p=0.047 OR=2.299(1.010-5.230) (Table 7).

Discussion

HNSCC is consequence of genetic and environmental factors, mainly tobacco smoking and alcohol consumption. Autophagy is a complex pathway, modulated by different molecular mechanisms with an important interest in HNSCC development[16]. To show the possible association of polymorphisms in autophagy genes and the susceptibility to suffer these tumours, a multicentre case-control study of head and neck squamous cell carcinoma was performed. Four polymorphisms were selected in ATG genes involved in phagosome generation. This was the case of the exonic missense polymorphisms *ATG2B* rs3759601, *ATG16L1* rs2241880 and *ATG10* rs1864183, and the intronic mutation in *ATG5* rs2245214 which involves changes in the recognition sites for SRp40 transcription factor. ATG5, *ATG10* and *ATG16L1* code for proteins that form the Atg5-Atg12-Atg16L1 conjugation complex[17], while *ATG2B* is necessary for closure of isolation membranes of autophagosomes[18].

Analysis of laryngeal cancer shows an association between the less common allele genotypes (CT+TT) in *ATG10* rs1864183 and a higher risk to develop it. It has been described that a lower expression of autophagy genes accelerate the tumor development due to a diminution in autophagy process[19]. *ATG10* rs1864183 C>T variant in exon 4 leads a catalytic change in the protein (Thr212Met) which causes a dysregulation in the autophagosome formation and a higher risk to develop breast cancer[20]. In this situation the cell cannot degrade a damaged organ, collecting damaging substances that cause an increase in DNA damage and carcinogenesis. Although this polymorphism has never been studied in HNSCC, this result indicates the importance of the autophagy pathway in laryngeal tumor. We could hypothesize that less common allele genotypes (CT+TT) could be related with a lower autophagy and accumulation of DNA damage, related with a higher risk to develop laryngeal squamous cell carcinoma.

Though only associated in *ATG2B* rs3759601 heterozygosity (CG), there was a statistically significant result related with a lower risk of develop laryngeal cancer. However this result is difficult to explain due to its non-significance in dominant models. Nevertheless, there was a positive association between the homozygous GG genotype in the same polymorphism and an increase risk to suffer from pharyngeal squamous cell carcinoma. In mammals, there are two ATG2 genes which are functionally redundant[21]. *ATG2B* is an essential protein in the autophagy process due to it is essential for the autophagosome and lipid droplets formation [19,22]. Mutations in *ATG2B* gene have been associated with colorectal and gastric cancer[14]. *ATG2B* rs3759601 C>G SNP in exon 25 produces a protein change p.Gln1382Glu which could result in diminished autophagy and a higher risk to suffer pharyngeal cancer in our sample.

We did not find any significant result in the intronic *ATG5* rs2245214 SNP distribution and HNSCC susceptibility. This result can be related with the position of this polymorphism in the

intronic region 6 of the *ATG5* gene and the consequence of ineffective change in the protein function.

Finally, we found an association in the distribution of CC genotypes in the dominant and recessive models of *ATG16L1* rs2241880 polymorphism and a higher risk to suffer from oral cavity squamous cell carcinoma. Autophagy-related 16-like 1 (ATG16L1) gene is a central adaptor in Atg5-Atg12-Atg16L1 complex formation and elongation of the autophagosome[23]. *ATG16L1* variant rs2241880, a nonsynonymous 898T>C polymorphism that encodes a threonine-to-alanine change (T300A), is associated with a decreased autophagy in Chron´s disease and higher inflammation[23]. In these studies CC genotype increases the secretion of TNF-α and IL-1β promoting a higher inflammation[23]. It has been also described that T300A variant enhances Atg16L1 cleavage by caspase 3, resulting in defective autophagy[24] and chronic inflammatory state which increase Crohn´s disease susceptibility[25] and colorectal cancer[26,27]. Our results show that CC genotypes are associated with an increased susceptibility to develop oral cavity squamous cell carcinoma maybe due to lower autophagy and a higher inflammation, a very important pathway implied in the etiology of this tumor[28].

In conclusion, this study provides evidence of the putative role of some polymorphisms in autophagy genes as a genetic susceptibility factor in head and neck squamous cell carcinogenesis. This is the first autophagy susceptibility study in which cases and controls are matched by their risk factors, only taking into account their genetic background. Our finding emphasize the importance of autophagy in these tumours, the same as the heterogeneity between locations include under the same term of head and neck cancer. Additional studies in larger groups should be done and would be necessary to confirm our results.

Material and methods

Study design

The data presented here is part of a multicentre study of three years of duration coordinated by the Medical Oncology Department of the University Hospital of Salamanca with the collaboration of 20 Spanish hospitals, all of them belonging to the Spanish Head & Neck Cancer Cooperative Group (TTCC).

The recruitment period extended from January 2012 to December 2014. The inclusion criteria were: adults diagnosed of HPV negative squamous cell carcinoma of larynx, pharynx or oral cavity. They were recruited in different Spanish hospitals that participate in the project after signing a written informed consent designed for this project according to local rules. The protocol of TTCC-2010-05 was initially approved by the TTCC Executive Committee, and then by the local institutional review board of University Hospital of Salamanca, according to country regulations. The research was conducted in full accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and was consistent with Good Clinical Practice guidelines and the applicable local regulatory requirements.

455 patients diagnosed of HNSCC were included in the study. Controls were hospitalized patients without personal or familial history of cancer trying to be paired with cases by age,

sex, smoking and alcoholism habit. They were recruited in different departments of the 20 hospitals. The initial sample size calculated for the control group was the same than the number of patients included in the study. However, this size was not reached due to the restricted inclusion criteria, so finally only 259 controls were included.

The information into socio-demographic and data informed by patient questionnaires were collected by auto-application, being supervised by the member of the research team with the objective of correct filled. Tumour clinic-pathological data were collected by oncologists following the TNM grading system reported by the American Joint Committee on Cancer (AJCC). All data were treated with the security measures establish in compliance with the Protection of Personal Data Organic Law 15/1999, 13th December, and safe-keeping by the University Hospital of Salamanca in its specific hospital server. Global study recruitment procedures and data collection have been previously described[29].

DNA isolation and genotyping

DNA was extracted from leukocytes of peripheral blood tube by phenol-chloroform method. Four polymorphisms in important ATG genes (ATG2B rs375901, ATG5 rs224514, ATG10 rs1864183 and ATG16L1 rs2241880) were selected according to the following criteria: previously described association with illness susceptibility, >5% minor allele frequency in Caucasian population and published evidence of functionality. Genotyping of selected polymorphisms (Table 1) were analyzed by the allelic discrimination assay by TaqMan® probes (Applied biosystems), with specific oligonucleotides to amplify the polymorphic sequences and two labelled probes with the fluorochrome VIC and FAM to detect both alleles of each polymorphism. The reaction was performed using the specific PCR Master Mix in the Step-One Plus Real-Time PCR system (Applied biosystems)[30]. To ensure the reproducibility, a 5% of random samples were re-genotyping. A total of 11 samples (5 patients and 6 controls) cannot be amplified due to low DNA quality rate and were excluded of the study.

Statistical analysis

Control group was tested for assumption of the Hardy-Weinberg equilibrium (HWE) by chi-squared test for each polymorphism (Table 1). The association between the different clinical and molecular variables was analyzed by cross tabs and the X^2 test of Pearson. The Odds ratio (OR) and 95% confidence intervals were calculated by a logistic regression analysis. It was considered the existence of statistically significant differences where the P-value was < 0.05. These analyses were performed with the statistical software SPSS v.21.0 (IBM).

Because of the lower inclusion of matched controls, the statistical analysis was realized in two different ways. Firstly, patients were stratified according to its location (larynx, pharynx and oral cavity) comparing with the global control group (Table 2). To take into account the possible confounding variables, it was made a statistical adjustment for sex, and the continuous variables of age, packs of tobacco per year (PPY) and standard drink units of alcohol per week (SDU/week).

Secondly, we used the Propensity Score method (PS), a statistical term applied to the potent matching technique to equate groups in a cohort study [31]. Through a logistic regression

analysis introducing the confounders as predictive variables, the method provides a numeric probability of each predictor group [32]. PS allows to pair the cases with the controls through the selection of a control sample with the same characteristics than patients regarding sex, tobacco and alcohol consumption. In this way both groups are matched according to: packs of tobacco consumed per year (PPY): no smokers, <20PPY and >20PPY, standard drink units of alcohol per week (SDU/week): <14 SDU/week and >14 SDU/week and sex (Table 4). As the Propensity Score method did not include the age of the individuals, in the second analysis age was introduced in the logistic regression as adjustment variable only in laryngeal carcinoma where this variable was statistically significant (p>0.05 by ANOVA test).

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Author contributions statement

The study conception and design was elaborated by JFM, RST, EDB, JCAK, JJCH & RGS, the analysis and interpretation was performed by JFM, RST, JCAK, JJCH & RGS. MPB, EPR, RM, EDB, CSC, ARD, JCD, EFR and JJCH collected and assembled the data. JFM, RST did the statistical analysis and the study supervision or coordination was done by JJCH & RGS. JFM did the drafting of the article. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper.

Competing financial interest

The authors declare no competing financial interest.

Table 1. Autophagy polymorphisms analysed in the study.

Gene	SNP ID	Base change	Protein change	Chr. location	Assay ID	HWE*
ATG2B	rs3759601	C>G	Q1383E	14:96311131	c_9690166_10	>0.05
ATG5	rs2245214	C>G	Intronic	6:106214866	c_3001905_20	>0.05
ATG10	rs1864183	C>T	T212M	5:82253397	c_11953871_20	>0.05
ATG16L1	rs2241880	T>C	T300A	2:233274722	c_9095577_20	>0.05

** Hardy-Weinberg equilibrium (HWE) calculated in the control group.*

Table 2. Descriptive case-control study.

	PATIENTS N=450		CONTROLS N=253		P-value	LARYNX N=213		P-value	PHARYNX N=165		P-value	ORAL CAVITY N=72		P-value
Characteristics	N	%	N	%		N	%		N	%		N	%	
Age (years)	61.97±9.242		52.18±12.752		**0.000**	62.96±8.987		**0.000**	61.00±9.086		**0.000**	61.29±10.232		**0.000**
Sex														
Female	52	11.6	130	51.4	**0.000**	13	6.1	**0.000**	23	13.9	**0.000**	16	22.2	**0.000**
Male	398	88.4	123	48.6		200	93.9		142	86.1		56	77.8	
Tobacco smoking														
Never	22	4.9	23	9.1		7	3.3		8	4.8		7	9.7	
<20 PPY	62	13.8	72	28.5	**0.030**	20	9.4	**0.000**	30	18.2	0.013	12	16.7	0.162
>20 PPY	352	78.2	146	57.7		180	84.5		121	73.3		51	70.8	
Missing	14	3.1	12	4.7		6	2.8		6	3.6		2	2.8	
Packs per year	57.00±36.512		31.88±28.861		**0.000**	61.17±35.498		**0.000**	54.91±36.947		**0.000**	49.55±37.366		**0.000**
Alcohol drinking														
Never	105	23.3	153	60.5		53	24.9		27	16.4		25	34.7	
<14 SDU/week	94	20.9	46	18.2	**0.000**	44	20.7	**0.000**	31	18.8	**0.000**	19	26.4	**0.000**
>14 SDU/week	238	52.9	48	19.0		114	53.5		96	58.2		28	38.9	
Missing	13	2.9	6	2.4		2	0.9		11	6.7		0	0	
SDU/week	30.07±39.349		9.02±21.213		**0.000**	27.81±37.008		**0.000**	36.27±40.710		**0.000**	23.43±41.553		**0.000**

P-values related to controls. Statistically significant results in bold.

Table 3. Comparative results in selected ATG polymorphism distribution in laryngeal, pharyngeal and oral cavity cancer related to controls.

	Genotype	Control		Larynx				Pharynx				Oral cavity			
		N	%	N	%	P-value	OR (CI 95%)	N	%	P-value	OR (CI 95%)	N	%	P-value	OR (CI 95%)
ATG2B **rs3759601**	CC	106	41.9	98	46.0	/	1.00	63	38.2	/	1.00	28	38.9	/	1.00
	CG	119	47.0	88	41.3	**0.049**	**0.607 (0.369-0.999)**	80	48.5	0.749	1.091 (0.640-1.858)	36	50.0	0.720	1.120 (0.603-2.080)
	GG	28	11.1	27	12.7	0.921	1.041 (0.472-2.296)	22	13.3	**0.016**	**2.613 (1.200-5.690)**	8	11.1	0.522	1.391 (0.506-3.821)
Recessive	CC+CG	225	88.9	186	87.3	/	1.00	143	86.7	/	1.00	64	88.9	/	1.00
	GG	28	11.1	27	12.7	0.444	1.339 (0.635-2.825)	22	13.3	**0.013**	**2.493 (1.212-5.129)**	8	11.1	0.583	1.301 (0.508-3.333)
Dominant	CC	106	41.9	98	46.0	/	1.00	63	38.2	/	1.00	28	38.9	/	1.00
	CG+GG	147	58.1	115	54.0	0.103	0.675 (0.421-1.083)	102	61.8	0.282	1.317 (0.789-2.175)	44	61.1	0.630	1.158 (0.637-2.106)
ATG5 **rs2245214**	CC	104	41.1	82	38.5	/	1.00	72	43.6	/	1.00	31	43.1	/	1.00
	CG	124	49.0	105	49.3	0.369	1.256 (0.763-2.068)	79	47.9	0.624	0.879 (0.524-1.474)	31	43.1	0.348	0.744 (0.401-1.380)
	GG	25	9.9	26	12.2	0.274	1.551 (0.707-3.401)	14	8.5	0.638	0.810 (0.337-1.946)	10	13.9	0.611	1.272 (0.503-3.216)
Recessive	CC+CG	228	90.1	187	87.8	/	1.00	151	91.5	/	1.00	62	86.1	/	1.00
	GG	25	9.9	26	12.2	0.406	1.364 (0.656-2.837)	14	8.5	0.743	0.870 (0.380-1.993)	10	13.9	0.380	1.480 (0.617-3.552)
Dominant	CC	104	41.1	82	38.5	/	1.00	72	43.6	/	1.00	31	43.1	/	1.00
	CG+GG	149	58.9	131	61.5	0.272	1.307 (0.811-2.107)	93	56.4	0.574	0.867 (0.527-1.426)	41	56.9	0.531	0.831 (0.465-1.485)
ATG10 **rs1864183**	CC	93	36.8	70	32.9	/	1.00	46	27.9	/	1.00	27	37.5	/	1.00
	CT	115	45.4	116	54.4	0.059	1.648 (0.981-2.770)	86	52.1	0.127	1.537 (0.885-2.670)	34	47.2	0.452	1.274 (0.678-2.392)
	TT	45	17.8	27	12.7	0.946	1.026 (0.493-2.133)	33	20.0	0.201	1.594 (0.780-3.260)	11	15.3	0.875	0.931 (0.384-2.257)
Recessive	CC+CT	208	82.2	186	87.3	/	1.00	132	80.0	/	1.00	61	84.7	/	1.00
	TT	45	17.8	27	12.7	0.415	0.760 (0.392-1.472)	33	20.0	0.517	1.232 (0.656-2.312)	11	15.3	0.612	0.812 (0.363-1.817)
Dominant	CC	93	36.8	70	32.9	/	1.00	46	27.9	/	1.00	27	37.5	/	1.00
	CT+TT	160	63.2	143	67.1	0.118	1.484 (0.905-2.434)	119	72.1	0.100	1.552 (0.920-2.618)	45	62.5	0.587	1.180 (0.649-2.147)
ATG16L1 **rs2241880**	TT	72	28.5	58	27.2	/	1.00	44	26.7	/	1.00	18	25.0	/	1.00
	TC	130	51.3	108	50.7	0.597	1.157 (0.674-1.988)	81	49.1	0.551	1.194 (0.667-2.137)	31	43.1	0.860	1.066 (0.524-2.168)
	CC	51	20.2	47	22.1	0.312	1.414 (0.722-2.769)	40	24.2	0.166	1.647 (0.813-3.335)	23	31.9	**0.039**	**2.304 (1.043-5.093)**
Recessive	TT+TC	202	79.8	166	77.9	/	1.00	125	75.8	/	1.00	49	68.1	/	1.00
	CC	51	20.2	47	22.1	0.389	1.288 (0.724-2.292)	40	24.2	0.205	1.469 (0.810-2.666)	23	31.9	**0.017**	**2.214 (1.150-4.263)**
Dominant	TT	72	28.5	58	27.2	/	1.00	44	26.7	/	1.00	18	25.0	/	1.00
	TC+CC	181	71.5	155	72.8	0.436	1.226 (0.735-2.046)	121	73.3	0.332	1.313 (0.758-2.276)	54	75.0	0.321	1.393 (0.724-2.682)

P value & OR adjusted by sex, age, packs per year and SDU per week. Statistically significant results in bold.

Table 4. Descriptive case-control study matched by the Propensity Score method.

	LARYNX N=126		CONTROL N=126		P-value	PHARYNX N=100		CONTROL N=100		P-value	ORAL CAVITY N=70		CONTROL N=70		P-value
Characteristics	N	%	N	%		N	%	N	%		N	%	N	%	
Age (years)	63.02±8.566		56.30±12.803		**0.000**	59.96±8.41		59.52±10.044		0.742	60.92±10.008		62.24±8.88		0.412
Sex															
Female	13	10.3	13	10.3	1.000	20	20.0	22	22.0	0.728	16	22.9	17	22.9	1.000
Male	113	89.7	113	89.7		80	80.0	78	78.0		54	77.1	54	77.1	
Tobacco smoking															
Never	7	5.5	7	5.5		7	7.0	8	8.0		7	10.0	7	10.0	
<20 PPY	20	15.9	22	17.5	0.944	22	22.0	23	23.0	0.943	12	17.1	12	17.1	1.000
>20 PPY	99	78.6	97	77.0		71	71.0	69	69.0		51	72.9	51	72.9	
Missing	0	0	0	0		0	0	0	0		0	0	0	0	
Alcohol drinking															
Never	53	42.1	51	40.5		26	26.0	27	27.0		23	32.9	23	32.9	
<14 SDU/week	28	22.2	31	24.6	0.904	30	30.0	30	30.0	0.985	19	27.1	19	27.1	1.000
>14 SDU/week	45	35.7	44	34.9		44	44.0	43	43.0		28	40.0	28	40.0	
Missing	0	0	0	0		0	0	0	0		0	0	0	0	

P-values related to controls. Statistically significant results in bold.

Table 5. Comparative results in selected ATG polymorphism distribution in risk factor-matched laryngeal cancer and controls.

	Genotype	**Larynx**		**Control**		**P-value***	**OR (CI 95%)**
		N	%	N	%		
ATG2B **rs3759601**	CC	59	46.8	46	36.5	/	1.00
	CG	52	41.3	69	54.8	**0.028**	**0.535 (0.307-0.935)**
	GG	15	11.9	11	8.7	0.904	1.058 (0.423-2.644)
Recessive	CC+CG	111	88.1	115	91.3	/	1.00
	GG	15	11.9	11	8.7	0.375	1.479 (0.624-3.506)
Dominant	CC	59	46.8	46	36.5	/	1.00
	CG+GG	67	53.2	80	63.5	0.063	0.604 (0.355-1.028)
ATG5 **rs2245214**	CC	47	37.3	49	38.9	/	1.00
	CG	63	50.0	66	52.4	0.725	1.105 (0.633-1.931)
	GG	16	12.7	11	8.7	0.269	1.662 (0.675-4.089)
Recessive	CC+CG	110	87.3	115	91.3	/	1.00
	GG	16	12.7	11	8.7	0.294	1.567 (0.677-3.627)
Dominant	CC	47	37.3	49	38.9	/	1.00
	CG+GG	79	62.7	77	61.1	0.533	1.186 (0.693-2.031)
ATG10 **rs1864183**	CC	38	30.2	50	39.7	/	1.00
	CT	70	55.5	58	46.0	**0.020**	**2.004 (1.114-3.608)**
	TT	18	14.3	18	14.3	0.312	1.531 (0.671-3.494)
Recessive	CC+CT	108	85.7	108	85.7	/	1.00
	TT	18	14.3	18	14.3	0.985	1.007 (0.481-2.110)
Dominant	CC	38	30.2	50	39.7	/	1.00
	CT+TT	88	69.8	76	60.3	**0.026**	**1.888 (1.078-3.308)**
ATG16L1 **rs2241880**	TT	40	31.7	40	31.7	/	1.00
	TC	62	49.3	66	52.4	0.930	1.027 (0.570-1.848)
	CC	24	19.0	20	15.9	0.415	1.382 (0.635-3.010)
Recessive	TT+TC	102	81.0	106	84.1	/	1.00
	CC	24	19.0	20	15.9	0.381	1.359 (0.684-2.701)
Dominant	TT	40	31.7	40	31.7	/	1.00
	TC+CC	86	68.3	86	68.3	0.723	1.106 (0.633-1.935)

**P value & OR adjusted by age. Statistically significant results in bold.*

Table 6. Comparative results in selected ATG polymorphism distribution in risk factor-matched pharyngeal cancer and controls.

	Genotype	**Pharynx**		**Control**		**P-value**	**OR (CI 95%)**
		N	%	N	%		
ATG2B **rs3759601**	CC	35	35.0	44	44.0	/	1.00
	CG	48	48.0	49	49.0	0.494	1.231 (0.678-2.235)
	GG	17	17.0	7	7.0	**0.026**	**3.053 (1.139-8.182)**
Recessive	CC+CG	83	83.0	93	93.0	/	1.00
	GG	17	17.0	7	7.0	**0.035**	**2.721 (1.075-6.887)**
Dominant	CC	35	35.0	44	44.0	/	1.00
	CG+GG	65	65.0	56	56.0	0.194	1.459 (0.825-2.580)
ATG5 **rs2245214**	CC	47	47.0	39	39.0	/	1.00
	CG	45	45.0	52	52.0	0.265	0.718 (0.401-1.286)
	GG	8	8.0	9	9.0	0.567	0.738 (0.260-2.092)
Recessive	CC+CG	92	92.0	91	91.0	/	1.00
	GG	8	8.0	9	9.0	0.800	0.879 (0.325-2.379)
Dominant	CC	47	47.0	39	39.0	/	1.00
	CG+GG	53	53.0	61	61.0	0.254	0.721 (0.411-1.265)
ATG10 **rs1864183**	CC	30	30.0	38	38.0	/	1.00
	CT	51	51.0	47	47.0	0.316	1.374 (0.738-2.559)
	TT	19	19.0	15	15.0	0.264	1.604 (0.700-3.676)
Recessive	CC+CT	81	81.0	85	85.0	/	1.00
	TT	19	19.0	15	15.0	0.452	1.329 (0.633-2.792)
Dominant	CC	30	30.0	38	38.0	/	1.00
	CT+TT	70	70.0	62	62.0	0.233	1.430 (0.794-2.575)
ATG16L1 **rs2241880**	TT	27	27.0	34	34.0	/	1.00
	TC	48	48.0	49	49.0	0.522	1.234 (0.648-2.347)
	CC	25	25.0	17	17.0	0.130	1.852 (0.835-4.108)
Recessive	TT+TC	75	75.0	83	83.0	/	1.00
	CC	25	25.0	17	17.0	0.167	1.627 (0.816-3.247)
Dominant	TT	27	27.0	34	34.0	/	1.00
	TC+CC	73	73.0	66	66.0	0.283	1.393 (0.761-2.551)

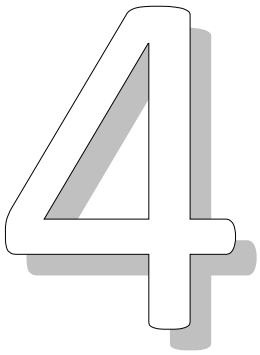
Statistically significant results in bold.

Table 7. Comparative results in selected ATG polymorphism distribution in risk factor-matched oral cavity cancer and controls.

	Genotype	**Oral cavity**		**Control**		**P-value**	**OR (CI 95%)**
		N	%	N	%		
ATG2B **rs3759601**	CC	27	38.6	27	38.6	/	1.00
	CG	36	51.4	39	55.7	0.823	0.923 (0.458-1.859)
	GG	7	10.0	4	5.7	0.413	1.750 (0.459-6.679)
Recessive	CC+CG	63	90.0	66	94.3	/	1.00
	GG	7	10.0	4	5.7	0.352	1.833 (0.512-6.568)
Dominant	CC	27	38.6	27	38.6	/	1.00
	CG+GG	43	61.4	43	61.4	1.000	1.000 (0.506-1.975)
ATG5 **rs2245214**	CC	31	44.3	25	35.7	/	1.00
	CG	31	44.3	35	50.0	0.356	0.714 (0.349-1.460)
	GG	8	11.4	10	14.3	0.421	0.645 (0.222-1.878)
Recessive	CC+CG	62	88.6	60	85.7	/	1.00
	GG	8	11.4	10	14.3	0.614	0.774 (0.286-2.094)
Dominant	CC	31	44.3	25	35.7	/	1.00
	CG+GG	39	55.7	45	64.3	0.301	0.699 (0.354-1.379)
ATG10 **rs1864183**	CC	26	37.1	26	37.1	/	1.00
	CT	33	47.2	33	47.2	1.000	1.000 (0.483-2.069)
	TT	11	15.7	11	15.7	1.000	1.000 (0.369-2.710)
Recessive	CC+CT	59	84.3	59	84.3	/	1.00
	TT	11	15.7	11	15.7	1.000	1.000 (0.402-2.485)
Dominant	CC	26	37.1	26	37.1	/	1.00
	CT+TT	44	62.9	44	62.9	1.000	1.000 (0.504-1.985)
ATG16L1 **rs2241880**	TT	18	25.7	26	37.1	/	1.00
	TC	31	44.3	33	47.2	0.441	1.357 (0.625-2.947)
	CC	21	30.0	11	15.7	**0.035**	**2.758 (1.072-7.096)**
Recessive	TT+TC	49	70.0	59	84.3	/	1.00
	CC	21	30.0	11	15.7	**0.047**	**2.299 (1.010-5.230)**
Dominant	TT	18	25.7	26	37.1	/	1.00
	TC+CC	52	74.3	44	62.9	0.147	1.707 (0.829-3.517)

Statistically significant results in bold.

Epidermal growth factor receptor (EGFR) pathway polymorphisms as predictive markers of cetuximab toxicity in locally advanced head and neck squamous cell carcinoma (HNSCC) in a Spanish population



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Artículo 4: "Epidermal growth factor receptor (EGFR) pathway polymorphisms as predictive markers of cetuximab toxicity in locally advanced head and neck squamous cell carcinoma (HNSCC) in a Spanish population"

El tratamiento del CECC tiene un abordaje multidisciplinario incluyendo cirugía, radioterapia, quimioterapia basada en platino y el uso de fármacos frente a nuevas dianas terapéuticas, como es el caso del cetuximab, anticuerpo monoclonal IgG1 contra EGFR. Este fármaco desencadena sus efectos antitumorales a través de tres mecanismos diferentes: inhibición competitiva del ligando debido a su unión al dominio III extracelular del receptor, disminución del receptor en la membrana a través de endocitosis y degradación en el lisosoma; y, por último, la inducción de la citotoxicidad celular dependiente de anticuerpo (ADCC) a través de la interacción de la fracción constante del cetuximab con el receptor gamma (FcgR) portado por las células inmunes.

Aunque el uso de cetuximab está aprobado para el tratamiento de CECC localmente avanzado, el beneficio clínico y una baja toxicidad está restringido a un subgrupo de pacientes. La relación entre la presencia de toxicidad ante el tratamiento con cetuximab y una mejor respuesta ha sido ampliamente descrita. Para definir el subgrupo de pacientes que pueden beneficiarse de este tratamiento, se planteó un estudio de asociación entre los polimorfismos en la vía de señalización de EGFR y la toxicidad en pacientes con CECC.

Se estudiaron 110 pacientes con CECC localmente avanzado. La mayoría recibió cetuximab en concomitancia con quimioterapia o radioterapia administrada en una dosis inicial de 400mg/m^2 seguido por dosis semanales de 250mg/m^2 hasta la progresión de la enfermedad o toxicidad severa. Los datos de toxicidad fueron aportados en el cuestionario de recogida de datos según los criterios del NCI-CTCAE versión 3.0. Para realizar el análisis de los polimorfismos se extrajo el DNA de un tubo de sangre periférica estudiando los siguientes SNPs con sondas Taqman®: *EGFR* rs2227983, rs28384375 y rs17336639; *KRAS* rs61764370, *FCGR2A* rs180127 y *FCGR3A* rs396991, mientras que por PCR y posterior digestión con enzimas de restricción se analizó el SNP en *CCND1* rs603965.

La toxicidad específica tras el tratamiento con cetuximab se presentó como *rash* acneiforme en un 55.5%, sequedad de piel con un 45.5% y prurito en un 20.9%. Esta toxicidad fue independiente de los ciclos de cetuximab recibidos ($p>0.05$). Los pacientes de este estudio solo presentaban el alelo común en los SNPs rs28384375 y rs17336639 de *EGFR* y fueron descartados del estudio.

El análisis estadístico demostró una asociación estadísticamente significativa entre el alelo G del SNP rs61764370 de *KRAS* y una menor aparición de sequedad de piel o toxicidad global (considerando la presencia de cualquier tipo de toxicidad). Este hecho puede ser debido a la presencia de este SNP en una zona de unión de miRNA de la familia let-7 en el extremo 3´-UTR del gen *KRAS*, asociado a una menor inhibición y mayor expresión del gen. La presencia de menor toxicidad puede relacionarse con peor respuesta al tratamiento y progresión tumoral, por un aumento de la expresión del oncogén *KRAS*.

De modo similar, el polimorfismo rs2227983 de *EGFR* demostró una asociación entre el alelo A y un menor riesgo a desarrollar prurito. Una posible explicación podría ser que el SNP produce un cambio de lisina a arginina en la región extracelular, produciendo un cambio estructural en el receptor, disminuyendo la interacción del cetuximab, asociándose a una menor efectividad de la diana terapéutica y a menor toxicidad.

Aunque no significativo (p=0.051), se observó una tendencia entre el genotipo TT del SNP *FCGR2A* y un menor riesgo a desarrollar sequedad de piel. Esto podría ser debido a que el polimorfismo causa un cambio de histidina a arginina, teniendo el alelo T, codificante para histidina, una menor afinidad por el cetuximab, causando menor toxicidad debido a una menor citotoxicidad antitumoral mediada por anticuerpos (ADCC). El resto de polimorfismos no mostraron asociación con ningún tipo de toxicidad producida por el cetuximab.

Este estudio aporta una evidencia preliminar de biomarcadores en genes implicados en la vía de señalización de EGFR, prediciendo la toxicidad y respuesta ante el tratamiento de CECC con cetuximab, lo que permitiría identificar aquellos pacientes que se beneficiarían de este tratamiento o deberían ser alternados a otras opciones terapéuticas.

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Epidermal growth factor receptor (EGFR) pathway polymorphisms as predictive markers of cetuximab toxicity in locally advanced head and neck squamous cell carcinoma (HNSCC) in a Spanish population

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ABSTRACT

Objectives: To examine the relationship between polymorphisms of the epidermal growth factor receptor (EGFR) pathway and toxicity in head and neck squamous cell carcinoma (HNSCC) patients treated with cetuximab.

Material and methods: Multicenter, retrospective, observational pilot study which included 110 patients with histologically-confirmed human papillomavirus (HPV) negative HNSCC in locally advanced stages (III-IVA-B) and who were treated with chemotherapy and radiotherapy plus cetuximab between 2003 and 2013. Genetic analyses for single nucleotide polymorphisms (SNP) in genes EGFR, CCDN1, FCGR2A, FCGR3A and KRAS-LCS6 were performed though available allelic discrimination assay and/or polymerase chain reaction-restriction fragment length polymorphism methods.

Results: Acneiform rash was observed in 55.5% of patients, dry skin in 45.5% and pruritus in 20.9%. A significant association with dry skin and global cetuximab-related toxicity was observed for the KRAS-LCS6 (rs61764370) variant ($p < 0.05$); carriers of the G allele (genotypes TG + GG) in the dominant model were observed to have a decreased susceptibility of developing dry skin (OR = 0.287 [95%CI = 0.119–0.695]). Carriers of the A (GA + AA) allele for EGFR (rs2227983) showed a decreased risk of suffering from pruritus (OR = 0.345 [0.124–0.958]). Similarly, KRAS (rs1801274) was related with lower global cetuximab-related toxicity (OR = 0.266 [0.114–0.622]).

Conclusion: This pilot study provides preliminary evidence supporting genetic variation of EGFR (rs2227983), KRAS (rs61764370) and FCGR2A (rs180127) as useful biomarkers for predicting reduced skin toxicity in HNSCC patients treated with a cetuximab-based therapy. Alternative therapeutic options should be explored for these patients.

Introduction

Head and Neck Carcinoma (HNC) includes a large number of tumors located in different anatomical regions of the upper aerodigestive tract. More than 90% of HNC tumors have a squamous cell histology [1] and are classified as head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide [2]. Tobacco use and alcohol consumption are the most relevant

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etiological factors showing additive effect [3,4]. Nevertheless, in the last decades viral infection by the Epstein-Barr virus (EBV) or human papillomavirus (HPV) have been suggested as a cause of nasopharyngeal and oropharyngeal cancer, respectively. These EBV or HPV positive tumors appear to be clinically and molecularly different from negative virus carcinomas [5]. In addition, genetic variation in the germinal cell line has been found to modify the risk of disease and patient survival [3,6]. Despite their common squamous origin, the prognosis of these tumors primarily depends on their size and the presence of cervical lymph node and/or distant metastasis. Treatment options for HNSCC includes surgery, radiotherapy, platinum-based chemotherapy and targeted therapeutic agents [7]. However, these patients usually achieve an advanced-staged diagnosis that compromises first line response rates [8].

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that plays a fundamental role in signal transduction pathways involved in DNA repair, tumor cell survival (PI3K-PTEN-AKT pathway), cell proliferation (RAS-RAF-MAPK pathway) and metastasis [9]. More than 95% of HNSCC patients have EGFR overexpression and it has been associated with a decreased response to therapy, reduced disease-free and overall survival (OS) [5]. Due to its prevalence and crucial role in pathogenesis, targeting EGFR has become a rational approach for HNSCC treatment.

Cetuximab, a chimeric mouse/human IgG1-type monoclonal antibody (MAb), is an anti-EGFR therapy approved for the treatment of locally advanced HNSCC [10,11]. Cetuximab in combination with radiotherapy or platinum-containing chemotherapy regimens has already shown significant improvement of treatment outcomes in metastatic and relapsed disease [7,10–15].

Cetuximab can cause antitumor effects through three different mechanisms: firstly, it specifically binds to the extracellular domain III of the EGFR as a competitive inhibitor of the natural EGF ligands and downstream pathway activation [16]. Secondly, cetuximab decreases the number of EGFRs in the tumor cell membrane though EGFR-cetuximab complex endocytosis and destruction by lysosomes. Thirdly, cetuximab can induce antibody-dependent cell-mediated cytotoxicity (ADCC) [17] through the interaction of the Fc region of the monoclonal antibody with the Fc gamma receptor (FcgR) carried by macrophages and natural killer cells [18,19].

Clearly, clinical benefit and low toxicity with EGFR-targeting antibodies seems to be restricted to a particular subgroup of HNSCC patients [20]. Although critically required for managing the high cost of this type of therapy and their anticipated integration in other clinical regimens, no validated predictive factors are currently available to improve treatment decision making [20]. Thus, it appears necessary to better define the subpopulation of patients who truly benefits from cetuximab treatment and its toxicity. Single nucleotide polymorphisms (SNP) may affect pharmacodynamics of anti-EGFR therapies introducing inter-patient variability at the level of the EGFR target itself, the downstream cascade, as well as at the ADCC. It has been reported that two SNPs located in the coding region of the FcgR have been associated with differences in the response and toxicity to cetuximab: a histidine (H)/arginine(R) polymorphism at position 131 of FCGR2A (rs1801274) and a valine(V)/phenylalanine(F) polymorphism at position 158 in FCGR3A (rs396991) [18,19,21,22]. At least three functional EGFR variants have been associated with EGFR regulation: rs2227983 [23], rs28384375 and rs17336639 [24,25] coding for amino acids located at the extracellular domain.

Moreover, some downstream effectors of EGFR signaling such as cyclin-D1 gene (CCDN1) may also plays a role in modulating cetuximab activity, given that CCND1 A870G (rs603965) polymorphism is positively correlated with HNSCC patient survival [26]. Finally, microRNA (miRNAs) – small non-coding RNAs able to suppress translation through their binding to the gene 3′-untanslated region (UTR) or inducing mRNA degradation – [27], can regulate KRAS activity, i.e. let-7 miRNA. A polymorphism in LCS6 (rs617764370) modifies let-7 binding affinity and it was associated with increased KRAS expression in an *in vitro* model, reducing survival in oral cancer and improving patient response to cetuximab [28].

Several studies have also found a relationship between skin toxicity, the most relevant cetuximab-related secondary effect [10,29], and a better response [30–32].

Therefore, the main objective of this study was to examine the possible associations between polymorphisms at genes coding for EGFR, CCDN1, FCGR2A, FCGR3A and KRAS-LCS6 and toxicity in HNSCC patients treated with cetuximab.

Materials and methods

Patients and treatment

A total of 110 patients with histologically-confirmed HPV-negative HNSCC were enrolled in a multicenter retrospective observational pilot study coordinated by the Medical Oncology Service of the University Hospital of Salamanca, Spain. All patients included in the study were diagnosed with locally advanced stages (III-IVA-B) and treated with chemotherapy and radiotherapy plus cetuximab between 2003 and 2013. The study was carried out after ethics committee approval and collection of informed consent from each patient. Patient tumor characteristics (location and stage) and data related to treatment (radiotherapy, chemotherapy and EGFR targeted therapy) and specific toxicity were compiled in a case report form (CRF) questionnaire by a medical oncologist.

The inclusion criterion was patients with a confirmed oral cavity, larynx, hypopharynx or oropharynx HNSCC diagnosis and who were treated with cetuximab. Cetuximab alone (n = 2) or with chemotherapy (n = 6), radiotherapy (n = 21), or radiochemotherapy (n = 81) was administered at an initial dose of 400 mg/m^2 followed by subsequent weekly dose of 250 mg/m^2 until disease progression or severe toxicity. Toxicity was recorded according to National Cancer Institute Common Toxicity Criteria (NCI-CTCAE), version 3.0. Exclusion criteria were uncertain or debatable diagnosis, benign tumors, and HPV-positive HNSCC confirmed by either PCR or immunohistochemistry.

DNA isolation and polymorphisms genotyping

Samples were obtained by venipuncture of a peripheral vein. DNA was extracted from leukocytes by phenol-chloroform extraction. Genetic analysis was performed using TaqMan® Allelic Discrimination Assay (Applied Biosystems) for SNPs for which TaqMan® probes were designed (Table 1). In these cases, 40 ng/μl of each sample were added to 6.25 μl of Taqman® Universal PCR Master Mix and 12.5 μl of reaction was combined with specific forward and reverse primers, and allele-specific VIC (allele 1) and FAM (allele 2) labeled probes. The assay was performed in a 96 well plate and the detection was measured in the Applied Biosystems Step One Plus instrument where the thermal cycling and detection was carried out. Negative and positive controls were always added [33].

The CCDND1 A870G polymorphism (rs603965) was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The forward primer was: 5′-GTGAAGTT CATTTCCAATCCGC-3′ and the reverse: 5′-GGGACATCACCCTCACT TAC-3′. Digestion was made by the restriction enzyme *ScrFI*. The PCR products were run on 3% Syber-safe stained agarose gel and visualized under UV light [34].

Table 1
Polymorphisms analyzed by TaqMan® probes in HNSCC patients.

SNP	RefSNP	Location	Context sequence [VIC/FAM]
EGFR R521K	rs2227983	Chr.7: 55161562	GAGGGCTGCTGGGGCCCGGAGCCCA**[A/G]**GGACTGCGTCTCTTGCCGGAATGTC
EGFR V592A	rs28384375	Chr.7: 55233025	CACTACATTGACGGCCCCCACTGCG**[C/T]**CAAGACCTGCCCGGCAGGAGTCATG
EGFR P266R	rs17336639	Chr.7: 55154060	GCCACGTGCAAGGACACCTGCCCCC**[C/G]**ACTCATGCTCTACAACCCCACCACG
KRAS-LCS6	rs61764370	Chr.12:25207290	F:5′-GCCAGGCTGGTCTCGAA-3′ R:5′-CTGAATAAATGAGTTCTGCAAAACAGGTT-3′ CTCAAGTGAT**[T/G]**CACCAC
FCGR2A H131R	rs1801274	Chr.1: 161509955	AATGGAAAATCCCAGAAATTCTCCC**[A/G]**TTTGGATCCCACCTTCTCCATCCCA
FCGR3A V18F	rs396991	Chr.1: 161544752	TCTGAAGACACATTTTTACTCCCAA**[C/A]**AAGCCCCCTGCAGAAGTAGGAGCCG

Statistical analysis

Statistical analysis compared categorical parameters and polymorphism status by the Chi-square test. P-values were considered statistically significant when $p < 0.05$. Significant variables were included in the logistic regression analysis and size effects were indicated by odds ratio (OR) with their 95% confidence interval (95% CI). Dose and toxicity influence was analyzed by a Mann-Whitney *U* test due to the non-parametric distribution of the variables. Secondary endpoint was cetuximab-related toxicity. All these tests were conducted using SPSS software 21.0 version for Windows (SPSS Inc., Chicago).

Toxicity was graded between 0 = absence and grade 4 = severe, grouped as low (1–2) or high (3–4) grade, and classified as "present" or "absent". The term global toxicity was applied if the patients presented some grade of toxicity to the treatment.

Results

A total of 110 locally advanced HNSCC patients were analyzed. Most of the patients included in the study were men with a median age of diagnosis of 59.63 years old. Regarding tumor characteristics, the most common were laryngeal neoplasias (45.5%), followed by pharyngeal (41.8%) and oral cavity (12.7%); the majority of tumors were stage IV (70.0%), followed by stage III (30.0%) (Table 2). As for the therapy received in combination with cetuximab, 6 (5.5%) patients were treated with chemotherapy, 21 (19.1%) with radiotherapy and 81 (73.6%) with radiochemotherapy (Table 3). Statistical association was not found in the comparison between patients undergoing radiochemotherapy and cetuximab versus the remaining therapies ($p > .05$), thus indicating that the toxicity between the two groups was likely produced by the cetuximab treatment itself (Table 3).

Regarding the specific toxicity of cetuximab, 55.5% of HNSCC patients presented acneiform rash, 46.4% of them in low grade; while dry skin was present in 45.5% of cases and pruritus in 20.9% (Table 4). Despite a variation in the number of cetuximab cycles, mean 10.54 ± 15.04, no statistically-significant relationship was observed between accumulated doses and toxicity in the Mann-Whitney *U* test (dry skin $p = 0.116$, pruritus $p = 0.787$ and rash $p = 0.284$).

Table 2
Characteristics of HNSCC patients.

		N	%
Sex	Male	100	90.9
	Female	10	9.1
Location	Larynx	50	45.5
	Oropharynx	30	27.3
	Hypopharynx	16	14.5
	Oral cavity	14	12.7
Stage	III	33	30.0
	IVA	65	59.1
	IVB	12	10.9

The genotype distribution of EGFR rs2227983, rs28384375 and rs17336639, FCGR2A rs1801274, FCGR3A rs396991, KRAS-LCS6 rs61764370, and CCDN1 rs603965 polymorphisms are shown in Table 5. EGFR polymorphisms rs28384375 and rs17336639 had only the major allele variant in our sample (Table 5), though in the European population rs28384375 has been described as having a distribution of 84% CC, 14.2% CT and 1.2% TT, and for rs17336639: 98.9% CC and 1.1% CG. Thus they were not included in subsequent analyses. The remaining SNPs were analyzed according to the most common toxicity produced by monoclonal antibodies treatment: dry skin, pruritus and acneiform rash.

Statistical analyses using the Chi-square test showed significant association between dry skin and the KRAS-LCS6 (rs61764370) variant ($p < 0.05$). Moreover, global cetuximab-related toxicity was also associated with this polymorphism, and may be due to the association with dry skin toxicity (Table 6). These results showed that being a carrier of the G allele (genotypes TG + GG) of the KRAS-LCS6 rs61764370 polymorphism in the dominant model decreases the susceptibility to develop dry skin after cetuximab treatment ($p = 0.006$, OR = 0.287 [95% CI = 0.119–0.695]) (Table 7). Although not significant, a tendency in the recessive model of FCGR2A rs1801274 where the TT genotype was close to being associated with a decreased risk of dry skin, $p = 0.051$ OR = 0.380 (0.144–1.003), was observed (Table 7). Secondly, the EGFR rs2227983 polymorphism showed an association with pruritus toxicity. Carriers of GA + AA genotypes were found to have a decreased risk of suffering from pruritus: $p = 0.041$, OR = 0.345 (0.124–0.958) (Table 7). Regarding patients with global cetuximab-related toxicity, the KRAS (rs61764370) variant was less susceptible to global toxicity related to MAb treatment ($p = 0.002$, OR = 0.266 [95% CI = 0.114–0.622]) (Table 7).

Discussion

Cetuximab combined with radiotherapy or chemotherapy improves locoregional control and survival in HNC patients, but only a subset of all patients are able to benefit from anti-EGFR monoclonal antibodies [35]. Thus, the detection of predictive biomarkers and of beneficial patient profiles is crucial. Several studies have correlated clinical outcome and toxicity to IgG1 cetuximab treatment with polymorphisms in the EGFR pathway with conflicting results [18,19,21,22,26,28]. In this proof of concept study we evaluated the possibility of an association between cetuximab toxicity and polymorphism distribution in the EGFR pathway, looking for predictive biomarkers of toxicity.

Skin toxicity is a frequent side effect of EGFR targeting agents and it correlates with a better treatment efficacy [32]. It causes some cutaneous changes such as acneiform rash, dry skin and itching. Although these toxicities can negatively impact on the patient quality of life, the identification of new biomarkers may contribute

Table 3
Treatment and cetuximab-related toxicity.*

			Acneiform Rash		Dry skin		Pruritus		Global toxicity	
	N	%	N	%	N	%	N	%	N	%
Cetuximab alone	2	1.8	19	65.5	15	51.7	4	13.8	22	75.9
Cetuximab + Radiotherapy	21	19.1								
Cetuximab + Chemotherapy	6	5.5								
Cetuximab + Radiochemotherapy	81	73.6	42	51.9	35	43.2	19	23.5	51	63.0

* The data represents only patients who developed toxicity. A Chi-Square test was performed between patients undergoing radiochemotherapy plus cetuximab versus the remaining therapies and there were not statistical differences between both groups ($p > .05$) (data not shown).

Table 4
Toxicity caused by cetuximab therapy, clustered by low (1–2) and high (3–4) grade.

	Acneiform rash		Dry skin		Pruritus	
	N	***%***	***N***	***%***	***N***	***%***
Absence	49	44.5	60	54.5	87	79.1
G1-2	51	46.4	41	37.3	22	20.0
G3-4	10	9.1	9	8.2	1	0.9

Table 5
Distribution of polymorphism genotypes in this sample.

SNP	Genotype frequency		
EGFR rs2227983	GG	GA	AA
	60 (54.5%)	43 (39.1%)	7 (6.4%)
EGFR rs28384375	CC	CT	TT
	110 (100%)	0 (0%)	0 (0%)
EGFR rs17336639	CC	CG	GG
	110 (100%)	0 (0%)	0 (0%)
FCGR2A rs1801274	CC	CT	TT
	17 (15.5%)	68 (61.8%)	25 (22.7%)
FCGR3A rs396991	TT	TG	GG
	39 (35.5%)	55 (50.0%)	16 (14.5%)
KRAS-LCS6 rs61764370	TT	TG	GG
	75 (68.2%)	31 (28.2%)	4 (3.6%)
CCDN1 rs603965	AA	AG	GG
	29 (26.4%)	57 (51.8%)	24 (21.8%)

to being able to predict the patient who will develop toxicity and thus have a better response to treatment. EGFR is normally found in keratinocytes of the epidermis, follicular epithelium and sweat glands. This receptor has an important function in skin homeostasis and its inhibition drives to an abnormal proliferation and differentiation of the epithelium [36].

In this study no relationship between genotype distribution of EGFR rs28384375, rs17336639, FCGR2A rs1801274, FCGR3A rs39661 and CCDN1 rs603965 gene polymorphisms and cetuximab toxicity was observed for this patient population. However, an association between EGFR rs2227983 and pruritus development after cetuximab treatment was observed. The EGFR SNP rs2227983 G > A in exon 13 produces a change of arginine to lysine in the position 521 (R521K). Previous reports have noted that carriers of the A allele (AA or GA) were associated with a lower incidence of skin rash compared with the GG genotype in advanced HNC [32]. In this study AA + GA was associated with a lower risk of developing pruritus ($p = 0.041$; OR = 0.345 [0.124–0.958]). Although this relationship remains unclear, this SNP is located in the extracellular region, where the monoclonal antibody and the EGF ligand interact, so structural changes at codon 521 could provoke a modification of EGF interaction with the receptor. In conclusion, AA genotype could be related with decreased cetuximab binding, low effectiveness of the monoclonal antibody and less toxicity, also clearly related with a lower response [32].

In this study, KRAS rs617764370 SNP was observed to be associated with lower dry skin and global cetuximab-related toxicity. MicroRNA SNPs are arising as relevant molecular markers in personalized medicine. The KRAS-LCS6 variant has a functional impact on let-7 miRNA joining to 3′-UTR of KRAS gene [28], causing less inhibition and an increased KRAS expression [28]. KRAS, a downstream EGFR effector, is involved in cell proliferation and maintain skin homeostasis [36]. KRAS rs61764370 has been also associated with reduced OS in oral cancer [28]. Our results show a lower risk of developing skin or global toxicity in variant carriers probably due to a higher KRAS activity. If early skin toxicity predicts better outcome and response after cetuximab treatment [30,32], these results indicate that lower toxicity is related with worse response and tumor progression after cetuximab treatment, associated to higher KRAS expression. Moreover, EGFR inhibition has been previously associated with higher grade of skin toxicity, due the lower activity of downstream signal, inducing inflammatory response [36]. As KRAS is an important effector on the pathway that maintains skin homeostasis, the increase of KRAS expression due to variant rs61764370 could activate important transcription factors to keep skin homeostasis and reducing skin toxicity [36].

Table 6
P-values of different polymorphism selected comparing grade of toxicity (as shown in Table 3) and the presence or absence of the event.

		Rash acneiforme		Dry skin		Pruritus		Global toxicity
Polymorphism	Gene	Grade	Yes/no	Grade	Yes/no	Grade	Yes/no	Yes/no
rs2227983	EGFR	0.808	0.863	0.917	1.000	0.172	0.079	0.853
rs1801274	FCGR2A	0.474	0.410	0.101	0.051	0.620	0.446	0.138
rs396991	FCGR3A	0.285	0.274	0.497	0.185	0.646	0.694	0.292
rs61764370	KRAS	0.389	0.135	**0.039**	**0.013**	0.657	0.411	**0.003**
rs603965	CCDN1	0.437	0.512	0.974	0.819	0.357	0.147	0.688

Statistically significant results in bold.

Table 7
Distribution of genotypes associated to skin/global toxicity.

SNP	Genotype	Patients with toxicity	Patients without toxicity	p-Value	OR (95% IC)
KRAS rs61764370 in association with dry skin	TT	41 (82.0%)	34 (56.7%)	/	1.00
	TG	8 (16.0%)	23 (38.3%)	**0.008**	**0.288 (0.114–0.727)**
	GG	1 (2.0%)	3 (5.0%)	0.275	0.276 (0.027–2.780)
	TT + TG	49 (98.0%)	57 (95.0%)	/	1.00
	GG	1 (2.0%)	3 (5.0%)	0.419	0.388 (0.039–3.849)
	TT	41 (82.0%)	34 (56.7%)	/	1.00
	TG + GG	9 (18.0%)	26 (43.3%)	**0.006**	**0.287 (0.119–0.695)**
FCGR2A rs1801274 in association with dry skin	CC	6 (12.0%)	11 (18.3%)	/	1.00
	CT	37 (74.0%)	31 (51.7%)	0.164	2.188 (0.726–6.595)
	TT	7 (14.0%)	18 (30.0%)	0.616	0.713 (0.190–2.678)
	CC + CT	43 (86.0%)	42 (70.0%)	/	1.00
	TT	7 (14.0%)	18 (30.0%)	**0.051**	**0.380 (0.144–1.003)**
	CC	6 (12.0%)	11 (18.3%)	/	1.00
	CT + TT	44 (88.0%)	49 (81.7%)	0.363	1.646 (0.562–4.823)
EGFR rs2227983 in association with pruritus toxicity	GG	17 (73.9%)	43 (49.4%)	/	1.00
	GA	6 (26.1%)	37 (42.6%)	0.090	0.410 (0.147–1.148)
	AA	0 (0.0%)	7 (8.0%)	0.832	0.999 (0.000-)
	GG + GA	23 (100.0%)	80 (92.0%)	/	1.00
	AA	0 (0.0%)	7 (8.0%)	0.999	0.000 (0.000-)
	GG	17 (73.9%)	43 (49.4%)	/	1.00
	GA + AA	6 (26.1%)	44 (50.6%)	**0.041**	**0.345 (0.124–0.958)**
KRAS rs61764370 in association with global toxicity	TT	60 (77.9%)	24 (50.0%)	/	1.00
	TG	16 (20.8%)	21 (43.8%)	**0.007**	**0.296 (0.123–0.715)**
	GG	1 (1.3%)	3 (6.2%)	0.058	0.105 (0.010–1.076)
	TT + TG	76 (98.7%)	45 (93.8%)	/	1.00
	GG	1 (1.3%)	3 (6.2%)	0.115	0.115 (0.016–1.569)
	TT	60 (77.9%)	24 (50.0%)	/	1.00
	TG + GG	17 (22.1%)	24 (50.0%)	**0.002**	0.266 (0.114–0.622)

Statistically significant results in bold.

ADCC is one of the secondary pathways through cetuximab exerts its antitumor effect. Recently, two FCGR SNPs have been identified that affect the binding strength to IgG1, varying ADCC function and affecting clinical tumor response [22]. In this study no association was observed between FCGR3A rs39661 and cetuximab toxicity. However, rs180127 (FCGR2A H131R) was close to being associated with skin toxicity (p = 0.051). These results show that genotypes with the allele variant T (TT), which encodes for histidine, could emerge as a possible predictor of reduced cetuximab skin toxicity (OR = 0.380 [0.144–1.003]). A putative explanation could be that FCGR2A expressing macrophages would play an important role in restoring tumor immune surveillance as predicted in preclinical models [21]. It is known that FCGR2A-131 H/H genotype has higher affinity to human IgG2 than131R allele [19] and it is associated with longer progression-free survival in cetuximab monotherapy. Cetuximab is a IgG1 antibody, and 131H has been related with low affinity binding to murine IgG1 [22]. Thus, in those situations IgG1 binds more strongly to FCGR2A 131R, and ADCC antitumor response can be less effective. As a direct relationship between skin toxicity and better MAb response has been documented [30–32], these results suggest that lower affinity to IgG1 recognition in patients with T allele could result in less toxicity to MAb treatment probably due to lowest antitumor cytotoxicity.

Lastly, cyclin-D1 gene (CCDN1), a downstream effector of EGFR is also involved in cetuximab activity. No association was observed between the CCND1 870A > G (rs603965) polymorphism and EGFR MoAb toxicity, an association that has been documented elsewhere with survival in patients with colorectal cancer treated with cetuximab [37].

Conclusions

This pilot study provides preliminary evidence supporting EGFR rs2227983, KRAS rs61764370 and FCGR2A rs180127 as useful biomarkers for predicting reduced skin toxicity in HNSCC patients receiving cetuximab-based therapy. This could indicate that patients with these genetic variants could have less toxicity and a poor prognosis, being better scheduled in another therapeutic alternative. Although these polymorphisms are checked in HNSCC cetuximab-related toxicity in this study for the first time, they should be interpreted carefully. The statistical power of this study is limited due to the moderate number of analyzed patients. Studies in larger groups should be performed and would be necessary to confirm these results and validate our findings.

Conflict of interest statement

All authors declare no conflict of interest in relation to this manuscript.

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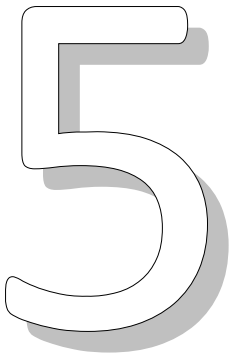
Mutational burden and prognostic factors in a cohort of homogenously treated Spanish HNSCC patients



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Artículo 5: "Mutational burden and prognostic factors in a cohort of homogenously treated Spanish HNSCC patients"

La secuenciación de nueva generación ha permitido definir el conjunto de alteraciones genéticas características de muchos tipos de tumores, transformando tanto su diagnóstico como las aproximaciones terapéuticas. Gracias a diversos estudios, destacando el *Cancer Genome Atlas,* se describió el espectro de los genes frecuentemente mutados en CECC. Sin embargo, la relevancia clínica de estos datos es desconocida debido a la falta de homogeneidad en el tratamiento o la naturaleza heterogénea de los estudios.

Para contribuir a la comprensión de cómo las mutaciones somáticas pueden influir en el tratamiento del CECC, se realizó un estudio mutacional en 26 de los genes más frecuentemente alterados en cáncer, correlacionándolo con el perfil de HPV y la respuesta y supervivencia al tratamiento.

Se seleccionaron 150 bloques tumorales en parafina de pacientes pretratados, pertenecientes al ensayo clínico TTCC-2007-01. En este estudio se incluyeron pacientes con CECC localmente avanzados irresecables que recibían el tratamiento actual de elección de quimioterapia de inducción (TPF) seguido, tras respuesta, de una posterior randomización a radioterapia convencional con cisplatino o cetuximab. Se realizaron cortes seriados de las parafinas para medir el porcentaje tumoral así como el estado de HPV por inmunohistoquímica de $p16^{INK4a}$. Tras la desparafinización y extracción del DNA se realizó el análisis mutacional mediante el panel TruSight® Tumor 26 (Illumina).

Los resultados de nuestra serie confirmaron los datos anteriormente descritos que muestran que *TP53* es el gen más mutado, con un mayor porcentaje en tumores HPV-; seguido de *PIK3CA*, más mutado en HPV+. El tercer gen más mutado en tumores HPV+ fue *PTEN*, mientras que en HPV- fue *FBXW7*. Genes mutados en menor porcentaje como *MET* y *APC* corroboraron los porcentajes previamente reportados por otros autores. El análisis comparativo entre mutaciones y características clínicas de los pacientes y respuesta al tratamiento no mostró ningún resultado estadísticamente significativo.

El análisis de supervivencia global (SG) no manifestó diferencias entre ambos brazos de tratamiento, mientras que en supervivencia libre de progresión (SLP) el brazo de radioterapia convencional con platino obtuvo mejores resultados. La relación entre supervivencia y el estatus de HPV, mostró que los pacientes con tumores HPV+ presentaron mayor SG y SLP ($p<0.05$).

En relación con el estado mutacional, los pacientes con tumores sin mutación presentaron mayor SG que aquellos mutados, sin diferencias en SLP. De modo similar, existió una correlación entre el número de mutaciones, donde aquellos pacientes con tumores que portaron más de una mutación frente a los no mutados presentaron una SG estadísticamente significativa. Estos resultados podrían asociarse con la agresividad tumoral, relacionado con un mayor número de mutaciones somáticas. Aunque se ha descrito una menor supervivencia en aquellos pacientes con mutación en *TP53* mutados frente a los germinales, nuestros resultados no exhibieron esta relación, tal vez debido al bajo número de pacientes sin mutación en *TP53*.

En conclusión, nuestros datos corroboran y expanden los datos publicados sobre la carga mutacional del CECC. Así mismo, definimos el perfil mutacional de CECC HPV+ en la población española, observando mutaciones frecuentes en *PIK3CA* y *PTEN*, definiéndolos como posibles dianas terapéuticas. Además, la presencia de mutaciones en el tumor puede ser un biomarcador importante de supervivencia global en CECC, especificando un posible grupo que podría beneficiarse de un tratamiento más personalizado.

Title page

Mutational burden and prognostic factors in a cohort of homogeneously treated Spanish HNSCC patients.

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Abstract

Objectives: To examine the mutational spectrum in homogenously treated locally advanced head and neck squamous cell carcinoma (HNSCC) and evaluate its influence in response to treatment and survival.

Material and methods: Next generation sequencing (NGS) in the 26 most frequent mutated genes in cancer were studied in 150 locally advanced HNSCC FFPE blocks from a multicenter clinical trial. Human papillomavirus (HPV) status was measured by $p16^{INK4a}$ immunohistochemistry. Clinicopathological features and response to treatment were measured and compared with the sequencing results.

Results: TP53 was the most mutated gene in locally advanced HNSCC. We did not find any association between mutations and response to treatment ($p>0.05$). We showed the differences between HPV positive and negative tumors in which HPV- were more mutated. Mutational and HPV status were correlated with survival, being mutated or HPV negative tumors associated with lower overall survival ($p<0.05$).

Conclusion: This study confirmed and expand previous published mutational burden in HNSCC. Survival analysis showed that non mutated HNSCC tumor define better prognosis, being an important biomarker in HNSCC.

Keywords: Head and Neck Neoplasms, DNA Sequence, Mutation, Survival, Response, Human papillomavirus, Biomarkers, Tumor

Highlights

- TP53 is the most mutated gene in HNSCC, with a higher incidence in HPV negative tumors.

- Although mutations were not correlated with response to treatment, they were associated with lower survival ($p<0.05$).

- HPV positive tumors are associated with better survival than negative ones, confirming the better prognostic.

- Mutational status is a biomarker of survival in HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common neoplasia in the developed world[1,2]. It groups a heterogeneous mixture of tumor locations in the upper aerodigestive tract. Tobacco smoking and alcohol consumption are the most classical risk factors[3] although viral etiology is an established factor too[4]. However, those risk factors induce cancer through different pathways and represent distinct clinical and epidemiological entities[5].

Most of HNSCC are diagnosed in locally advanced disease (stage III or IV). Treatment of these stages includes surgery, radiotherapy (RT), biotherapy (BT) and chemotherapy (CT). In 2009, data from a large meta-analysis established chemoradiotherapy (CTRT) as the standard of care for locally advanced HNSCC. The addition of cetuximab, an IgG1 chimeric monoclonal antibody against EGFR, concomitant with RT resulted in longer progression-free survival (PFS) and overall survival (OS) compared to RT alone, although a direct comparison with CTRT has not yet been published[6].

The role of induction chemotherapy has remained a subject of controversy[7]. The combination of cisplatin-docetaxel and 5-fluorouracil (TPF) has emerged as the most active regimen in locally advanced disease, showing better results than cisplatin-5-fluorouracil (PF)[8–10], although it has not show a convincing survival benefit in induction regimens compared with historical data of treatment with chemo/bioradiotherapy alone.

Induction chemotherapy to improve larynx preservation and survival in larynx and hypopharynx cancer may be an alternative to CTRT. The use of cetuximab added to radiation therapy (RTBT) in patients with laryngeal cancer stage III and IVA that respond to TPF could improve functional larynx preservation[11], although randomized phase III trials did not find that induction chemotherapy provide benefit in time-to-treatment failure or OS[12–14]. On the other hand, a randomized phase II-III study done by Paccagnella and colleagues[15] suggested that adding TPF induction chemotherapy to CTRT results in higher rate of radiological complete response compared with concurrent CTRT alone, improving PFS and OS.

Analysis from *Cancer Genome Atlas* described the molecular landscape of HPV-positive and HPV-negative HNSCC, improving specificity at diagnosis and therapeutic approaches[16]. Massively parallel sequencing, known as next-generation sequencing (NGS), has helped to identified a burden of genetic alterations, characterizing many cancer types[17], including head and neck squamous cell carcinomas[18]. Since the first description of recurrently mutated genes in HNSCC[19], additional studies have included further genes, being the most frequent: *TP53, NOTCH1, PIK3CA, CDKN2A, CCDN1, HRAS, FAT1, FBXW7* and *FGFR3*[20,21]. For this reason targeted sequencing has became an easier and cheaper tool to study those mutated genes previously reported in HNSCC[21]. However, the clinical relevance of data obtained from NGS is unknown due to the lack of homogeneity in the treatment.

To contribute to the understanding on how somatic mutations influence the outcome of HNSCC, we have studied a 26 genes panel by next-generation sequencing in a homogenously treated locally advanced HNSCC Spanish cohort. In this study we analyzed mutations from

formalin-fixed paraffin-embedded (FFPE) HNSCC tumors evaluating the mutational burden according to HPV profile as well as response to treatment and survival.

Materials and Methods

Patients

150 formalin-fixed paraffin-embedded (FFPE) blocks from pretreated HNSCC patients were included in this study. All of them belong to the clinical trial TTCC-2007-01: "Open Label Randomized, Multi-centre phase III trial of TPF plus concomitant treatment with cisplatin and radiotherapy versus concomitant cetuximab and radiotherapy in locally advanced, unresectable head and neck cancer", ClinicalTrials.gov identifier: NCT00716391[22]. It was a non-inferiority, randomized and controlled study with a parallel assignment intervention model and an endpoint of safety/efficacy, carried out between 2008 and 2013 with a total recruitment of 530 patients. The follow-up of the clinical trial finished on November 2016. According to protocol, written informed consent was obtained from subjects alive. This study was approved by the ethical committee of each hospital.

Eligible patients: histologically or cytologically confirmed, previously untreated unresectable locally advanced (Stage III-IV) tumors (from oral cavity, oropharynx, larynx, hypopharynx), ECOG performance status 0–1. Unresectable disease was determined by Northern California Oncology Group (NCOG) in measurable disease. Treatment: Docetaxel, cisplatin, 5-fluorouracil (TPF)- based induction chemotherapy (T 75 mg/m^2 d1, P 75 mg/m^2 d1, F 750 mg/m^2 CI d 1–5 q 21 d + G-CSF & ciprofloxacin, by 3 cycles; then, if objective response achieved, they were randomized to: conventional radiotherapy (RT) up to 70 Gy + P 100 mg/m^2 d 1–22-43 vs conventional RT up to 70 Gy + cetuximab 400/250 mg/m^2 weekly until the completion of RT, and they were stratified by primary tumor site (TS). Surgery after RT (neck dissection) was allowed. The primary endpoint was non-inferiority of cetuximab-radiotherapy versus cisplatin-radiotherapy in terms of overall survival. Response Rate (RR), loco-regional control (LRC) and toxicity in both arms were considered secondary objectives.

Clinical data was compiled in a case report form (CRF) by medical oncologists involved in the clinical trial, including history of tobacco and alcohol use.

DNA extraction

Percentage of tumor cells was measured in hematoxylin-eosin tumor sections by central pathologist. Between five and ten 10µm FFPE section from diagnosis blocks were treated with deparaffinization solution (Qiagen, Heidelberg, Germany) and DNA extraction was done using QIAamp DNA FFPE Tissue kit (Qiagen, Heidelberg, Germany).

DNA quality evaluation and targeted NGS

Following TruSight® Tumor 26 Reference Guide (Illumina, San Diego, USA), DNA quality was measured by qPCR. Comparing FFPE-gDNA amplification potential with a reference non-FFPE gDNA (QCT), delta Cq value was used to predict the dilution required for each sample.

TruSight®Tumor 26 panel includes a set of 174 amplicons in complete exons of 26 cancer-associated genes. Following steps of hybridization with the oligo pool, removing unbound oligos and extension and ligation with bound oligos, an amplification of the libraries were performed. PCR products were checked on a 4% TBE agarose gel and finally the libraries were cleaned up by magnetic beads. PCR products were quantified using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and libraries were normalized at 4nM in a final pool. Sequencing was performed in a NextSeq 500 System (Illumina, San Diego, USA).

Data were transformed in BaseSpace platform and the VCF format files were read in the Variant Studio Software (Illumina, San Diego, USA). Only somatic variants over 5% of frequency with a quality score >500 in the bi-directional sequencing quality filter and considered from the software of PASS filter were reported. Those variants of uncertain significance were considered pathogenic if at least two *in silico* prediction tools (SIFT and PolyPhen) classified them as deleterious/probably damaging[23], and they were defined as likely pathogenic in the Catalogue Of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic) or the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/clinvar) databases.

Assessment of HPV status

FFPE sections were deparaffinized and exposed to 10mM citrate buffer antigen retrieval at 92ºC for 30 minutes. HPV status was carried out by p16 immunohistochemistry (IHC), a surrogate marker for HPV infection[24], using a $p16^{INK4a}$ mouse monoclonal antibody (Cell Marque, Rocklin, CA). Percentage of p16 staining was measured and only those tumors >70% nuclear and cytoplasmic p16+ were considered positive.

Statistical analyses

Statistical analysis compared categorical parameters and mutational status by the Chi-square test. P-values were considered statistically significant when $p < 0.05$. Significant variables were included in the logistic regression analysis and size effects were indicated by odds ratio (OR) with their 95% confidence interval (95% C.I.). Mutational status was classified as presence or absence of mutations, number of mutations (none, one or more than one) and the status of TP53 (mutant or wild-type). Response was divided in two groups of treatment: induction chemotherapy and chemo/bioradiotherapy. In both groups response was classified in complete response versus others (partial response, stable disease and progression).

Survival analysis was done according to the overall survival (OS) and progression-free survival (PFS) by Kaplan-Meier plots and log-rank test p-values were calculated in all the curves. Median was indicated in those plots in which it was achieved while in the others mean was shown. Hazard-ratio was calculated to measure the risk of the event with its 95% confidence

interval (95% C.I.) by Cox regression. Median follow-up in OS was 24.31 months while in PFS it was 10.87 months.

All these tests were conducted using SPSS software version 21.0 (SPSS Inc., Chicago) and GraphPad Prism software version 6.0 (GraphPad Software Inc., California).

Results

150 FFPE blocks were included in this study. Most were from men (89.3%), tobacco and alcohol consumers, with HPV- oropharyngeal squamous carcinoma (43.3%), diagnosed in tumor stage IV-A (Table 1), with an average of 58±7 years old.

130 HNSCC FFPE blocks (86.7%) presented mutations whereas 20 (13.3%) did not carry any mutation in the selected genes. 191 pathogenic mutations were found in the sequencing of the 150 FFPE blocks. The average of the mutated fragment coverage was 18693 (1000-120097) reads. Globally, the most mutated gene was *TP53* followed by *PIK3CA* (Figure 1). 106 out of 130 tumors with mutation (81.5%) had *TP53* mutations (alone or with others). Most of the mutations were missense (61.78%), followed by nonsense (15.18%) and frameshift (11.51%) (Supplementary table 1).

Comparison of categorical variables such as sex, alcohol and tobacco consumption or tumor characteristics such as stage, location and HPV status with the presence or absence of mutation, did not show any statistically significant difference ($p>0.05$) (Table 2). We also compared the categorical variables with the number of mutations (none, one or more than one) and with those tumors carrying a mutation in *TP53* or in other genes. Our results did not find any association ($p>0.05$) (data not shown).

Clinical data from HPV groups are described in Table 3. Most of HPV+ tumors were located in oropharynx n=11 (44%), followed by larynx n=7 (28.0%), hypopharynx n=4 (16%) and oral cavity n=3 (12%).

Mutational plot shows differences between HPV negative and positive tumors (Figure 2). In both groups *TP53* was the most frequently mutated gene (74.4% in HPV- and 60% in HPV+) followed by far from PIK3CA (12.8% versus 16%). *PTEN* (12%) was the third most commonly mutated gene in HPV+ tumors whereas in HPV- the third most frequently mutated gene was *FBXW7* (6.4%) (Figure 2). Simultaneous mutations in different genes were more frequent in HPV- tumors. Although not statistically significant, HPV+ samples were less mutated than HPV- (16.0% versus 12.8%, $p=0.667$) (Table 3). When we consider only mutated tumors, *TP53* mutations were less frequent in HPV+ (71.4% versus 83.5% in HPV-, $p=0.192$) while *PIK3CA* alterations were more frequent within HPV+ tumors (19.0% versus 14.7% in HPV- samples, $p=0.611$) (Table 3).

Data from treatment are indicated in Table 1. 26 samples (17.3%) of our study were not randomized and only received induction chemotherapy based on TPF regimen. 68 patients (45.4%) were also treated with chemoradiotherapy and 56 (37.3%) with bioradiotherapy. After TPF, 14% of the patients (n=21) had complete response whereas after concomitant

radiotherapy it increased up to 45.2% (n=56) (Table 4). We did not found any statistically significant differences between the response and the mutational burden (p>0.05) (Table 4) independently of their HPV profile and the randomization (data not shown).

In our sample, survival analysis between RTBT and RTCT treatment groups showed no differences in overall survival (OS) (p=0.161) while in progression free survival (PFS) concurrent cisplatin had better progression-free survival than bioradiotherapy (p=0.010, HR=1.783 (1.114-2.777)) (Figure 3).

Finally, OS and PFS were correlated with the mutational status. Patients with non mutated tumors had a better OS with a median of 69.914 months versus 21.684 months in patients with mutated tumors (p=0.021, HR=2.198 (1.106-4.367)) (Figure 4A). Nevertheless, there were no differences in PFS (p=0.191) (Figure 4B). We also found correlation with the number of mutations, observing that those patients that carry tumors with more than 1 mutation had lower OS than patients with non mutated tumors (p=0.009, HR=2.660 (1.284-5.511)) (Figure4C). However, the differences between patients with tumors with one or more than one mutation were not statistically significant (p=0.147). There was no difference in OS or PFS between patients with wild-type or mutated *TP53* tumors (Supplementary figure 1A-B), neither between those mutated in *TP53* nor in other genes (OS p=0.659 and PFS p=0.726). Lastly, patients with HPV+ tumors showed higher OS and PFS compared with HPV- (p=0.005 and p=0.019 respectively) (Figure 4E-F).

Discussion

Most of the head and neck cancers are diagnosed at a locally advanced stage. In the last years, combined therapies that include induction chemotherapy have shown benefits in organ preservation without a clear improvement in survival but implying higher toxicity, mostly in concurrent radiotherapy with high doses cisplatin. At present, biomarkers predicting response to treatment have yet to be defined. For that reason we proposed to study with NGS the mutational status of 26 of the most common altered genes in cancer in a homogeneously treated sample of HNSCC from the the clinical trial TTCC-2007-01[22].

The 150 patients included in our series presented epidemiological characteristics common to HNSCC in our region: the ratio between sexes was 9:1 in detriment of men, subjects were heavy smokers and drinkers, and most of the patients were diagnosed in stage IV[25]. p16 IHC, a surrogate of HPV infection showed that 16% carried HPV, a lower percentage than that previously reported in Europe[26]. Nevertheless, the HPV cases showed similar location than in other countries from Southern Europe, mostly in oropharynx[27].

Globally, the most mutated gene in our series was *TP53* (67.02%). We observed a lower percentage of mutated *TP53* in HPV+ tumors (71.4%) than in HPV- (83.5%) as it has been previously reported in HNSCC[28], although it was not statistically significant (p=0.192). These results could be explained if *TP53* sequestration by the viral oncoprotein E6 prevents from selective pressure of gaining mutations in this gene[29,30]. The lack of statistically significant

result in *TP53* distribution between HPV groups could be explained by the concurrence of viral infection and tobacco smoking and alcohol consumption in the majority of our patients.

PI3K has been reported as the most mutated pathway in HNSCC. *PIK3CA* gene, that encodes for the catalytic subunit of the family, has been reported with an average mutational rate of 10.53% in HNSCC[31] exhibiting laryngeal tumors higher percentage[32]. Mutations in this gene have been also related with HPV+ tumors[5]. Our results corroborated than *PIK3CA* was more frequently mutated in HPV+ tumors but we did not see an increased percentage in laryngeal carcinoma. In fact, 16.7% of pharyngeal tumors had *PIK3CA* mutations compared with 12.5% of larynx and oral cavity. We also observed a highest percentage of *PIK3CA* mutations in HPV+ tumors, being the second most mutated gene in our series.

Mutations in *FBXW7*, coding for an E3 ubiquitin ligase member of the F-box protein family, have been previously observed in HNSCC[19]. This tumor suppressor gene targets for *NOTCH1*, being an important protein in cell proliferation control. Previous studies found it mutated in 5% of HNSCC[33,34], a concordant result with our results. Interestingly, FBXW7 was most mutated in HPV- tumors.

Other genes mutated in our series, such as *MET*, *PTEN* and *APC,* have been reported in HNSCC in varied percentages[29,34,35]. We found 6 patients (4%) with mutations in *PTEN,* incidence lower than in other studies that reported around 10%[17]. Our results showed that PTEN mutations were presented in a higher percentage in HPV+ patients as previously described by other groups[5,29].

The lack of association between the mutational status and the presence of HPV can be explained due to the small number of HPV+ cases and the fact that most of them were smokers. Nevertheless, mutational burden had an impact on survival that should be considered as an important prognostic factor.

In our series, OS was similar between patients treated with conventional radiotherapy plus cisplatin or cetuximab. Conversely, PFS was better in the group treated with cisplatin. Survival analysis showed that the presence of mutation in the tumors was associated with a poor prognosis displayed by lower OS. This data could be related with tumor aggressiveness, as it has been reported in other series[36]. Moreover, the number of mutations could be an indicative of OS, because carriers of tumors with more than one mutation had lower OS than those with non mutated tumors. Previous studies indicated that *TP53* mutations have been associated with decreased OS[37]. Our study did not show statistically significant relationship in OS and PFS between patients with tumors wild-type or mutant *TP53* (p=0.217).

Finally, HPV+ HNSCC has been associated with better prognosis and better OS and PFS than HPV- tumors[26,38–40]. Our results confirmed that patients with HPV+ tumors showed better OS and PFS, with increased survival.

Overall, our data strongly support and expand previous published mutational burden in HNSCC. We have also defined the mutational profile of HPV+ HNSCC in Spanish population showing, apart from *TP53* mutations, frequent alterations in *PIK3CA* and *PTEN* genes, defining possible pathways for targeted therapy. Moreover, survival analysis showed that mutational

status in the tumor could define prognosis of the patient, being an important biomarker in HNSCC. Although we cannot find any linkage between mutations and response to treatment, the association in survival could give us some important data to continue with, giving a step further into a personalized treatment for patients suffering from this type of cancer.

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Competing financial interest

The authors declare no competing financial interest.

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Tables

Table 1: Summary of selected patient characteristics and clinicopathological data.

Characteristic	Group	N=150	%
Sex	Male	134	89.3
	Female	16	10.7
Location	Larynx	27	18.0
	Hypopharynx	39	26.0
	Oropharynx	65	43.3
	Oral cavity	19	12.7
Stage	III	10	6.7
	IVA	109	72.6
	IVB	31	20.7
Tobacco	Non smoker	19	12.7
	Smoker	131	87.3
Alcohol	Non drinker	39	26.0
	Drinker	111	74.0
HPV status (p16INK4a IHC)	Negative	125	83.3
	Positive	25	16.7
Group of treatment	Induction TPF alone	26	17.3
	TPF+RT-Cisplatin	68	45.4
	TPF+RT-Cetuximab	56	37.3

Table 2: Mutation state versus clinicopathological features.

Characteristic	Group N=150	Non mutated N=20	Mutated N=130	p-value
Sex	Male	16 (80.0%)	118 (90.8%)	0.146
	Female	4 (20.0%)	12 (9.2%)	
Location	Larynx	3 (15.0%)	24 (18.5%)	0.856
	Hypopharynx	4 (20.0%)	35 (26.9%)	
	Oropharynx	10 (50.0%)	55 (42.3%)	
	Oral cavity	3 (15.0%)	16 (12.3%)	
Stage	III	1 (5.0%)	9 (6.9%)	0.849
	IVA	14 (70.0%)	95 (73.1%)	
	IVB	5 (25.0%)	26 (20.0%)	
Tobacco	Non smoker	4 (20.0%)	15 (11.5%)	0.290
	Smoker	16 (80.0%)	115 (88.5%)	
Alcohol	Non drinker	6 (30.0%)	33 (25.4%)	0.661
	Drinker	14 (70.0%)	97 (74.6%)	
HPV status (p16INK4a IHC)	Negative	16 (80.0%)	109 (83.8%)	0.667
	Positive	4 (20.0%)	21 (16.2%)	

Table 3: Differences between HPV+ and HPV- in our sample.

Characteristic	Group N=150	HPV negative N=125	HPV positive N=25	p-value
Sex	Male	112 (89.6%)	22 (88.0%)	0.813
	Female	13 (10.4%)	3 (12.0%)	
Location	Larynx	20 (16.0%)	7 (28.0%)	0.418
	Hypopharynx	35 (28.0%)	4 (16.0%)	
	Oropharynx	54 (43.2%)	11 (44.0%)	
	Oral cavity	16 (12.8%)	3 (12.0%)	
Stage	III	8 (6.4%)	2 (8.0%)	0.802
	IVA	90 (72.0%)	19 (76.0%)	
	IVB	27 (21.6%)	4 (16.0%)	
Tobacco	Non smoker	17 (13.6%)	2 (8.0%)	0.442
	Smoker	108 (86.4%)	23 (92.0%)	
Alcohol	Non drinker	33(26.4%)	6 (24.0%)	0.803
	Drinker	92 (73.6%)	19 (76.0%)	
Mutational status	Non mutated	16 (12.8%)	4 (16.0%)	0.667
	Mutated	109 (87.2%)	21 (84.0%)	
TP53 status (only mutated patients)	Non mutated	18 (16.5%)	6 (28.6%)	0.192
	Mutated	91 (83.5%)	15 (71.4%)	
PIK3CA status (only mutated patients)	Non mutated	93 (85.3%)	17 (81.0%)	0.611
	Mutated	16 (14.7%)	4 (19.0%)	

Table 4: Analysis of treatment response and mutations in three difference groups: presence/absence of mutations (mutational status), number of mutations and TP53 status in mutated patients.

		TPF response			TPF+RT-CDDP/Cetuximab		
Characteristic	**Group**	**CR N=21**	**Others N=129**	**p-value**	**CR N=56**	**Others N=68**	**p-value**
Mutational status	Non mutated	4 (19.0%)	16 (12.40%)	0.406	7 (12.5%)	10 (14.7%)	0.722
	Mutated	17(81.0%)	113 (87.6%)		49 (87.5%)	58 (85.3%)	
Number of mutations	None	4 (19.0%)	16 (12.4%)		7 (12.5%)	10 (14.7%)	
	1 mutation	13 (61.9%)	67 (51.9%)	0.297	32 (57.1%)	35 (51.5%)	0.816
	> 1 mutation	4 (19.0%)	46 (35.7%)		17 (30.4%)	23 (33.8%)	
TP53 status (only mutated patients)	Non mutated	4 (23.5%)	20 (17.7%)	0.563	9 (18.4%)	10 (17.2%)	0.879
	Mutated	13 (76.5%)	93 (82.3%)		40 (81.6%)	48 (82.8%)	

Figures

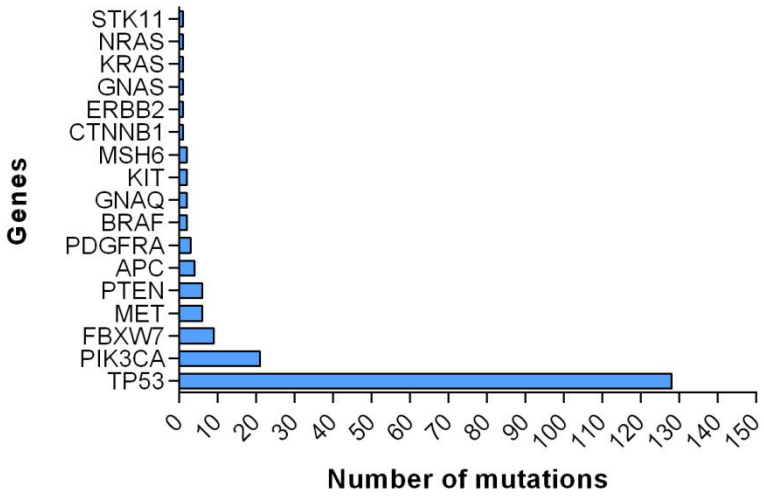

Figure 1: Number of mutations found in the sequencing of 150 HNSCC by TruSight Tumor 26 panel.

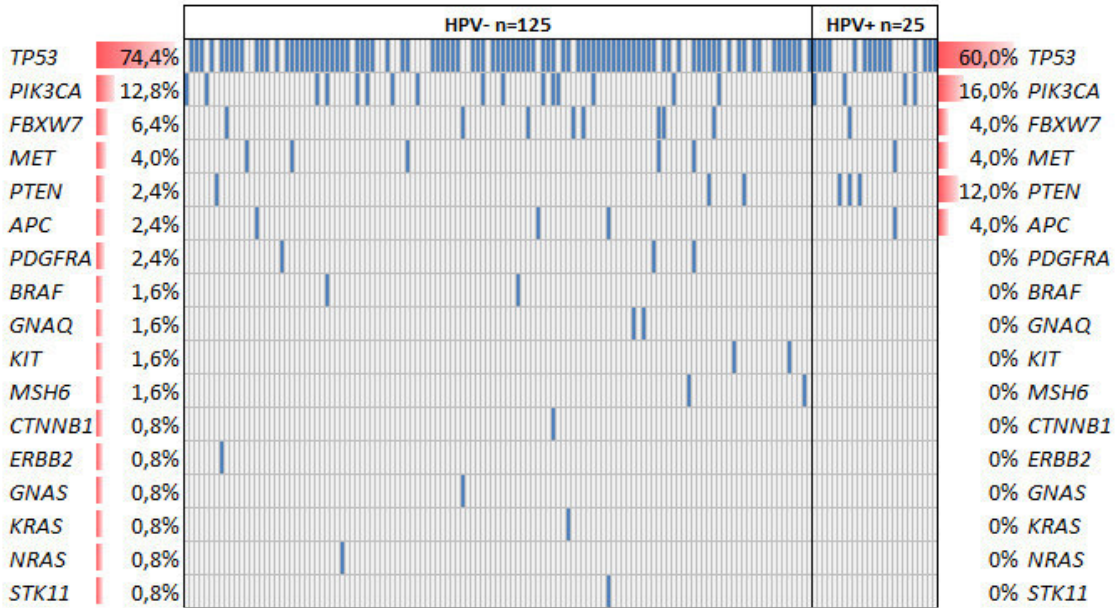

Figure 2: Mutational plot divided in HPV positive and negative HNSCC tumors. Blue rectangle indicates presence of mutations in each patient. Percentage of mutations in each gene divided per HPV group is indicated in the border of the table and red line represents its proportion.

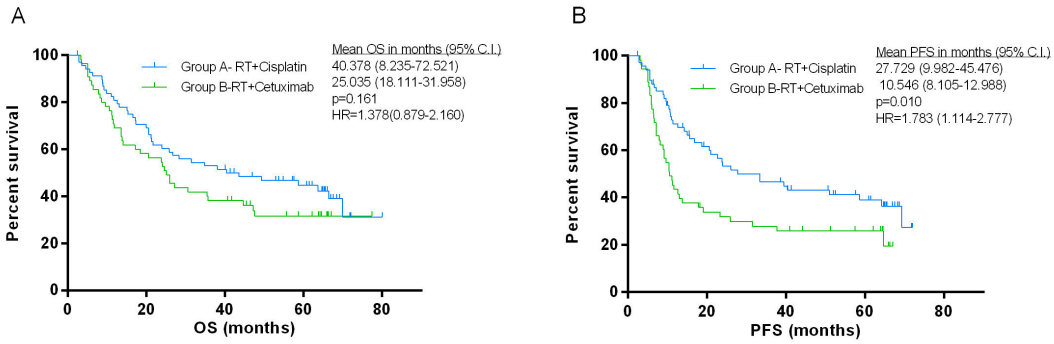

Figure 3: Kaplan-Meier survival curves in the 150 selected patients from the TTCC-2007-01 clinical trial. A) OS, B) PFS according to its treatment option. Median, log rank test p-values and hazard ratios are shown in each plot.

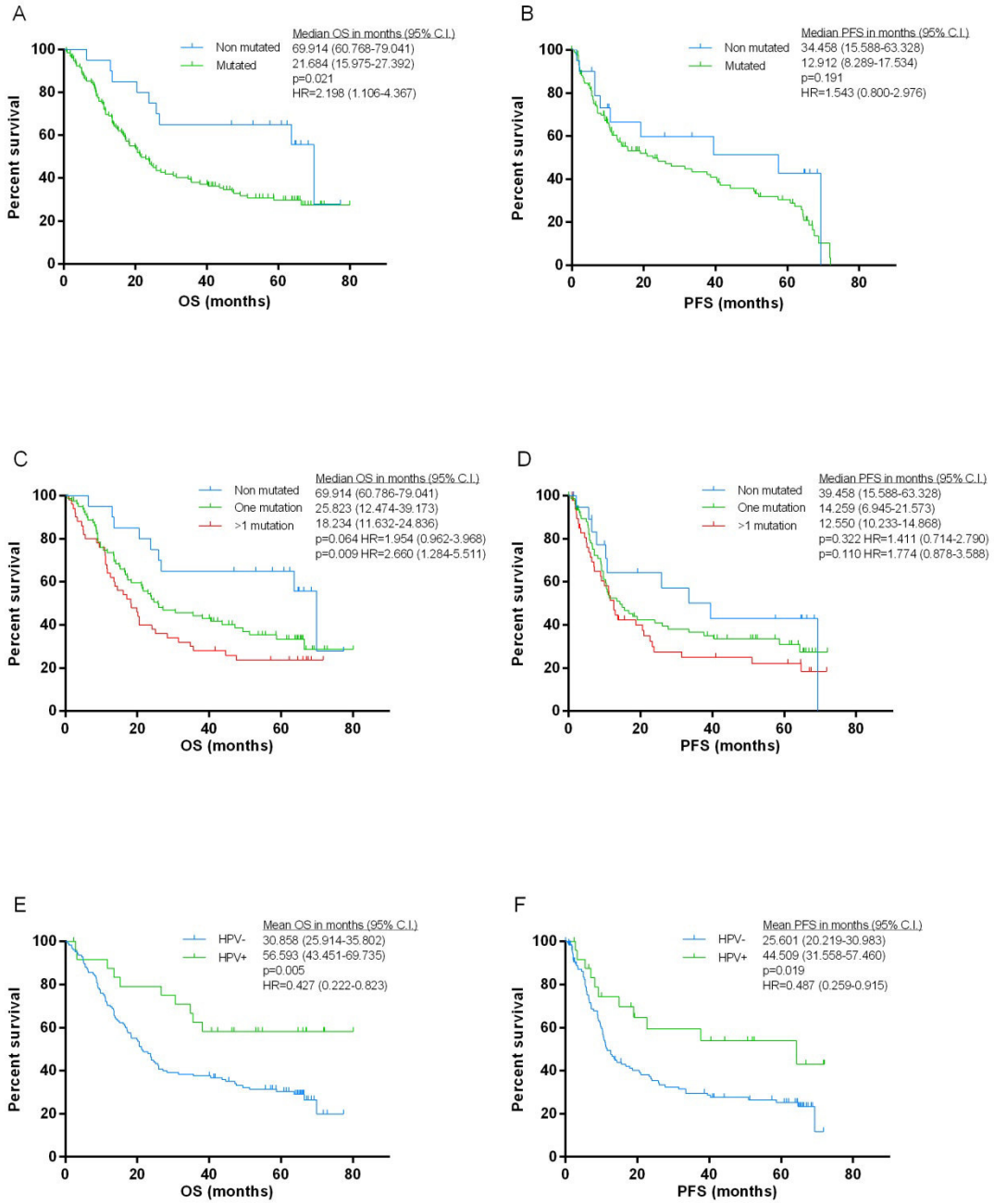

Figure 4: Kaplan-Meier survival curves. Mutational status and overall survival (A) and progression-free survival (B), number of mutations and their overall survival (C) and PFS (D). Lastly, HPV status and OS (E) and PFS (D). Median (mean in E and F), log rank test p-values and hazard ratios are shown in each plot.

Supplementary material

Supplementary table 1: Description of the pathogenic mutations found in our sample.

Gene	Consequence	HGVSc	HGVSp
APC	missense_variant	NM_000038.5:c.4283G>A	NP_000029.2:p.Gly1428Glu
	missense_variant	NM_000038.5:c.3755C>T	NP_000029.2:p.Ser1252Phe
	missense_variant	NM_000038.5:c.4237A>G	NP_000029.2:p.Met1413Val
	missense_variant	NM_000038.5:c.3790G>A	NP_000029.2:p.Val1264Ile
BRAF	missense_variant	NM_004333.4:c.1429C>T	NP_004324.2:p.His477Tyr
	missense_variant, splice_region_variant	NM_004333.4:c.1859T>C	NP_004324.2:p.Met620Thr
CTNNB	missense_variant	NM_001098210.1:c.110C>T	NP_001091680.1:p.Ser37Phe
ERBB2	missense_variant	NM_004448.2:c.2404C>T	NP_004439.2:p.Pro802Ser
FBXW7	missense_variant	NM_033632.3:c.1322G>A	NP_361014.1:p.Arg441Gln
	missense_variant	NM_033632.3:c.1513C>T	NP_361014.1:p.Arg505Cys
	missense_variant	NM_033632.3:c.1528G>A	NP_361014.1:p.Asp510Asn
	missense_variant	NM_033632.3:c.1556A>G	NP_361014.1:p.Tyr519Cys
	missense_variant	NM_033632.3:c.1315A>G	NP_361014.1:p.Thr439Ala
	stop_gained	NM_033632.3:c.1217G>A	NP_361014.1:p.Trp406Ter
	frameshift_variant, feature_truncation	NM_033632.3:c.1819delG	NP_361014.1:p.Asp607IlefsTer21
	missense_variant	NM_033632.3:c.1787C>G	NP_361014.1:p.Ser596Cys
	missense_variant	NM_033632.3:c.2038A>G	NP_361014.1:p.Thr680Ala
GNAQ	missense_variant	NM_002072.3:c.560C>T	NP_002063.2:p.Thr187Ile
	missense_variant	NM_002072.3:c.772A>G	NP_002063.2:p.Ile258Val
GNAS	missense_variant, splice_region_variant	NM_080425.2:c.2516A>G	NP_536350.2:p.Asp839Gly
KIT	frameshift_variant, feature_truncation	NM_000222.2:c.1537delA	NP_000213.1:p.Glu514SerfsTer13
	missense_variant	NM_000222.2:c.1921C>T	NP_000213.1:p.Leu641Phe
KRAS	3_prime_UTR_variant	NM_033360.2:c.*73T>C	
MET	missense_variant	NM_001127500.1:c.3076C>T	NP_001120972.1:p.Pro1026Ser
	missense_variant	NM_001127500.1:c.3029C>T	NP_001120972.1:p.Thr1010Ile
	missense_variant	NM_001127500.1:c.3029C>T	NP_001120972.1:p.Thr1010Ile
	missense_variant	NM_001127500.1:c.3029C>T	NP_001120972.1:p.Thr1010Ile
	missense_variant	NM_001127500.1:c.3776C>T	NP_001120972.1:p.Thr1259Ile
	missense_variant	NM_001127500.1:c.1030G>A	NP_001120972.1:p.Gly344Arg
MSH6	missense_variant	NM_000179.2:c.3245C>T	NP_000170.1:p.Pro1082Leu
	missense_variant	NM_000179.2:c.3226C>T	NP_000170.1:p.Arg1076Cys
NRAS	missense_variant	NM_002524.4:c.95A>G	NP_002515.1:p.Tyr32Cys
PDGFR	stop_gained	NM_006206.4:c.2482C>T	NP_006197.1:p.Gln828Ter
	missense_variant	NM_006206.4:c.1984G>A	NP_006197.1:p.Gly662Arg
	missense_variant	NM_006206.4:c.1936A>G	NP_006197.1:p.Lys646Glu
PIK3CA	missense_variant	NM_006218.2:c.1624G>A	NP_006209.2:p.Glu542Lys

	missense_variant	NM_006218.2:c.1637A>G	NP_006209.2:p.Gln546Arg
	missense_variant	NM_006218.2:c.1352G>T	NP_006209.2:p.Gly451Val
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.3140A>T	NP_006209.2:p.His1047Leu
	missense_variant	NM_006218.2:c.1624G>A	NP_006209.2:p.Glu542Lys
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.1258T>C	NP_006209.2:p.Cys420Arg
	missense_variant	NM_006218.2:c.3049G>C	NP_006209.2:p.Asp1017His
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.1624G>A	NP_006209.2:p.Glu542Lys
	missense_variant	NM_006218.2:c.1624G>A	NP_006209.2:p.Glu542Lys
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.1624G>A	NP_006209.2:p.Glu542Lys
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	missense_variant	NM_006218.2:c.1357G>A	NP_006209.2:p.Glu453Lys
	missense_variant	NM_006218.2:c.1633G>A	NP_006209.2:p.Glu545Lys
	stop_gained	NM_006218.2:c.10C>T	NP_006209.2:p.Arg4Ter
	missense_variant	NM_006218.2:c.1624G>A	NP_006209.2:p.Glu542Lys
PTEN	missense_variant	NM_000314.4:c.389G>A	NP_000305.3:p.Arg130Gln
	stop_gained	NM_000314.4:c.617_618delTCinsAA	NP_000305.3:p.Phe206Ter
	stop_gained	NM_000314.4:c.49C>T	NP_000305.3:p.Gln17Ter
	missense_variant, splice_region_variant	NM_000314.4:c.494G>A	NP_000305.3:p.Gly165Glu
	missense_variant	NM_000314.4:c.74T>C	NP_000305.3:p.Leu25Ser
	missense_variant	NM_000314.4:c.574_575delGCinsAA	NP_000305.3:p.Ala192Lys
STK11	missense_variant	NM_000455.4:c.182G>A	NP_000446.1:p.Gly61Asp
TP53	splice_acceptor_variant	NM_000546.5:c.560-1G>A	
	inframe_deletion	NM_000546.5:c.797_811delGACGGAACAGCTTTG	NP_000537.3:p.Gly266_Phe271del
	missense_variant	NM_000546.5:c.763A>T	NP_000537.3:p.Ile255Phe
	missense_variant	NM_000546.5:c.763A>T	NP_000537.3:p.Ile255Phe
	frameshift_variant, feature_elongation	NM_000546.5:c.455dupC	NP_000537.3:p.Pro153AlafsTer28
	stop_gained	NM_000546.5:c.438G>A	NP_000537.3:p.Trp146Ter
	missense_variant	NM_000546.5:c.997C>T	NP_000537.3:p.Arg333Cys
	stop_gained	NM_000546.5:c.1024C>T	NP_000537.3:p.Arg342Ter
	missense_variant	NM_000546.5:c.734G>T	NP_000537.3:p.Gly245Val
	missense_variant	NM_000546.5:c.743G>A	NP_000537.3:p.Arg248Gln
	stop_gained	NM_000546.5:c.586C>T	NP_000537.3:p.Arg196Ter
	inframe_deletion	NM_000546.5:c.685_690delTGTACC	NP_000537.3:p.Cys229_Thr230delinsdel
	missense_variant	NM_000546.5:c.725G>A	NP_000537.3:p.Cys242Tyr
	frameshift_variant, feature_elongation	NM_000546.5:c.444_445insA	NP_000537.3:p.Ser149IlefsTer32
	missense_variant	NM_000546.5:c.814G>T	NP_000537.3:p.Val272Leu

	stop_gained	NM_000546.5:c.438G>A	NP_000537.3:p.Trp146Ter
	splice_donor_variant, coding_sequence_variant, feature_truncation	NM_000546.5:c.671_672+1delAGG	
	stop_gained	NM_000546.5:c.438G>A	NP_000537.3:p.Trp146Ter
	splice_donor_variant, coding_sequence_variant	NM_000546.5:c.375_375+1delGGinsTT	
	splice_donor_variant	NM_000546.5:c.782+1G>A	
	missense_variant	NM_000546.5:c.743G>T	NP_000537.3:p.Arg248Leu
	missense_variant	NM_000546.5:c.742C>T	NP_000537.3:p.Arg248Trp
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	missense_variant	NM_000546.5:c.817C>T	NP_000537.3:p.Arg273Cys
	missense_variant	NM_000546.5:c.332T>A	NP_000537.3:p.Leu111Gln
	frameshift_variant, feature_truncation	NM_000546.5:c.812_813delAG	NP_000537.3:p.Glu271GlyfsTer34
	missense_variant	NM_000546.5:c.503A>T	NP_000537.3:p.His168Leu
	missense_variant	NM_000546.5:c.710T>A	NP_000537.3:p.Met237Lys
	frameshift_variant, feature_truncation	NM_000546.5:c.717_727delCAGTTCCTGCA	NP_000537.3:p.Ser240GlyfsTer20
	stop_gained	NM_000546.5:c.702C>A	NP_000537.3:p.Tyr234Ter
	missense_variant	NM_000546.5:c.332T>A	NP_000537.3:p.Leu111Gln
	missense_variant	NM_000546.5:c.817C>T	NP_000537.3:p.Arg273Cys
	frameshift_variant, feature_truncation	NM_000546.5:c.754delC	NP_000537.3:p.Leu252SerfsTer93
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	missense_variant	NM_000546.5:c.763A>T	NP_000537.3:p.Ile255Phe
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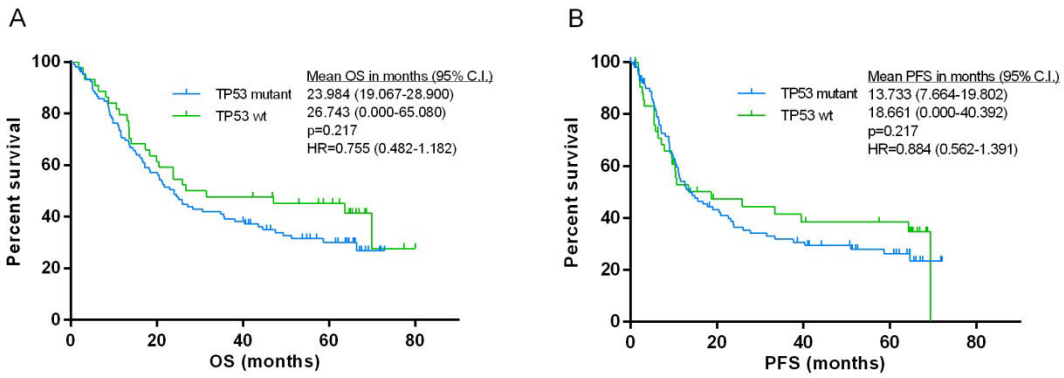

Supplementary Figure 1: Kaplan-Meier survival curves. Mutant and wild-type TP53 and overall survival (A) and progression-free survival (B). Median, log rank test p-values and hazard ratios are shown in each plot.

Autophagy modulation in head and neck squamous cell carcinoma cell lines: a new target for cancer treatment

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Artículo 6: "Autophagy modulation in head and neck squamous cell carcinoma cell lines: a new target for cancer treatment"

El uso de moduladores de la autofagia está emergiendo como una diana potencial para la terapia del cáncer. La combinación de modificadores autofágicos con un agente quimioterápico ha demostrado efectos sinérgicos. A pesar de la gran variedad de combinaciones de terapias empleadas en el CECC, la supervivencia a los 5 años no ha demostrado grandes cambios. Por ello, nos proponemos el estudio de fármacos y su modulación en la autofagia como nueva vía de tratamiento en líneas comerciales y establecidas de CECC de diferentes localizaciones con el propósito de definir una terapia antitumoral novedosa que mejore las pobres tasas de respuesta observadas en los CECC.

Tras el establecimiento de tres líneas celulares obtenidas de biopsias quirúrgicas de tumores de laringe (32860), valécula (32816) y orofaringe HPV+ (32816) y el uso de la línea comercial CAL33 como representativa de tumores de cavidad oral, éstas se caracterizaron tanto a nivel morfológico (H&E) como a nivel molecular mediante el estudio de variaciones en el número de copias y cariotipado. Posteriormente, se realizó un ensayo de análisis de fármacos en dos etapas: viabilidad celular mediante MTT y ciclo celular por citometría de flujo. El análisis de la autofagia se estudió mediante western-blot de las proteínas fosfo-mTOR, p62, beclin1 y LC3B. Se emplearon los fármacos cloroquina, decitabina, metformina, paclitaxel y panobinostat.

Aunque se observaron algunas diferencias en el fenotipo, todas las líneas celulares presentaron características epiteliales. Los resultados de caracterización permitieron confirmar las diferencias moleculares entre las distintas localizaciones tumorales de las líneas celulares, a pesar de que estas diferencias fueron mayores en el cariotipo que en el número de variación de copias, donde se asemejaban a la descripción general surgida de la caracterización de diferentes líneas celulares de CECC.

La adición de tratamientos mostró una disminución en la viabilidad celular dosis-dependiente, excepto en el caso de la decitabina, sin efecto en la proliferación celular. Esto permitió definir las concentraciones óptimas de 25µM de cloroquina, 7.5 µM de decitabina, 8mM de metformina, 10nM de paclitaxel y 50nM de panobinostat para los estudios posteriores de citometría y extracción de proteínas.

A pesar de las variaciones entre las diferentes líneas celulares utilizadas, globalmente el estudio del ciclo celular mostró cómo el inhibidor autofágico cloroquina, agente lisosomotrópico, producía una parada en G0/G1, con una disminución de G2/M y un pequeño incremento en la muerte celular. La cloroquina produjo un aumento en la expresión de p62 y una disminución de las proteínas específicas de autofagia (beclin1 y LC3B).

Por el contrario, el inhibidor de histona deacetilasas e inductor de la expresión génica de la vía autofágica decitabina, produjo un descenso en G0/G1 con un aumento en fase S y muerte. El análisis de proteínas corroboró el aumento de autofagia por sobreexpresión de las dos proteínas específicas estudiadas.

El resultado con metformina, fármaco antidiabético con propiedades antitumorales e inhibidor de autofagia mediante la inhibición de mTOR, mostró los resultados más llamativos con un descenso en G0/G1 y un aumento en fase S y en G2/M. El tratamiento produjo una gran disminución de los niveles de fosfo-mTOR, produciendo un aumento leve de LC3B-II.

La adición de paclitaxel, principio activo utilizado para el tratamiento de CECC, inhibidor de la formación de microtúbulos y por lo tanto del transporte de las vesículas autofágicas, obtuvo el mayor efecto citotóxico con una media de muerte del 35%, excepto en la línea de laringe 32860, que parece más resistente al paclitaxel. El efecto en la autofagia de esta droga no fue uniforme según la línea celular con un aumento leve de beclin1 y LC3B-I.

Por último, el inhibidor de histonas deacetilasas panobinostat produjo una disminución en G0/G1 y un importante incremento en G2/M, produciendo también un pequeño aumento en muerte celular. Al igual que en los ensayos de viabilidad celular, la línea celular 32816 tuvo un comportamiento diferente, con mayor sensibilidad a todos los tratamientos e inducción uniforme en la expresión de beclin1 y LC3B en todas las líneas celulares.

Este análisis preclínico confirma la utilidad de fármacos moduladores de autofagia en el tratamiento de CECC ya sea como monoterapia o en poliquimioterapia. Esta sinergia parece ser más apropiada en tumores HPV+, puesto que las líneas celulares derivadas de este tipo tumoral han resultado más sensibles al tratamiento.

Autophagy modulation in head and neck squamous cell carcinoma cell lines: a new target for cancer treatment

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Abstract

Head and neck squamous cell carcinomas (HNSCC) are treated with multidisciplinary options but their survival rates remain limited. Autophagy pathway has a dual role in carcinogenesis and its use as therapeutic target could define a new treatment alternative that could improve the outcomes in HNSCC. In this study four cell lines from different locations (laryngeal, pharyngeal, tongue and vallecula tumours) were used for autophagy modulation drug screening. Three of these cell lines were newly established and characterized, showing molecular and phenotypic differences between them. Chloroquine, decitabine, metformin, paclitaxel and panobinostat were tested by MTT cell viability assay, cell cycle analysis by flow cytometry and autophagy expression by western blot of phospho-mTOR, p62, beclin1 and LC3B proteins. The results showed that paclitaxel, chloroquine and panobinostat were the most effective drugs in HNSCC cell line viability and cell cycle modification, reaching paclitaxel the highest death rates. Moreover, all treatments showed a modification of autophagy proteins expression in different ways. Differences between tumour locations were notable between cell lines and confirm the diversity in HNSCC. In conclusion, autophagy modulation could be a promising target therapy especially in HPV positive HNSCC, because of the highest sensibility in the HPV+ tumour derived cell line.

Keywords: Head and neck cancer, drug screening, autophagy, cell lines, establishment

Introduction

Head and neck squamous cell carcinoma (HNSCC) includes epithelial malignancies of the oral cavity, pharynx and larynx[1]. It is the sixth most common tumour with an incidence of 600.000 cases per year worldwide[2]. Most important risk factors are tobacco and alcohol consumption, although virus infection, mainly human papillomavirus (HPV), is also an important etiological cause[3] defining a different molecular entity than caused by tobacco and alcohol. HNSCC treatment options comprise a multidisciplinary approach including surgery or/and radiotherapy for early-stages, whereas locally advanced tumours also include chemotherapy (induction chemotherapy and/or chemoradiation) and biotherapy[4]. Disappointingly, 5-years survival rates remain below 50%, without a significant improve in the last decade[5].

Most common events along HNSCC development have been described[5]. These tumours usually have complex karyotypes with frequent copy number gains on 3q, 8q, 9q, 11q and 20q chromosomal arms, as well as losses of 3p, 4q, 9p and 18q[6–8]. Moreover, genetic alterations in HNSCC are abundant and include lot of different pathways[9], being classified according to their expression profiles in different groups[10]. HNSCC cell lines are good preclinical models to test novel treatment therapies. In spite of more than 300 HNSCC cell lines reported[11], most of them did not come from the primary tumour site and molecular characterization and clinical features are not available.

Macroautophagy (referred from now as autophagy) is one of the most conserved cellular degradation pathway[12]. Its role in cancer is controversial, acting both in promotion and inhibition of the tumourigenesis[13]. Alterations in autophagic signaling pathways are frequently observed in cancer[13], so the use of autophagy modulators is emerging as a potential target for cancer therapy[14]. The combination of autophagy modifiers with a chemotherapy agent has shown benefits in treatment effects[15]. Although molecular process of autophagy is well described[16], understanding its role in cancer, may allow us to develop novel therapeutic strategies to enhance the effects of chemotherapy and improve the clinical outcome of cancer patients[17]. Certain proteins status such as conversion from LC3B-I to LC3B-II, phospo-mTOR and p62 down-regulation and Beclin-1 increase are considered the best indicators of active autophagy[18].

The purpose of this article was to study the use of some drugs and the autophagy modulation in commercial and new established molecularly characterized HNSCC tumour cell lines from all the main locations, to check their effect in viability and cell cycle modification. Alteration in autophagy protein expression was also studied to show the real effect of inducers or inhibitors in this pathway. This could define a promising novel cancer therapy, improving the poor response rates observed in HNSCC tumours.

Material and methods

Cell lines establishment and culture conditions

Surgically resected fresh HNSCC tumour biopsies from Othorhinolaryngology operating theatre were received in aseptic conditions, after signed informed consent by the patient. After three

washes with phosphate-buffered saline (PBS) + 1% penicillin and streptomycin (Gibco, Life Technologies, Carlsbad, CA) + 1% amphotericin B (Sigma-Aldrich, St. Louis, MO), pieces were cultured in a dish with enriched RPMI 1640 medium with 20% heat-inactivated fetal bovine serum (FBS) (Gibco-Life Technologies, Carlsbad, CA) + 2% penicillin/streptomycin. After mechanical degradation by tumour extrusion in the dish, they were incubated at 37ºC in a 5% of CO_2 atmosphere. Tumour cells were purified by removal of fibroblast in sequence passages. Cells were passaged twice a week by trypsinization and the tumour cell line was considered established after 30 passages. Finally from nine different tumours, we established three different squamous cell carcinomas cell lines from oropharyngeal (32816), laryngeal (32860) and vallecula, the region between pharynx and larynx (32661).

Because of the lack of an oral cavity tumour cell line, CAL33 from human tongue squamous cell carcinoma was purchased from American Type Culture Collection (ATCC, Rockaville, MD) and was cultured under the same conditions described above.

Cell lines characterization

Characterization of the different cell lines was done in different steps. First of all, trypsinized cells were fixed in 70% methanol and centrifuged in the cytospin. Hematoxylin and eosin staining was done in the slides to corroborate their tumourogenicity phenotype.

Cytogenetic analysis and FISH procedure

Chromosome preparation were processed as previously described[19]. Briefly, before harvesting, HNSCC cells in the logarithmic growth phase were incubated with 100 ng/ml colcemid (Gibco-Life Technologies, Carlsbad, CA) for 45 min. The cells were harvested by treatment with 0.025% trypsin-EDTA, suspended in 0.075 MKCl at room temperature for 20 min, and fixed with methanol/ acetic acid (3:1) three times. Chromosomes were identified by G-banding and karyotypes were described according to the ISCN, 2016[20]. Dual color FISH using Vysis whole chromosome painting (WCP) probes, a chromosome 1 painting probe (WCP 1) labelled in SpectrumOrange and chromosome 3 painting probe (WCP 3) labelled in SpectrumGreen (Abbott Molecular, VYSIS, Chicago, IL) were prepared according to the manufacturer's instructions.

Affymetrix CytoScan 750K

DNA was extracted from cell line using the QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany) and analysed on the Affymetrix® CytoScan 750K Array according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). The CytoScan 750K Array provides whole-genome coverage, with more than 750,000 markers for copy number analysis and over 200,000 SNPs for genotyping. Data analysis was performed using the Chromosome Analysis Suite (CHAS) (Affymetrix, Santa Clara, CA). All markers were used to determine copy number and SNP probes were used to calculate loss of heterozygosity (LOH) and genotype.

Cell viability assay

$10x10^3$ cells were seeded on a 24 well plate and were treated with a battery of autophagy modulators such as: Panobinostat, Decitabine, Chloroquine, Paclitaxel and Metformin.

Concentrations of the drugs are indicated in table 1. Treatments were done for 72 hours, measuring each 24 hours the viability of the cells by cellular metabolic function using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO; 98% purity). MTT salt dissolved in PBS, pH7.2, at 5mg/ml was added to the cells (110ul/well). MTT was reduced via mitochondrial dehydrogenases converting into formazan. After incubation at 37ºC for 1 hour, the medium was aspirated. The formazan crystals were dissolved in 500ul of dimethylsulfoxide (DMSO) and the absorbance was measured at 570nm in a plate reader (Ultra Evolution, Tecan, Mannedorf, Switzerland). Blank and control without drugs were always used. All the experiments were performed in triplicate and were repeated at least three times under the same conditions. Curves of viability were calculated subtracting the background of the blank and converting the absorbance of the tetrazolium salt absorbance in terms of percentage respect to the control. Optimal concentrations were calculated according to the minimum inhibitory concentration in all the cell lines.

Flow cytometry

In order to analyze the effect on the cell cycle induced by selected drugs, flow cytometry was performed with the optimal concentration calculated by the viability assays. Cells were treated again for 72 hours and both supernatant and trypsin treated adherent cells in treated and controls were taken each 24 hours, 70% cold ethanol fixed and store at -20ºC until the last day. Last day, ethanol was removed by sequence of washes with PBS and finally they were resuspended in 1ml of PBS, stained with 50µg/ml propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and treated with 100µg/ml RNase, to removed RNA. After an overnight incubation at room temperature, 30.000 cells cycle was measured in FACScalibur flow cytometer (BD Biosciences, San Jose, CA). DNA histogram of cytometry data was analyzed in the CellQuest Pro software (BD Biosciences, San Jose, CA) and cell cycle phase percentage was calculated in WinMDI 2.9 software (Windows, Albuquerque, NM).

Protein extraction and western blot

Cells were collected with trypsin each day for 72 hours after treatment at specific concentrations. After 45 minutes incubation with cell lysis buffer on ice, lysates were centrifugated at 15000rpm during 15 minutes at 4ºC and recovered protein supernatants measured in spectophotometry (Nanodrop) at 260nm. 50ug of proteins were separated in 8% and 12% SDS-PAGE at 120V and immunoblotted with specific antibodies against LC3B (Novus, ref.NB600-1384, 1:1000) Beclin-1 (Cell signaling, ref. 3738S, 1:1000), p62 (Abcam, ref.ab109012, 1:1000) and mTOR (Cell Signalling, ref.2972S, 1:1000) in transferred polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Barcelona, Spain). Membranes were incubated with the corresponding horseradish peroxidase-conjugated (HRP) secondary antibody at 1:10000 (Santa Cruz Biotechnology, CA) and were developed using Pierce® ECL Western Blotting substrate (Thermo Scientific, Madrid, Spain). Developed signals were recorded on X-ray films (Fujifilm Spain, Barcelona, Spain).

Statistical analysis

Statistic comparison with one variable (treated vs. untreated cell cycle phase each 24 hours) was performed using a Student's two-tailed, paired t test or one-way analysis of variance

(ANOVA). The difference was considered to be statistically significant when $p < 0.05$. Results are expressed as mean ± standard deviation (SD).

All these tests were conducted using SPSS software version 21.0 (SPSS Inc., Chicago, IL) and GraphPad Prism software version 6.0 (GraphPad Softare Inc., CA).

Results

Clinical features of the biopsies are described in Table 2. All the patients were high tobacco and alcohol consumers. Importantly, 32816 cell line was derived from a histologically confirmed HPV positive tumour by $p16^{INK4a}$ immunohistochemistry.

Cell lines characterization

HNSCC tumour tissues culture produced epithelial outgrowths with the typical cobblestone patterns. Lines were morphological distinct. 32661 had a nest-like growth pattern while the rest of the cell lines were more spread. 32860 culture showed two main morphological cell types (a low dense big stem cell-like phenotype that differentiates in regular epithelial phenotype cells), whereas 32816 grew as a very uniform culture and CAL33 had more filamentous aspect. H&E staining confirmed tumour morphology showing the presence of pleomorphic cells, with nucleus-cytoplasm disproportion, eosinophil dense cytoplasm and very irregular hyperchromatic nucleus with the presence of atypical mitosis (Supplementary figure S1).

Karyotyping of the cells lines by G banding revealed a moderate hyperploidy, with an average number of 47-49 chromosomes per cell, except for CAL33 that showed a near-tetraploidy karyotype. Cytogenetics analysis of the cells lines detected rearrangements involving chromosomes 3, 7, 8, 9, 18, 20 and X. These changes were described as 3p+, i(7q), i(8q), der(X), deletion of 18 and gains of 20. However, conventional cytogenetics showed genomic changes specific for cell lines. For example, in 32816 cell line FISH analysis demonstrated that part of chromosome 3 was translocated on chromosome 12, showed an unbalanced translocation between chromosome 12 and 3 (Figure 1). In 32661 cell line at least two different sub clones were observed. The loss of chromosome 4 was presented in two clones; however, only one of them showed der(18)t(18;?)(p11;?) (See Figure).

DNA copy number aberrations were detected in all HNSCC cell lines analysed. The median number of copy number aberrations for tumour cell line was 97. DNA copy number gains were more frequently detected (median: 49, range: 21–91) than losses (median 48, range: 12–112). The higher number of copy number aberrations was presented in CAL33 and 32661 cell lines. Data obtained from aCGH analyses are summarized in supplementary table S1.

In all cell lines the most frequently, minimal overlapping regions of copy number gain were found on 3q11.2-3q29, 7p22.3-7p11.2, 8q11.1-8q24.3, 9p24-p22.3, 9q21.13-9q22.32, 9q31.1-9q31.1, 16p13.2-p11.2, 20p13-q13.2, Xp22.2. Respect to copy number losses, minimal overlapping regions, were detected on 3p14.3-p21, 4q34.3-q35.1, 12q24.31, 16q22.1, 18q12, 18q21, 18q22.1-q23. In CAL33 cell line, losses on 5p/q, 21q21 and gains on 13q were also

detected. Losses on 2p25 and 2p22.3-2p14 were detected only in 32661 cell line (Supplementary figure S2).

Cell lines response to treatments

Most of the cells had similar growths rates with lower split ratios in 32816 and 32661 cell lines. Viability curves are represented in the figure 2. Although each cell lines presented small differences, globally, chloroquine treatment produced an important decrease in cell viability in all the concentrations, so 25µM minimum concentration was chosen for following experiments. On the contrary, decitabine did not show any pronounced viability decrease and we decided to use the highest concentration of 7.5µM. Between this opposite results we found the effects with metformin, paclitaxel and panobinostat, with a dose-dependent activity. In these experiments, minimum visible decreased viability concentration was chosen for the cytometry and protein expression analysis (8mM metformin, 10nM paclitaxel and 50nM panobinostat) (Table 1). The highest changes were observed in the cell line derived from laryngeal cancer (32860) and the one derived from HPV+ oropharyngeal tumour (32816) in which cells seem to have more viability than the rest.

After the treatment with the selected concentrations obtained by the MTT viability assay, cell cycle cytometry was performed (Figure 3). Mostly, comparing with the untreated controls, chloroquine treatment produced a slight increase in G0/G1, a little decrease in G2/M and a reduced increment in cell death. This data was most accused in 32816 HPV+ oropharyngeal cell line. On the contrary, decitabine caused a G0/G1 descent and a higher percentage in S and death phases. In metformin treatment the most visible result is a G0/G1 drop, resulting in S phase rise. Metformin produced a G0/G1 descent and consequently, an increase in S and G2/M phases, although in 32816 these effects were not found. Paclitaxel is the treatment option that showed the highest death rates, increasing respect the untreated control with an average of 35%, except in 32860 laryngeal cell line which seem to be more resistant to taxol. Lastly, panobinostat produced a diminished G0/G1 and an important increment in G2/M cell cycle phase, producing also a little cell death rise. Again, 32816 showed different results. All this results were statistically significant ($p<0.05$).

Western Blot analysis had similar tendency in protein expressions varying on the drug treatment in the four cell lines. Treatment with autophagy inhibitors such as chloroquine produced higher expression of p62 (except in 32816 where the expression was constant) and a slight decrease in beclin1 and LC3B, especially in LC3B-I. Regarding paclitaxel treatment, it did not follow a tendency, changing phospo-mTOR expression depending on cell type whereas the rest of the proteins were invariable or just a little descent in beclin and LC3B-I in 32816 cell line was observed. Autophagy inductors had different behavior in autophagy proteins expression. Treatment with decitabine altered phospo-mTOR and p62 expression depend on the cell line, but showed increased beclin1 in all cell lines and higher LC3B-II levels except in CAL33 and 32816 cell lines. On the contrary, metformin produced a strong inhibition of phospo-mTOR, reduced p62 expression except in 32661 and rise in LC3B expression with the exception of 32816. Lastly, panobinostat induced mTOR, beclin1 and LC3B up-regulation, effect shown in all the cell lines (Figure 4).

Discussion

Despite multi-modality therapy, HNSCC continues to have a 5-year survival of just over 50%. The search of new drugs to treat this illness remains critical to improve long-term survival. New therapeutic approaches based on autophagy in HNSCC could give us this new alternative to limited current treatments. For that reason, we selected five approved drugs to treat different diseases that can be implicated in the modulation of the autophagolysosome formation.

Characterization of cell lines showed the molecular differences between tumour locations although it was higher in karyotyping than CNV analysis. Array CGH of all cell lines revealed gains on 3q, 7p, 8q, 9p, 9q, 16p, and 20 and losses on chromosome 3p, 8p, 12q, and 18. These data were in good concordance with published data[8] from HNSCC cases available on www.progenetix.org[21]. In addition, the results of array CGH matched the described karyotype determined by conventional cytogenetics. Isochromosome 7p and 8q were clearly verified by DNA gains of 7p and 8q in array CGH. 8q gains and i(8q) were some of the most common structural and copy number alterations in HNSCC and have been previously reported many times in varied studies[22]. Although it is much less frequently reported in the literature, the related gain of 7p has already been found in HNSCC. The distal part of chromosomal arm 3q was another chromosomal region subject of DNA copy number present in the cell lines. These changes were previously found in HNSCC associated with adverse patients outcome[22]. Several candidate proto-oncogenes reside inside the minimal overlapping region 3q25-qter detected in our HNSCC cell lines. One of these proto-oncogenes is *PIK3CA*. The PI3K signalling pathway is involved in multiple cancer-related functions such as cell survival, proliferation and cell migration[22], as well as an important autophagy effector. An activation of this pathway was considered to play a crucial role in HNSCC tumourigenesis founding genomic amplification and consecutive high PIK3CA protein expression in precancerous dysplasias of HNSCC and in primary and metastatic oral squamous cell carcinomas[23]. Further attractive candidate oncogenes in this region, as *SEC62* and *SOX2,* have been shown to be overexpressed in HNSCCs, affecting the metastatic potential of cancer cells[24].

Chloroquine is one the most specific autophagy inhibitor[25]. It is a lysosomotropic agent able to pass through the lysosome membrane increasing the pH of the organule, preventing from cellular degradation and inhibiting autophagy. Its origin was as antimalarial agent although nowadays it is used for immunological, reumatic and skin diseases[14,26]. The incorporation of chloroquine in chemotherapy and radiotherapy regimens has showed antitumour capacity in different neoplasias, potentiating their effects[27]. Our results with 25µM chloroquine showed a slight increase in G0/G1, a little decreased in G2/M and a reduced increment in cell death. This result was also reported in different *in vitro* studies in which viability reduction was only detected from 20µM and increment in death only appeared at high doses, such as colon[28] and hepatocellular carcinoma[29] with the same effect on G0/G1 cell cycle arrest[30]. Moreover, chloroquine treatment showed an enhanced autophagy, may be a promising drug for cancer therapy.

Metformin, a biguanide, is commonly used as first-line oral treatment for type 2 diabetes mellitus. Although its mechanism of action is diverse, metformin, through AMPK induction, suppress mTORC1 activation and signaling[31], an important negative autophagy regulator[32]. Last decade, some epidemiological studies have identified an association between metformin treatment and lower risk to cancer development included HNSCC[33]. For that reason, some clinical trials have been proposed, showing treatment benefit as well[34]. Our results indicated that treatment with 8mM metformin produced a total inhibition of cell viability, similar to previous studies where metformin alone reduce cell viability in a dose-dependent manner[35]. Cell cytometry analysis showed a G0/G1 drop, resulting in S and G2/M phases rise. Different tumour cell lines treated with metformin proved a parade in G0/G1[36] and G2/M[37]. The difference between these results could be related with the concentration of the drug, because in other series metformin was used at higher concentrations and with prolonged treatment. Anyway, metformin treatment showed an autophagy rise in our cell lines, could being used in concomitation with other drugs against HNSCC.

In our HNSCC cell lines, paclitaxel showed an important increment in cell death. The active ingredient obtained from *Taxus brevifolia* targets microtubule formation, important in processes of cellular structure maintain, motility and vesicle transport through cytoplasm[38]. Inhibiting microtubules disassembly, paclitaxel promotes G2/M arresting, favouring apoptosis[39]. Our results corroborate this effect, also defined in other HNSCC series with similar concentrations[39]. This drug has a high antitumour potential, being accepted in the use of different neoplasias, among them in HNSCC. In HNSCC combination taxol with platin and 5-fluorouracil (known as TPF) is the best induction chemotherapy regimen for locally advanced tumours[40]. Apart from apoptosis induction, paclitaxel regulates another programmed cell response such as autophagy through two different mechanisms: inhibition of autophagosome formation and trafficking[41]. However, autophagy protein expression did not show important alterations due to paclitaxel, maybe because of the main effect in apoptosis. So, the interplay between taxane treatment, autophagy and cell death should be taken into account as novel therapeutic implication of this widely used chemotherapy agent.

During tumourigenesis, many of the changes in gene expression are due to an altered epigenetic regulation such as DNA methylation and histone deacetylation[42]. Histone deacetilases (HDACs) are enzymes that regulate genetic expression through the removal of acetyl groups from lysines residues of histones producing a condense chromatin state and genetic silencing[43]. Panobinostat (LBH 589) is a potent pan-inhibitor of histone deacetilase class I, II and IV. Panobinostat acts through different pathways, allowing expression of tumour suppressor genes between others, causing an antiproliferative activity. It has been approved for some neoplasias, mainly in multiple myeloma[44]. HDACs inhibitors suppress cell proliferation by activation of cell-cycle checkpoints at G1/S or G2/M[44]. In our study it caused cell proliferation inhibition at 50mM, producing a G2/M arrest and cell death. This result has been reported in other *in vitro* studies like HNSCC[45]. Autophagy induction by HDACi was also described[46] as it was observed in our cell lines with an important autophagy stimulation after treatment with panobinostat.

DNA hypermethylation, by DNMTs, is one of the most studied epigenetic mechanisms in cancer[47]. So, the application of DNMTs inhibitors is a good therapeutic tool for cancer,

including HNSCC. Decitabine (5-aza-2´-deoxicitidine) is a cytidine analogue which inhibit DNMTs preventing the methylation[48]. Because decitabin incorporates during the synthesis, is it a specific S phase agent, as we showed in our results where decitabine produced a fall in G0/G1 and a higher percentage in S and death phases. Its use has been approved for myelodysplastic syndromes and acute myeloid leukemia[49]. However, decitabine is commonly used in combination of chemotherapy or radiotherapy, enhancing the effects of this treatments[50]. That could be explained the poor effects on the viability assay, where decitabine did not produced a decrease in cell viability. However, decitabine treatment caused an effect on autophagy, with a slight activation. So, the use of decitabine as an autophagy activator in combination with other drugs could be used as an alternative for current treatments.

Our results showed that use of modulators of autophagy such as metformin, panobinostat, paclitaxel and chloroquine had an inhibiting activity of cellular proliferation. These drugs and decitabin produced changes in cell cycle phases and a deregulation in the autophagic process. According to our preclinical results, these agents could be used in HNSCC in monotherapy or in combination with the current therapies to modify the autophagy mechanisms, an important pathway in these tumours. This synergy could be most appropriate in HPV+ tumours, due to the results from the oropharyngeal squamous carcinoma cell line.

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carcinoma. *PLoS One* **9,** e112880 (2014).

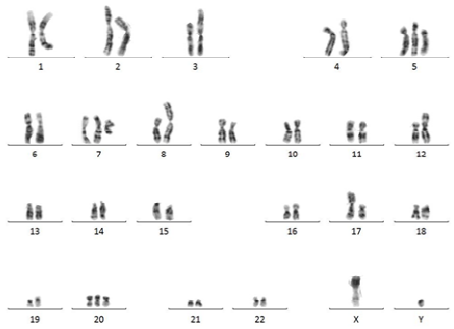
Table 1. Autophagy modulator drugs used in this study.

Drug	Trade name	Origin	Concentrations	IC50
Chloroquine	Aralen	Sigma-Aldrich	10, 25, 50, 75, 100, 125µM	25µM
Decitabine	Dacogen	Sigma-Aldrich	0.5, 1, 2, 3, 6, 7.5µM	7.5µM
Metformin	Glucophage	Sigma-Aldrich	0.5, 1, 2.5, 5, 6.5, 8 mM	8mM
Paclitaxel	Taxol	Sigma-Aldrich	2.5, 5, 10, 15, 20, 30nM	10nM
Panobinostat	Farydak	Novartis	10, 25, 50, 75, 100, 150nM	50nM

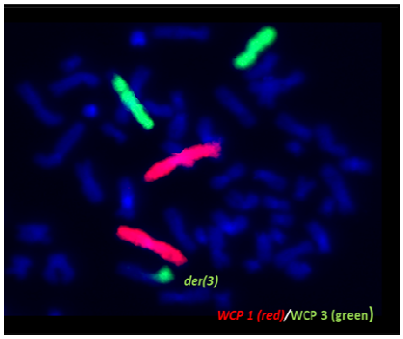
Table 2. Patient characteristics.

	CAL33	32661	32816	32860
Tumour site	Tongue	Vallecula	Tonsil (oropharynx)	Supraglotis (Larynx)
Sex	Male	Male	Male	Male
Age	69 years	71 years	73 years	68 years
Stage	NA	T4N2b	T3N2b	pT3N1
Tobacco	NA	Yes	Yes	Yes
Alcohol	NA	Yes	Yes	Yes
HPV	NA	Negative	Positive	Negative
Grade	Moderately differentiated	Moderately differentiated	Poorly differentiated	Poorly differentiated

A.

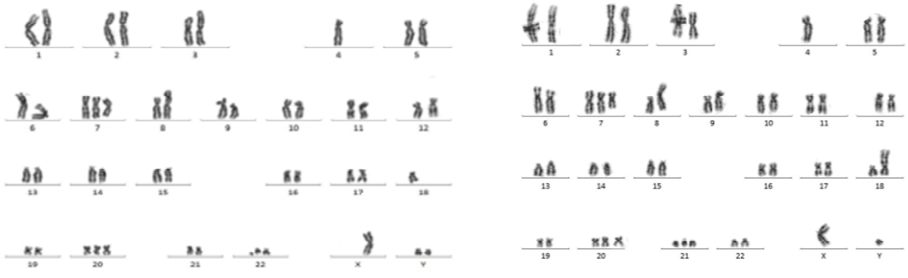


B.



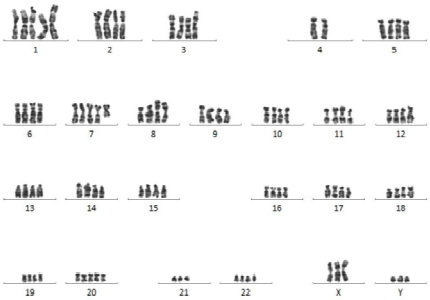
32816 karyotype: 47~49 YY, der(X)add(X)(p?);add(3)(p24),+7,+i(7)p10),i(8)(q10), +del(9)(p11-p24),der(12)t(3;12)(q11;p13),-18,+20 [cp10]

C.



32661 Karyotype: 47~50 YY, der(X)add(X)(p?);add(3)(p24),-4, +i(7)p10), i(8)(q10), del(9)(p11-p24),-18,+20.[cp10]/ 47~50 YY, der(X)add(X)(p?);add(3)(p24),-4, i(7)p10), i(8)(q10), del(9)(p11-p24),der(18)t(18;?)(p11;?),+20 [cp4]

D.



CAL33 karyotype: 96~ YYY, der(x)t(x;?)(p22;?)x3, + der(1)add(1)(p36), der(3)(p25), -4, -4, +i(7)(p10), i(8)(q10)X2, del(9)(p21), -18, +20, -21.

Figure 1. Representative karyotypes of the most different cell lines. A. 32816, C. 32661 and D. CAL33. Figure B demonstrated by FISH analysis the translocation between chromosome 3 and 12.

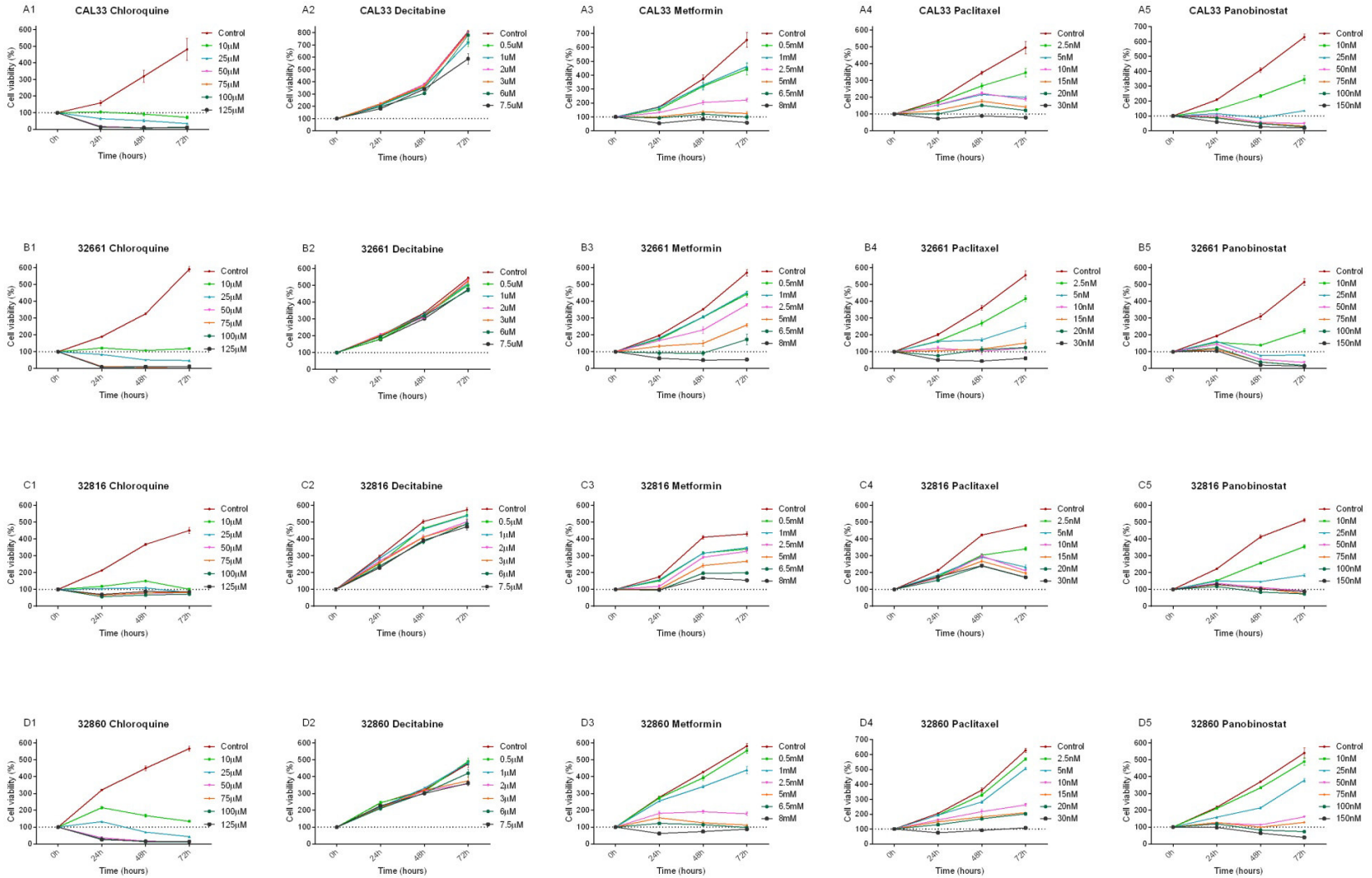


Figure 2. Cell viability graphs by MTT assay during 72 hours. Regarding cell lines A) CAL 33 (tongue), B) 32661 (vallecula), C)32816 (oropharynx), D) 32860 (larynx) and respect to drug treatment 1) chloroquine, 2) decitabine, 3) metformin, 4) paclitaxel and 5) panobinostat. Different drug concentrations are represented by different colours indicated in the right side of the graph. Control untreated cell line shows the normal viability. Y axis represents percentage of cell viability while X axis shows each 24 hours treatment for three days.

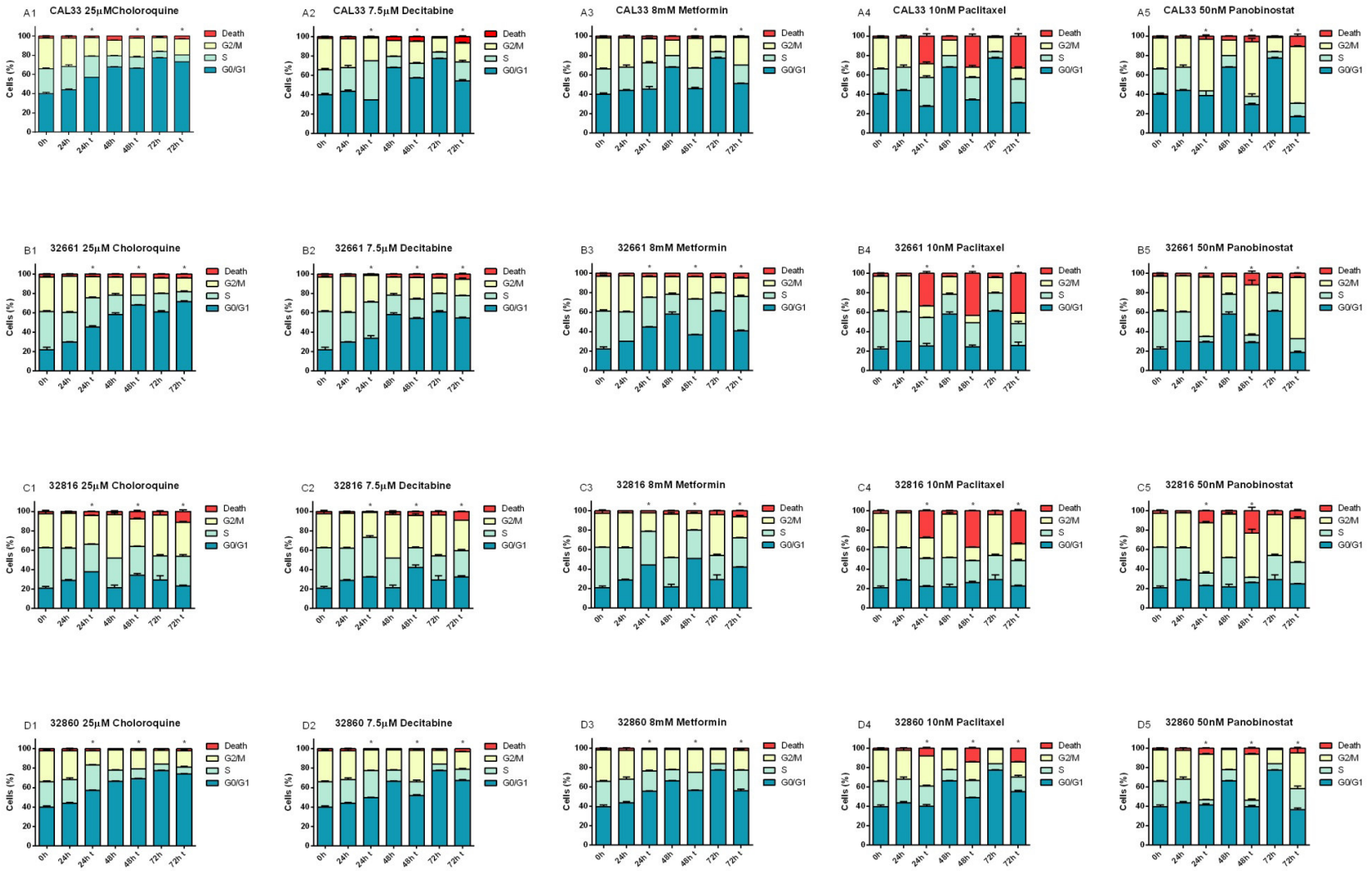


Figure 3. Cell cycle phase by flow cytometry assay during 72 hours in untreated and treated (t) cell cultures. Regarding cell lines A) CAL33 (tongue), B) 32661 (vallecula), C) 32816 (oropharynx), D) 32860 (larynx) and respect to drug treatment 1) 25µM chloroquine, 2) 7.5µM decitabine, 3) 8mM metformin, 4) 10nM paclitaxel and 5) 50 nM panobinostat. Cell cycle phases are indicated by different colours (right side of the graph). Y axis represents percentage of cell cycle phase while X axis shows each 24 hours treatment for three days. Statistically significant results respect the untreated controls are indicated by an asterisk.

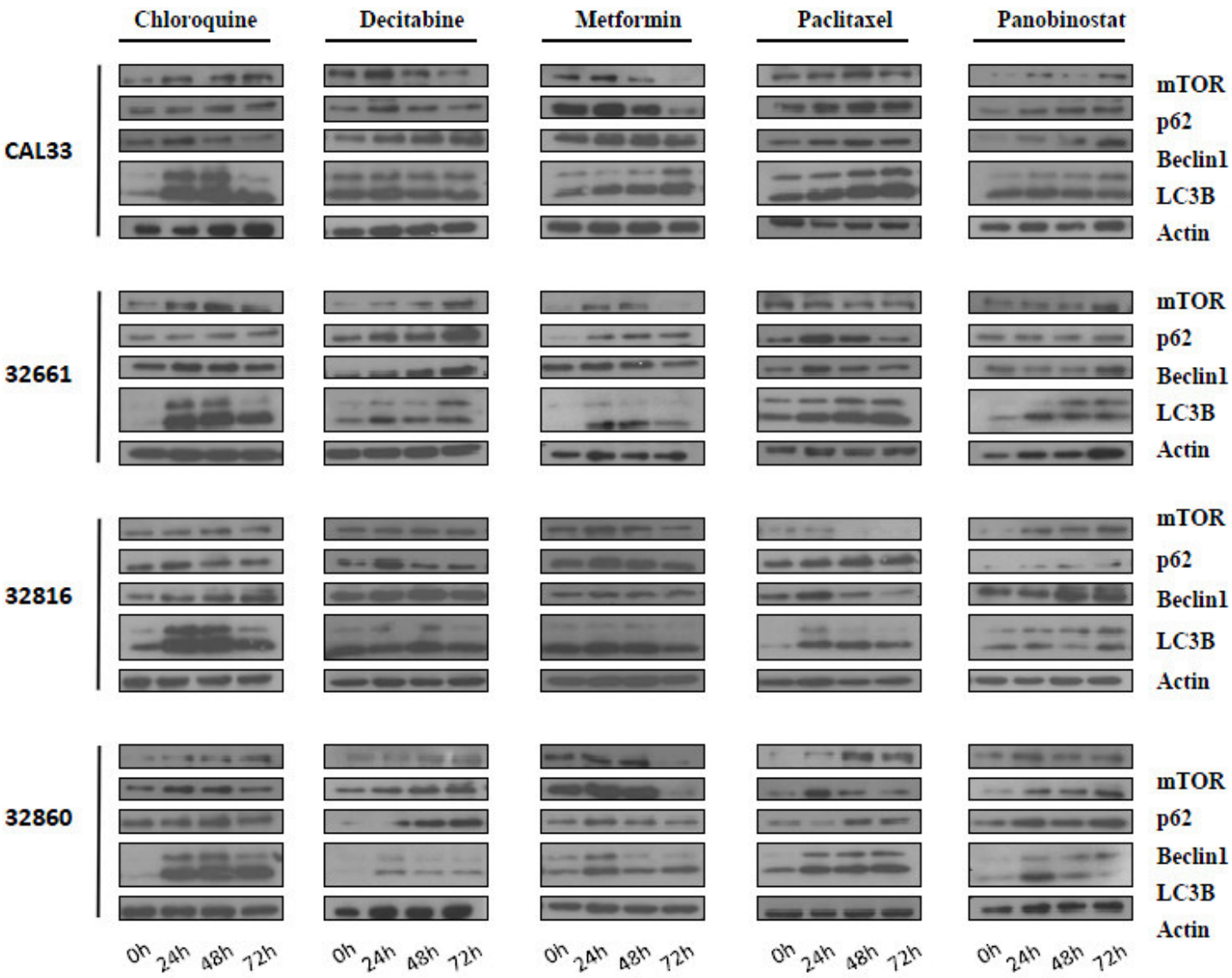
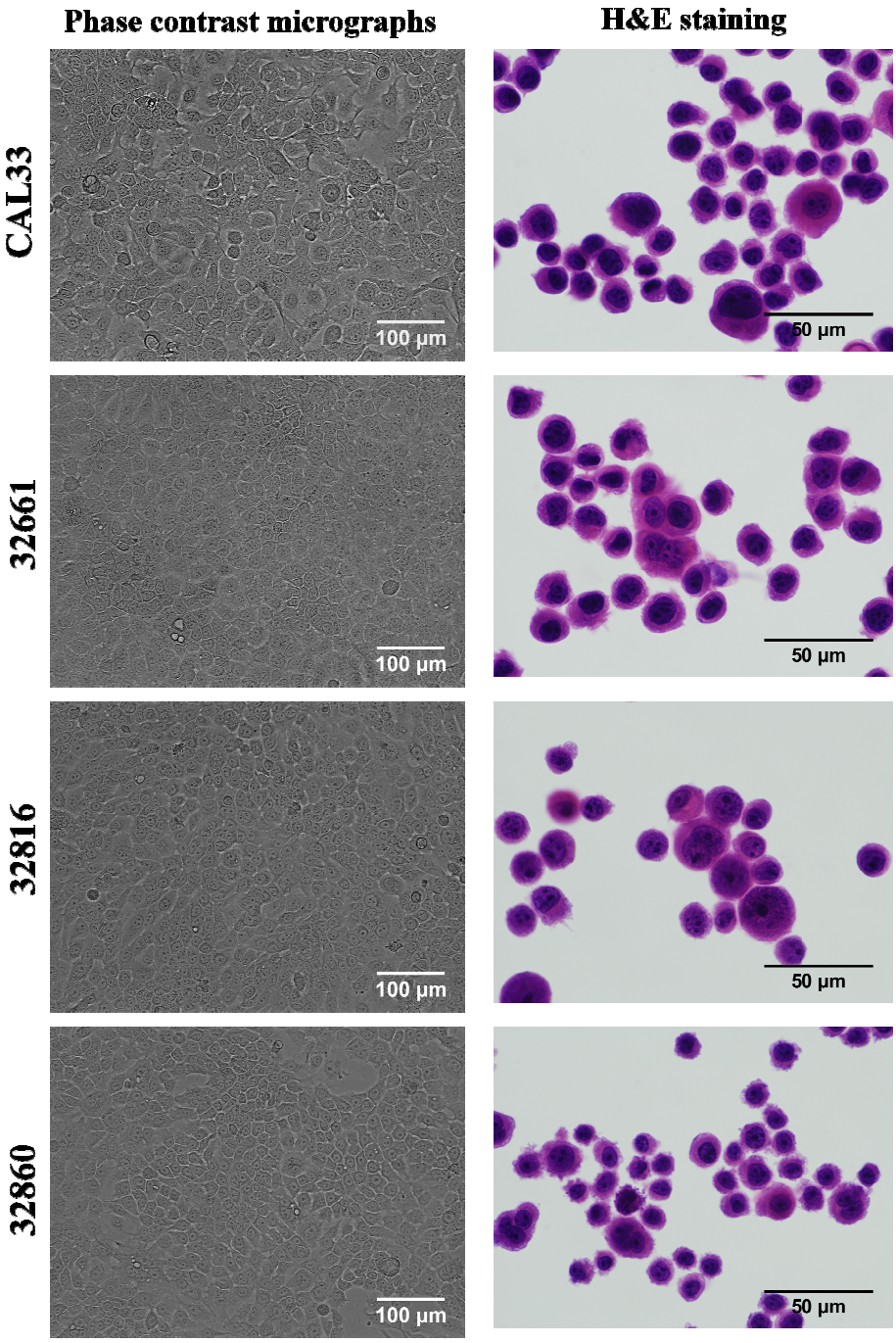


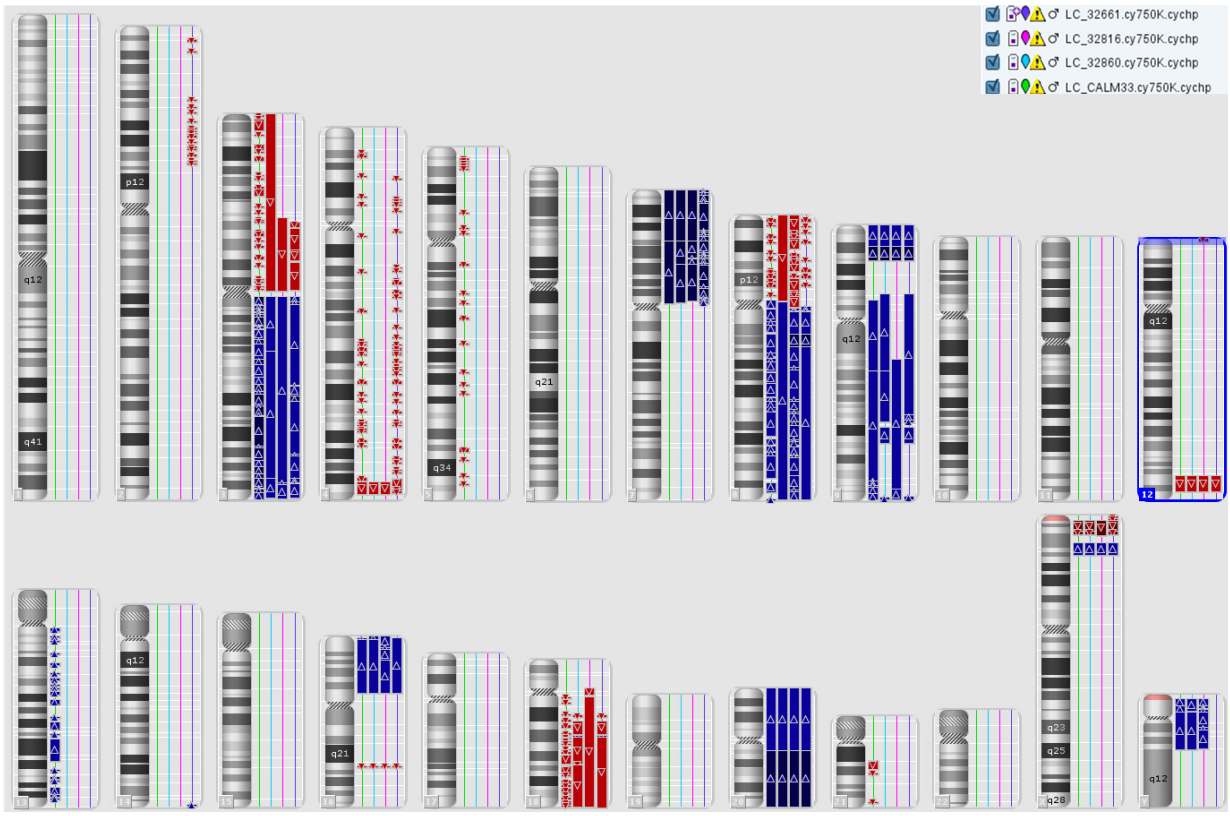
Figure 4. Western blot of autophagy related proteins: phosphor-mTOR, p62, beclin1 and LC3B (I and II). Actin served as a loading control. We show the effect after the treatment with different autophagy modulators during 72 hours in each HNSCC cell line.

Supplementary table S1. Karyotypes and chromosomal regions of DNA copy number aberrations detected by aCGH in four HNSCC cell lines.

Type Cell Line	Copy Number Changes Detected by array CGH		Karyotype
	Gains	Losses	
LC_32661	3q11.1-3q29,7p23.3-7p11.2, 8q12.1 8q24.3, 9p24.3-p22.2, 9p13.3-9q22.33, 9q31.1-9q31.3, 9q34, 14q32.33,16p13.3-16p11.2, 18q12.3, 20p13-q13.33, Xp22.2-p22.12	2p25.2-2p13.3, 3p14.3-3p11.1, 4p15, 4p14, 4p13, 4q12, 4q13.3, 4q21.1-q34.1, 4q34.3-4q35.2, 8p23.3-8p12, 8p11.23, 12q24.31-q24.33, 18q12.1, 18q12.2-18q12.3, 18q12.3-18q23, Xp22.33-p22.31	47~50 YY, der(X)add(X)(p?);add(3)(p24),-4, +i(7)p10), i(8)(q10), del(9)(p11-p24),-18,+20.[cp10]/ 47~50 YY, der(X)add(X)(p?);add(3)(p24),-4, i(7)p10), i(8)(q10), del(9)(p11-p24),der(18)t(18;?)(p11;?),+20 [cp4]
LC_32816	3q11.1-q29, 7p23.3-p11.2, 8q13.1-q24.3, 9p24.3-p22.2, 9q13-34.13, 9q34.2-q34.3, 16p13.33-p11.2, 20p13-q11.2, 20q11.2-q13.33, Xp22.2-p22.12, Yp11.31-p11.2, Yp11.2-q11.23	3p21.1-p11.2, 4q34.3-q35.2, 8p23.3-8p22, 8p21.3-8p21.1, 8p12, 12p13.33, 12q24.31-q24.33, 16q22.1,18p11.21-q11.1, 18q11.2-q23	47~49 YY, der(X)add(X)(p?);add(3)(p24),+7,+i(7)p10),i(8)(q10), +del(9)(p11-p24),der(12)t(3;12)(q11;p13),-18,+20 [cp10]
LC_32860	3q11.1-q13.33, 3q13.33-q27.3, 3q27.3-3q29, 7p23.3-p15.2, 7p15.2-p14.1, 7p14.1-p11.2, 8p11-18q24.3, 9p24.3-p23, 9p23-p22, 9p13.3q21.13, 9q21.13-q22.33, 9q31.1-q31.3, 9q34,16p13.3-p11.2, 18q12.3, 20p13-q11.22, 20q11,22-q13.33, Xp22.31-p22.2, Xp22.2-p22.13	3p26.3-p11.1, 4q34.3-q35.2, 8p23.3-p11.1, 12q24.31-q24.33, 16q22.1,18q12.2-q12.3,18q12.3-q21.31, 18q21.31-q23	47~49 YY, der(X)add(X)(p?);add(3)(p24),+7, +i(7)p10), i(8)(q10), del(9)(p11-p24), -18,+20
LC_CAL33	3q11.2-q29, 7p22.3-p15.2, 7p15.2-p11.2, 8p11.1-8q24.3, 9p24.3-p24.1, 9p13.1-q34.3,13q12.1-q13.3, 13q14.11, 13q14.12-, 13q14.3, 13q21.1, 13q21.33, 13q31.1, 13q31.3,13q32.3, 13q32.3, 13q33.2-q33.3, 16p13.3-p11.2, 20p13-q11.22, 20q11.2-q13.33, Xp22.31-p22.12	3p26.3-p36.1, 3p35.3, 3p25.1, 3p24.3, 3p23, 3p22, 3p21.31, 3p14.2, 3p14.1, 3p12.1, 4p15.33, 4p15.2, 4p14, 4q13.3, 4q22.1, 4q25, 4q28.2, 4q31.3, 4q34.3-q35.1, 5p15.33-p15.2, 5p12.2, 5p13, 5q12.1, 5q13.3,5q14.3,5q21.1, 5q23.1, 5q33.1-q33.3, 5q35, 8p23.3-p23.1, 8p22, 8p21.3, 8p12, 8p11.21, 12q24.31-q24.33, 16q22.1, 18q11.2, 18q12.1, 18q12.2, 18q12.3, 18q21.2, 18q22.1-q23, 21q21.1-q21.3, 21q21.3, 21q22.3	96~ YYY, der(x)t(x;?)(p22;?)x3, + der(1)add(1)(p36), der(3)(p25), -4, -4, +i(7)(p10), i(8)(q10)X2, del(9)(p21), -18, +20, -21.



Supplementary figure S1. Phase contrast microscope photographs and H&E from the commercial cell line CAL33 and the established cell lines 32661, 32816 and 32860.



Supplementary figure S2. – Whole genomic array CGH profile of the four HNSCC cell line. Karyoview of identified genomic abnormalities (right of each ideogram). Gains are shown in blue bars (regions of CN2–3 in light blue and CN≥3 in dark blue). Losses are shown analogically in red bars (regions of CN1–2 in light red and CN≤1 in dark red). Green line represents tongue carcinoma cell line CAL33, light blue laryngeal cell line 32860, pink oropharyngeal carcinoma cell line 32816 and purple vallecula cell line 32661.

CONCLUSIONES

Primera: las características epidemiológicas de nuestra serie confirman la importancia del consumo de tabaco y alcohol como factores etiopatogénicos de CECC en la población española y la escasa incidencia de tumores derivados de la infección del virus del papiloma humano, habitualmente acompañados del consumo de tabaco y alcohol, dando lugar a un modelo de carcinogénesis mixto.

Segunda: el estudio de asociación de genes candidatos pone de manifiesto que variaciones en genes implicados en reparación de DNA, inflamación, autofagia, oxidación y apoptosis podrían desempeñar un papel en la susceptibilidad a desarrollar carcinoma escamoso de cabeza y cuello en la población española variando su implicación de acuerdo con la localización tumoral, lo que confirma la diversidad de estos tumores.

Tercera: los tumores HPV negativos presentan un menor porcentaje de mutaciones en relación con los tumores HPV positivos. Dado que los tumores HPV positivos tienen una mejor evolución, el estudio de la carga mutacional podría ser utilizado como factor pronóstico.

Cuarta: nuestros resultados del efecto *in vitro* de moduladores de autofagia muestran que el uso de estos fármacos puede ser una opción terapéutica prometedora en el tratamiento de los CECC. El hecho de que estos fármacos hayan sido aprobados por la FDA o la EMA facilita el desarrollo de ensayos clínicos para comprobar estos efectos en humanos.

Quinta: nuestros resultados corroboran la heterogeneidad subyacente de los tumores incluidos bajo el término de CECC. El conocimiento de las características moleculares de cada localización permitirá un tratamiento individualizado de los mismos.

CONCLUSIONS

First: epidemiological analysis confirmed the importance of tobacco and alcohol consumption as pathogenic factors in HNSCC in Spanish patients and the low incidence of HPV infection derived tumours. In this case it is usually accompanied by tobacco and alcohol intake, giving rise to a mix carcinogenesis model.

Second: gene candidate association study showed that variants in genes implicated in DNA repair, inflammation, autophagy, oxidation and apoptosis could play a role in the susceptibility to develop HNSCC in the Spanish population, changing its implication according to tumour location, confirming the diversity of these tumours.

Third: HPV negative tumours present a low percentage of mutations in relation with HPV positive ones. As HPV positive have better evolution, the study of the mutational burden could be used as a prognostic factor.

Fourth: our results of the *in vitro* effect of autophagy modulation show that the administration of these drugs could be a promising therapeutic option in the treatment of HNSCC. The fact that these drugs have been previously approved by the FDA or EMA, facilitate the development of clinical trial to check these effects in humans.

Fifth: our results confirm the heterogeneity in these tumours included under the term of HNSCC. The knowledge of the molecular characteristics of each location would allow to define a more individualized treatment.

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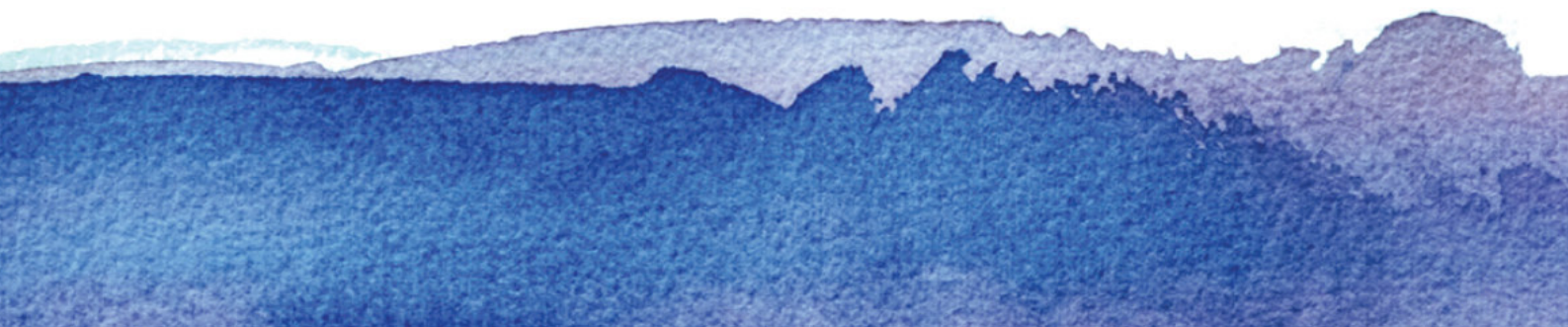
SUMMARY



UNIVERSITY OF SALAMANCA

DEPARTMENT OF MEDICINE

DOCTORAL THESIS-INTERNATIONAL DOCTORATE MENTION

MOLECULAR STUDY OF HEAD AND NECK SQUAMOUS CELL CARCINOMA IN THE SPANISH POPULATION

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INTRODUCTION

Head and neck cancer (HNC) includes a set of diverse tumours located in the lips, oral cavity, pharynx, larynx, salivary glands and thyroid glands among others[1]. Approximately 600.000 new cases are diagnosed per year, being the sixth cancer on incidence worldwide. Treatment of early stages includes surgery and/or radiotherapy, while locally advanced tumours are also treated with platin-based chemotherapy and biological therapies[2]. Only 40-50% of patients reach the five-years survival rate[3], causing and annual death of 271.000 patients[4]. Regarding its histological type, not all HNC are the same, prevailing with a 90% the squamous cell carcinomas[1].

Head and neck squamous cell carcinoma (HNSCC) is a multifactorial process associated with a variety of risk factors. At least 75% HNSCC are attributable to the combination of cigarette smoking and alcohol drinking, the most classic carcinogens[5]. Diverse epidemiological studies have also revealed the existence of other environmental and genetic related factors. Similar to other tumours, viral aetiology has shown an implication in the development of HNSCC, predominating Epstein-Barr virus (EBV) infection in nasopharynx and human papillomavirus (HPV), mainly subtype 16, in oral cavity and oropharynx tumours[6]. The carcinogenesis procedure triggered by viral infection define a different entity than that caused by tobacco and alcohol[8] classifying HNSCC in two main prognostic and therapeutic groups, being HPV negative tumours associated with an aggressive course and worst prognosis than HPV positive ones[9] (Table 1). Despite the defined role of environmental factors there is also an evidence of familial aggregation and increased cancer risks amongst HNSCC relatives[9], suggesting the existence of genetic predisposition factors[10].

Table 1. Main characteristics of HPV+ and HPV- HNSCC.

Characteristic	HPV negative HNSCC	HPV positive HNSCC
Incidence	Decreasing	Increasing
Aetiology	Tobacco and alcohol	Oral sex
Age	Above 60 years	40-60 years
Socioeconomic status	Low-medium	High
Field cancerization	Yes	Unknown
***TP53* mutations**	Frequent	Infrequent
Predilection site	None	Oropharynx
Stage	Advanced	Early
Prognosis	Poor	Favourable

HYPOTHESIS AND OBJECTIVES

Our hypothesis is that HNSCC represent a mixture of tumours with epidemiological and molecular differences according to its location. Thus, the aim of our study has been to characterized epidemiological factors as well as genetic alterations in HNSCC in relationship with susceptibility, response and toxicity to treatment. Because of the poor response rates, the study of new cancer related pathways, such as autophagy, could define a new targeted therapy for these tumours.

MATERIAL AND METHODS

Patients

Patients were recruited in two different clinical trials:

- 459 patients and 259 controls were included in the TTCC-2010-05 observational multicentre study. The inclusion criterion was histologically confirmed HPV negative HNSCC patients from larynx, oro/hypopharynx and oral cavity carcinomas. Controls were hospitalized patients without tumour history and paired by age, sex, smoking and alcoholism habit with the HNSCC cases.

DNA isolation and genotyping in TTCC-2010-05 clinical trial

DNA was extracted from leukocytes of peripheral blood by phenol-chloroform method. Genotyping was performed using the TaqMan® Allelic Discrimination Assay[11] (Thermo Fisher, Waltham, MA) in those single nucleotide polymorphisms (SNPs) where the probes are available while in the others, genotyping was analyzed by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP).

- 150 of 530 formalin-fixed paraffin-embedded (FFPE) blocks from pretreated HNSCC patients were included in this study (GEN-TTCC-2007-01). All of them belong to the clinical trial TTCC-2007-01 (NCT00716391), carried out between 2008 and 2013[12]. Eligible patients: previously untreated unresectable locally advanced (Stage III-IV) tumours (from oral cavity, oropharynx, larynx, hypopharynx), ECOG performance status 0–1. Treatment: docetaxel, cisplatin, 5-fluorouracil (TPF)-based induction chemotherapy by 3 cycles; then, if objective response achieved, they were randomized to: conventional radiotherapy (RT) + platin versus conventional RT + cetuximab.

DNA extraction in GEN-TTCC-2007-01

Percentage of tumour cells was measured in hematoxylin-eosin tumour sections from FFPE blocks by central pathologist. Between five and ten 10µm FFPE sections from diagnosis blocks were treated with deparaffinization solution and DNA extraction was done using QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany).

DNA quality evaluation and targeted NGS

Sequencing was performed by TruSight®Tumor 26 panel that includes a set of 174 amplicons in exons of 26 cancer-associated genes, following TruSight® Tumor 26 Reference Guide (Illumina, San Diego, CA). Sequencing was performed in a NextSeq 500 System and analyzed in the Variant Studio Software (Illumina, San Diego, CA). Only somatic variants over 5% of frequency with a quality score >500 in the bi-directional sequencing quality filter were reported. Those variants of uncertain significance were considered pathogenic if at least two *in silico* prediction tools (SIFT and PolyPhen) classified them as

deleterious/probably damaging[13], and they were defined as likely pathogenic in the Catalogue Of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic) or the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/clinvar) databases.

Assessment of HPV status

FFPE sections were deparaffinized and exposed to 10mM citrate buffer antigen retrieval at 92ºC for 30 minutes. HPV status was carried out by p16 immunohistochemistry (IHC), a surrogate marker for HPV infection[14], using a $p16^{INK4a}$ mouse monoclonal antibody (Cell Marque, Rocklin, CA). Percentage of p16 staining was measured and only those tumours >70% nuclear and cytoplasmic p16+ were considered positive.

Clinical data

Aetiological and clinicopathological data were recorded in specific questionnaires. The studies were approved by the University Hospital of Salamanca and the local ethics committees. All participants signed the provided written informed consent designed for this project. All data were treated with the security measures established in compliance with the Protection of Personal Data Organic Law 15/1999, 13th December, and safe-keeping by the University Hospital of Salamanca in its specific hospital server.

***In vitro* experiments**

Establishment of new HNSCC-derived cell lines

Surgically resected fresh HNSCC tumour biopsies from Othorhinolaryngology operating theatre were received in aseptic conditions. Pieces were cultured in a dish with enriched RPMI 1640 medium with 20% heat-inactivated fetal bovine serum (FBS) + 2% penicillin/streptomycin(Gibco-Life Technologies, Carlsbad, CA). After 30 passages, three different cell lines from laryngeal (32860), vallecula (32661) and HPV+ oropharyngeal (32816) tumours were established.

Cell characterization

Phenotype was characterized by hematoxylin and eosin staining, and molecular characterization was performed by copy number analysis Affymetrix® CytoScan 750K Array (Affymetrix, Santa Clara, CA) and karyotyping. These analyses were done in the three new established cell lines and the commercial cell line from tongue carcinoma CAL33 (ATCC, Rockaville, MD).

Drug screening

a) *Cell viability assay*

   $10x10^3$ cells were seeded on a 24 well plate and were treated with a battery of autophagy modulators such as: Panobinostat, Decitabine, Chloroquine, Paclitaxel and Metformin. Concentrations of the drugs are indicated in table 2. Treatments were done for 72 hours, measuring each 24 hours the viability of the cells by cellular metabolic function using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO).

b) Flow cytometry

It was performed with the optimal concentration calculated by the viability assays according to the minimum inhibitory concentration in all the cell lines, in order to analyze the effect on the cell cycle induced by selected drugs. Cells were seeded in Petri dishes and treated again for 72 hours. Both supernatant and trypsin-treated adherent cells were taken each 24 hours in treated and controls, 70% cold ethanol fixed and store at -20ºC until the last day when ethanol was removed and they were stained with 50µg/ml propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) in 1ml of PBS and treated with 100µg/ml RNAase, to removed RNA[15]. After an overnight incubation at room temperature, 30.000 cells cycle was measured in FACScalibur flow cytometer (BD Biosciences, San Jose, CA). DNA histogram of cytometry data was analyzed in the CellQuest Pro software and cell cycle phase percentage was calculated in WinMDI 2.9 software.

Table 2: Autophagy modulator drugs used in this study.

Drug	Trade name	Origin	Concentrations	IC50
Chloroquine	Aralen	Sigma-Aldrich	10, 25, 50, 75, 100, 125µM	25µM
Decitabine	Dacogen	Sigma-Aldrich	0.5, 1, 2, 3, 6, 7.5µM	7.5µM
Metformin	Glucophage	Sigma-Aldrich	0.5, 1, 2.5, 5, 6.5, 8 mM	8mM
Paclitaxel	Taxol	Sigma-Aldrich	2.5, 5, 10, 15, 20, 30nM	10nM
Panobinostat	Farydak	Novartis	10, 25, 50, 75, 100, 150nM	50nM

Protein extraction and western blot

Cells were collected and lysed each day for 72 hours after treatment at specific concentrations. After 45 minutes incubation with cell lysis buffer on ice, lysates were centrifugated. 50ug of proteins were separated in SDS-PAGE and immunoblotted with specific antibodies against LC3B (Novus, ref.NB600-1384, 1:1000), Beclin-1 (Cell signaling, ref. 3738S, 1:1000), p62 (Abcam, ref.ab2627, 1:1000) and phospho-mTOR (Cell Signalling, ref.2972S, 1:1000).

Statistical analysis

The statistical analysis to associate the relation between the different clinical and molecular variables was analyzed by cross tabs and the X^2 test of Pearson. The Odds ratio (OR) and 95% confidence intervals were calculated by logistic regression analysis. Quantitative variable distribution was analyzed by ANOVA test in those examples where the sample followed a parametric distribution ($p>0.05$ in Levene´s test); while in those with a non-parametric distribution; a Mann Whitney U test was applied. In association studies, Hardy-Weinberg equilibrium (HWE) was tested in control population by X^2 test. It was considered the existence of statistically significant differences where the two sided P-value was <0.05.

Propensity Score method (PS) was used to equate patients and controls according to: packs per year consumed (PPY): no smokers, <20PPY and >20PPY, standard unit of alcohol per week (SDU/week): <14SDU/week and >14SDU/week and sex.

Survival analysis was done according to overall survival (OS) and progression-free survival (PFS) by Kaplan-Meier plots and log-rank test p-values were calculated in all the curves. Hazard-ratio was calculated to measure the risk of the event with its 95% confidence interval (95% C.I.) by Cox regression. All these tests were conducted using SPSS software version 21.0 (SPSS Inc., Chicago) and GraphPad Prism software version 6.0 (GraphPad Softare Inc., California).

RESULTS

1. **Epidemiological characteristics of a Spanish cohort of patients diagnosed with squamous cell carcinoma of head and neck. Distribution of risk factors depending on the tumor location**

Major geographical differences in the incidence and location of the primary tumour in HNSCC are consequence of the diverse patterns of tobacco and alcohol consumption in the different regions, as well as genetic variances among populations. Our aim was to determine the epidemiological characteristics of head and neck squamous-cell carcinoma of the (HNSCC) in Spain and the distribution of risk factors based on the most frequent tumour locations (oral cavity, pharynx and larynx).

We included a total of 459 patients (75 oral cavity, 167 oro/hypopharyngeal and 217 laryngeal cancers). Median age at diagnosis was 59 years and the distribution of the patients by age was similar among the different tumour locations. Most of the tumours were diagnosed in locally advanced stages and in pharynx they were diagnosed with a larger locoregional extension (stage IV-A/B) and were more undifferentiated (histological grade 3), concordant with the reported most aggressive tumour location. Most of the patients were male with a similar frequency by location. The lower incidence of HNSCC in women compared to men in our series was explained by the difference in tobacco and alcohol consumption patterns, though this tendency is currently changing[16]. However, there was also a higher proportion of women with cancer in oral cavity and pharynx than in larynx ($p<0.05$). These locations are related with alcohol consumption[6] but after adjustment for this variable, it maintained statistically significant. This could be explained by underreported consumption or the stronger carcinogenic effects of alcohol due to a gender-specific risk in women[17].

Overall, there were not significant differences in epidemiological characteristics between patients who live in urban and rural areas. However, rural patients compared with urban patients were associated with a statistically significant increased risk of pharyngeal cancer regardless cigarette and alcohol intake. This could be related with a higher exposure to tobacco smoke in the oral cavity and pharynx than larynx in the rural patients smoking way or with HPV infection, associated with an earlier sexual debut and more sexual partners[18], implying differences in sexual practices between rural and urban areas.

Most patients had family support, a common fact in the Mediterranean area, and in terms of educational level, belonged to the lowest level (primary or no education)[19]. 166 patients had a family history of cancer, 47% of them HNSCC, and most of them were a first-degree relative of the patient suggesting an increased risk of develop HNSCC in subjects with a family history of the disease, mainly in those with a first-degree relative[9].

Clinical presentation of the disease was related to tumour location ($p<0.05$)[20]. The majority of the patients diagnosed with laryngeal cancer had hoarseness/dysphonia, while in pharyngeal cancer the most frequent sign was the presence of mass in the neck, followed by pain. Finally the presence of ulcerated lesions was the most frequent sign in the case of the oral cavity neoplasm. About half of the patients had symptoms during more than two months before diagnosis and 30.7% suffered weight loss. 35% of the patients had suggestive symptoms of gastroesophageal reflux, a linked risk factor, with no significant differences by tumour location.

Regarding the consumption pattern of tobacco, most of the patients were heavy smokers and began smoking at early age (10-15 years old). The kind of tobacco most consumed was black or a mixture of black and blond tobacco. With regard to alcohol consumption, most of the patients were heavy drinkers. However, the quantity in ethanol grams consumed per week was statistically significant depending on the tumour location, with higher alcohol consumption in patients diagnosed with pharyngeal cancer, the main etiological factor in this location[17].

To our knowledge, this is the first study with these characteristics conducted in Spanish population showing the epidemiological characteristics of HNSCC.

2. Identification of SNPs that influence HNSCC susceptibility

Not all individuals exposed to the carcinogens of tobacco and alcohol will suffer the disease so the identification of genetic variants in important signalling pathways could help to define tumour susceptibility. HNSCC carcinogenesis involves different pathways: carcinogen metabolism, DNA repair, cell cycle, immunity, apoptosis and inflammation[21]. Single Nucleotide Polymorphism (SNP) is the most abundant form of genetic variation, becoming an ideal genetic susceptibility marker[1].

Previously, diverse studies have evaluated the relationship between genetic variants and susceptibility to HNSCC[22–25]. However, the results were inconsistent because they were conducted in different populations with heterogeneity in the study design regarding risk factors, race or ethnicity. In this context, we present the first association study of polymorphisms in genes involved in the main cancer

pathways (Table 3) and the susceptibility to develop HNSCC within a Spanish population whose control group was totally paired in the most important risk factors (sex, tobacco and alcohol consumption), assuming the result into a greater extent to the SNPs.

Table 3. SNPs select in the study.

FUNCTION	GENE	RS	ID	Change
Oncogenes and tumour suppressor genes	*TP53*	1042522	C_2403545_10	Pro72Arg
	MDM2	2279744	PCR-RFLP	Thr309Gly
	KRAS-LC6	61764370	PCR-RFLP	Intronic
	EGFR	2227983	C_16170352_20	Lys521Arg
Base excision repair (BER)	*XRCC1*	25487	C_622564_10	Gln399Arg
		1799782	C_11463404_10	Arg194Trp
	APEX	1130409	C_8921503_10	Asp148Glu
Nucleotide excision repair (NER)	*ERCC2(XPD)*	13181	C_3145033_10	Lys751Gln
	ERCC1	11615	C_2532959_10	Asn118Asn
	XPC	2228000	C_16018061_10	Ala499Val
Double-strand break repair genes	*XRCC3*	861539	C_8901525_10	Thr241Met
		1799794	C_2983904_10	c.-316A>G
	KU70	2267437	C_15872242_20	c.-731C>G
Inflammatory genes	*IL1*	16944	C_1839943_10	c.-598T>C
	IL2	2069762	C_15859930_10	c.-385T>G
	IL6	1800795	C_1839697_20	c.-237C>G
	IL10	1800872	C_1747363_10	c.-627A>C
	TNFA	361525	C_2215707_10	c.-418G>A
Apoptotic genes	*NOD2*	2066844	C_11717468_20	Arg702Trp
		2066845	C_11717466_20	Arg908Gly
	BAX	4645878	C_27848291_10	Intronic
	BCL2	2279115	C_3044428_30	Intronic
Autophagy genes	*ATG2B*	3759601	C_9690166_10	Gln1383Glu
	ATG5	2245214	C_3001905_20	Intronic
	ATG10	1864183	C_11953871_20	Thr212Met
	ATG16L1	2241880	C_9095577_20	Thr300Ala
Carcinogen Metabolism genes	*CYP3A5*	776746	C_26201809_30	Intronic
	GSTP1	1695	C_3237198_20	Ile105Val
	GSTT1	N/A	PCR	Null/present
	GSTM1	N/A	PCR	Null/present
	NFE2L2 (NRF2)	13035806	C_11745134_10	3'-region
		2706110	C_11745133_10	3'-region
	KEAP1	1048290	C_9323035_10	Leu471Leu

After the application of the Propensity Score method in TTCC-2010-05 observational study, 126 larynx, 100 pharynx and 70 oral cavity squamous cell carcinomas were totally paired with the control group. Descriptive study of the analysis by locations did not show any statistically significant differences between sex, age, tobacco and alcohol intake respect the control group.

Less common genotypes in *ERCC1* rs11615 (p=0.011, OR=0.288 (0.110-0.751) in recessive model) and *ERCC2* rs13181 (p=0.046, OR=0.375 (0.143-0.982) in codominant model) were associated with lower risk

to suffer from laryngeal cancer. These genes are part of the nucleotide excision repair (NER) pathway, implicated in the repair of adducts produced by the tobacco, DNA oxidative damage, alkylating agents and thymine dimmers, and these polymorphisms were linked to a minor mRNA level or with a deficient DNA repair capacity respectively[26,27]. rs11615 and rs13181 variant genotypes have been also associated to a better response and longer survival for higher damage accumulation in patients treated with platinum[28] triggering cell death. A possible explanation of these results in our series could be the retrospective nature of our study, where those mutated allele carriers with less reparation and more aggressive illness could have been died along the follow-up and most of longest survivors with common allele would have been included in our series. This distribution was confirmed in our data.

Inflammation has been considered an important factor in the pathogenesis of a lot of human tumours[29], with a special interest in the context of oral cancer[30]. Variant allele in proinflammatory *IL6* rs1800795 (in dominant model), was related with a higher risk to suffer from laryngeal, p=0.002 OR=2.394 (1.376-4.163) and oral cavity tumours, p=0.018 OR=2.265 (1.148-4.653), whereas inflammatory gene *IL2* rs2069762 variant was associated with a lower risk of oral cavity cancer (GG p=0.039, OR=0.300 (0.096-0.940)). Our results can be related with the carcinogenesis induced by inflammatory process[29] in which *IL6* SNP produces a higher gene expression and largest inflammatory response[31], associated with higher tumour risk. On the contrary inflammatory *IL2* variant is associated with higher gene expression, so this interleukin should have more effect in self-reactive cells elimination than inflammatory response to tumour.

MDM2 gene is an important negative regulator of the tumour suppressor gene *TP53*[33]. In our sample the SNP rs2279744, located in the promoter P2 was associated with a higher risk to suffer from laryngeal cancer in those patients with the GG genotype in recessive model p=0.029 OR=2.413 (1.094-5.323), related with an increase *MDM2* expression and lower *TP53* tumour suppressing activity[34]. In contrast, *TP53* rs1042522 C mutant allele was associated with a decrease risk to develop from laryngeal p=0.002, OR=0.286 (0.119-0.607) and pharyngeal cancer p=0.001, OR=0.124 (0.035-0.476). This SNP Pro72Arg is located in the exon 4 of *TP53*, essential in the apoptotic response and the carcinogenesis inhibition. Arg allele is an apoptosis inductor[35] and is related with an increased risk to develop from different tumours, although its role is controversial. The contrary result in our study could be explained for the disequilibrium in HWE (p<0.05) or because of the association of the proline codon with longer survival[35], selecting those patient in our retrospective recruitment.

In addition to previous results, we found a statistically significant result in the CA+AA genotypes of the anti-apoptotic gene *BCL2* rs2279115 and a higher susceptibility to oral cavity cancer p=0.010, OR=2.753 (1.273-5.952). This SNP in the promoter increases the expression of this gene, decreasing apoptosis, related with a higher risk to HNSCC[36].

Lastly, rs1303586 variant genotypes GA+AA and CT+TT in rs2706110, both in the *NFE2L2* gene, were associated with a lower risk to suffer from laryngeal cancer, p=0.035 (OR=0.478 (0.240-0.949) and p=0.518 (OR=0.518 (0.299-0.900); while in pharyngeal carcinoma only *NFE2L2* rs2706110 less common genotypes CC+CT was related with a lower risk (p=0.043, OR=0.552). *NFE2L2* codifies for a transcription factor family (Nrf2) that induce many antioxidant genes under oxidative stress. SNPs in this gene have been reported with some diseases and cancer risk[37]. Functional analyses of these SNPs have not yet been described but our hypothesis is that these changes could increase antioxidant genes induction under stress, produced at high level in HNSCC by tobacco and alcohol consumption.

Autophagy takes part into the initiation and prevention of cancer, and its function can be altered during tumour progression[38]. *ATG2B* rs3759601 homozygous variant GG genotype was associated with a higher risk to develop pharyngeal cancer p=0.035, OR=2.721 (1.075-6.887) while *ATG10* rs1861483 allele T was also associated with a higher laryngeal cancer susceptibility p=0.026 OR=1.888 (1.708-3.308). These are essential proteins for the autophagosome formation and their SNPs could cause dysregulation in the autophagosome formation; result in diminished autophagy and higher accumulative DNA damage and carcinogenesis. Lastly, rs2241880 CC genotype in central adaptor *ATG16L1* was associated with a higher risk to develop oral carcinoma, p=0.047 OR=2.299 (1.010-5.230). This could be related with a defective autophagy and higher inflammation, as it is established in Chron´s disease[39].

Overall, this study showed the association between some polymorphisms in DNA repair, inflammation, metabolism, autophagy and apoptosis genes with a different susceptibility to develop HNSCC. The characteristics of the control group, favoured that this results were caused by their genetic background, avoiding confounder variables. In the same way, the differences found in this association study according to the location corroborate the heterogeneity in these tumours include under the same term of head and neck squamous cell carcinoma.

3. Mutational burden and prognostic factor in a cohort of homogeneously treated Spanish HNSCC patients

Analysis from *Cancer Genome Atlas* described the molecular landscape of HPV positive (HPV+) and HPV negative (HPV-) HNSCC, improving specificity at diagnosis and therapeutic approaches[40]. However, the clinical relevance of data obtained from next-generation sequencing (NGS) is unknown due to the lack of homogeneity in the treatment. To contribute to the understanding on how somatic mutations influence the outcome of HNSCC, we have studied a 26 genes panel by NGS in a homogenously treated locally advanced HNSCC Spanish cohort from the clinical trial TTCC-2007-01[12]. In this study we analyzed mutations from FFPE HNSCC tumours evaluating the mutational burden according to HPV profile as well as response to treatment and survival. From the 150 FFPE blocks included in this study, most were from

men (89.3%), high tobacco and alcohol consumers, with HPV- oropharyngeal squamous carcinoma (43.3%), diagnosed in tumour stage IV-A and with an average of 59 years old.

130 HNSCC FFPE blocks (86.7%) presented mutations whereas 20 (13.3%) did not carry any mutation in the selected genes. Comparison of presence or absence of mutations with categorical variables such as sex, alcohol and tobacco consumption, or tumour characteristics such as stage, location and HPV status, did not show any statistically significant difference (p>0.05) (Table 4). We also compared the categorical variables with the number of mutations (none, one or more than one) and with those tumours carrying a mutation in *TP53* or in other genes, with negative results (p>0.05).

Table 4: Mutation state versus clinicopathological features.

Characteristic	Group N=150	Non mutated N=20	Mutated N=130	p-value
Sex	Male	16 (80.0%)	118 (90.8%)	0.146
	Female	4 (20.0%)	12 (9.2%)	
Location	Larynx	3 (15.0%)	24 (18.5%)	0.856
	Hypopharynx	4 (20.0%)	35 (26.9%)	
	Oropharynx	10 (50.0%)	55 (42.3%)	
	Oral cavity	3 (15.0%)	16 (12.3%)	
Stage	III	1 (5.0%)	9 (6.9%)	0.849
	IV-A	14 (70.0%)	95 (73.1%)	
	IV-B	5 (25.0%)	26 (20.0%)	
Tobacco	Non smoker	4 (20.0%)	15 (11.5%)	0.290
	Smoker	16 (80.0%)	115 (88.5%)	
Alcohol	Non drinker	6 (30.0%)	33 (25.4%)	0.661
	Drinker	14 (70.0%)	97 (74.6%)	
HPV status (p16INK4a IHC)	Negative	16 (80.0%)	109 (83.8%)	0.667
	Positive	4 (20.0%)	21 (16.2%)	

16% of HPV+ cases in our series showed a lower percentage than previously reported in Europe[41], but similar than in other countries from Southern Europe where tobacco and alcohol are the most common carcinogens[42]. Mutational plot shows differences between HPV negative (N=125) and positive tumours (N=25) (Figure 1). In both groups *TP53* was the most frequently mutated gene (74.4% in HPV- and 60% in HPV+) followed by far from *PIK3CA* (12.8% versus 16%). *PTEN* (12%) was the third most commonly mutated gene in HPV+ tumours whereas in HPV- the third most frequently mutated gene was *FBXW7* (6.4%). Simultaneous mutations in different genes were more frequent in HPV- tumours, characterized with higher mutational burden. When we consider only mutated tumours, *TP53* mutations were less frequent in HPV+ (71.4% versus 83.5% in HPV-, p=0.192) maybe because *TP53* sequestration by the viral oncoprotein E6 prevents from selective pressure of gaining mutations in this gene in HPV+ tumours[43,44]. The lack of statistically significant result in *TP53* distribution between HPV groups could be explained by the concurrence of viral infection and tobacco and alcohol consumption in the majority of our patients. *PIK3CA* alterations were more frequent within HPV+ tumours (19.0% versus 14.7% in HPV- samples, p=0.611), confirming previous descriptions[45].

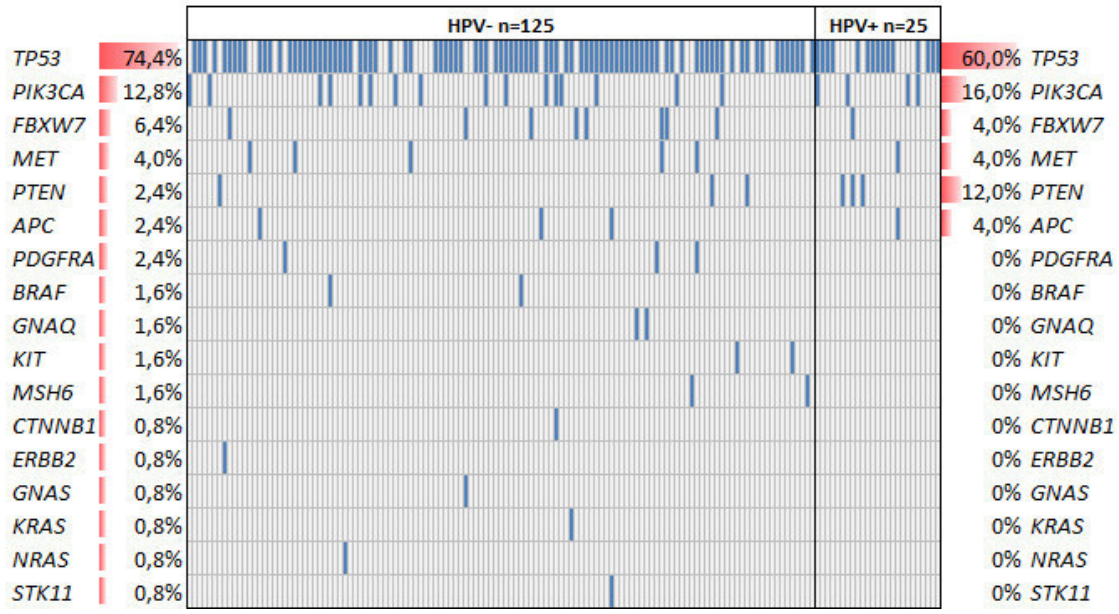

Figure 1: Mutational plot divided in HPV positive and negative HNSCC tumours. Blue rectangle indicates presence of mutations in each patient. Percentage of mutations in each gene divided by HPV group is indicated in the border of the table and red line represents its proportion.

26 samples (17.3%) of our study were not randomized and only received induction chemotherapy based on taxol-platin-5-fluorouracil (TPF) regimen. 68 patients (45.4%) were also treated with chemoradiotherapy (RTCT) and 56 (37.3%) with bioradiotherapy (RTBT). After TPF, 14% of the patients (n=21) had complete response whereas after concomitant radiotherapy it increased up to 45.2% (n=56). We did not find any statistically significant difference between the response and the mutational burden (p>0.05) independently of their HPV profile and the randomization.

Survival analysis between RTBT and RTCT treated groups showed no differences in overall survival (OS) (p=0.161). However, concurrent cisplatin had better progression-free survival (PFS) than bioradiotherapy (p=0.010, HR=1.783 (1.114-2.777)) (Figure 2).

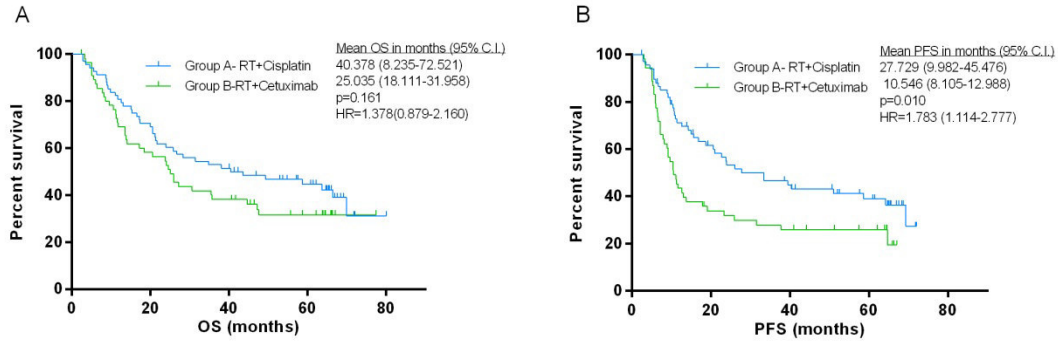

Figure 2: Kaplan-Meier survival curves in the 150 selected patients from the TTCC-2007-01 clinical trial. A) OS, B) PFS according to its treatment option. Median, log rank test p-values and hazard ratios are shown in each plot.

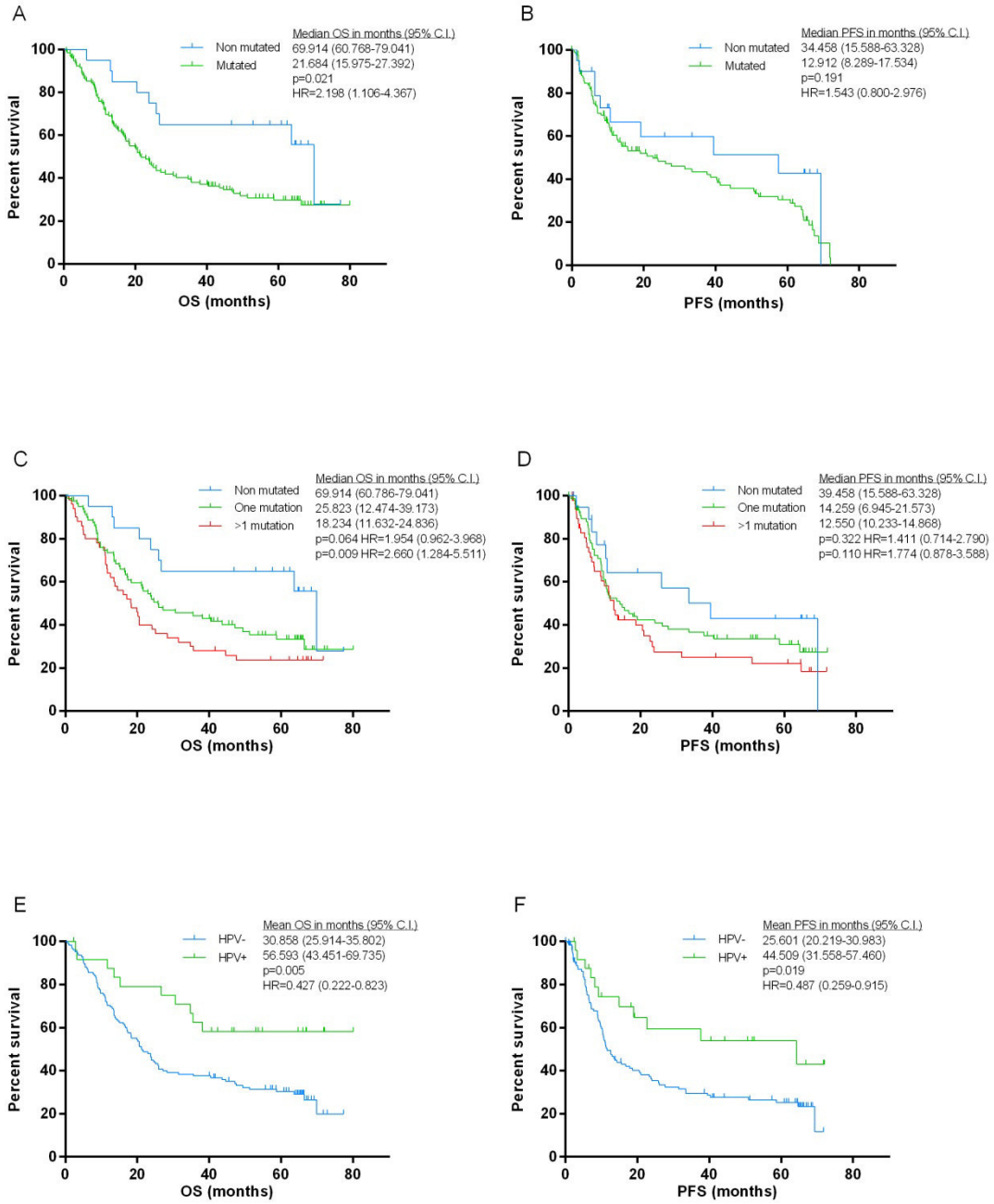

Figure 3: Kaplan-Meier survival curves. Mutational status and overall survival (A) and progression-free survival (B), number of mutations and their overall survival (C) and PFS (D). Lastly, HPV status and OS (E) and PFS (D). Median (mean in E and F), log rank test p-values and hazard ratios are shown in each plot.

Finally, OS and PFS were correlated with the mutational status. Patients with non mutated tumours had a better OS with a median of 69.914 months versus 21.684 months in patients with mutated tumours (p=0.021, HR=2.198 (1.106-4.367)) (Figure 3A). This data could be related with tumour aggressiveness as it has been reported in other series[46]. Nevertheless, there were no differences in PFS (p=0.191) (Figure 3B). We also found correlation with the number of mutations, observing that those patients that carry tumours with more than 1 mutation had lower OS than patients with non mutated tumours (p=0.009, HR=2.660 (1.284-5.511)) (Figure3C). However, the differences between patients with tumours with one

or more than one mutation were not statistically significant (p=0.147). There was no difference in OS or PFS between patients with wild-type or mutated *TP53* tumours, neither between those mutated in *TP53* nor in other genes (OS p=0.659 and PFS p=0.726). Lastly, patients with HPV+ tumours showed higher OS and PFS compared with HPV- (p=0.005 and p=0.019 respectively) (Figure 3E-F), corroborating previous reported results[47,48].

Overall, our data strongly support and expand previous published mutational burden in HNSCC. We have also defined the mutational profile of HPV+ HNSCC in Spanish population showing, apart from *TP53* mutations, frequent alterations in *PIK3CA* and *PTEN* genes, defining possible pathways for targeted therapy. Moreover, survival analysis showed that mutational status in the tumour could define prognosis of the patient, being an important biomarker in HNSCC. Although we cannot find any linkage between mutations and response to treatment, the association in survival could give us some important data to continue with, giving a step further into a personalized treatment for patients suffering from this type of cancer.

4. Epidermal Growth Factor Receptor (EGFR) pathway polymorphisms as predictive markers of cetuximab toxicity in locally advanced head and neck squamous cell carcinoma (HNSCC) in a Spanish population

More than 95% of HNSCC patients have EGFR overexpression[49]. Because of its prevalence and crucial role in pathogenesis, targeting EGFR with monoclonal antibody cetuximab has become a rational approach for HNSCC treatment. Cetuximab can cause antitumor effects through three different mechanisms: competitive inhibitor of the ligand EGF, decrease in the receptor through endocitosis of EGFR and lysosome degradation, and finally antibody-dependent cell-mediated cytotoxicity (ADCC)[50,51].

Cetuximab combined with radiotherapy or chemotherapy improves locoregional control and survival in HNSCC patients, but only a subset of all patients are able to benefit from anti-EGFR monoclonal antibodies [52]. Thus, the detection of predictive biomarkers and beneficial patient profiles is crucial. Several studies have correlated clinical outcome and toxicity to IgG1 cetuximab treatment with polymorphisms in the EGFR pathway with conflicting results. We have evaluated the possible association of cetuximab toxicity with polymorphism distribution in the EGFR pathway (Table 5), looking for predictive biomarkers of skin toxicity, a frequent side effect of EGFR targeting agents correlated with a better treatment efficacy[53].

110 locally advanced HNSCC patients treated with cetuximab from TTCC-2010-05 observational study were included. Most of the patients included in the study were men (90.9%) with a median age of diagnosis of 60 years old. Regarding tumour characteristics, the most common were laryngeal cancer

(45.5%), followed by pharyngeal (41.8%) and oral cavity (12.7%); the majority of tumours were stage IV (70.0%), followed by stage III (30.0%). According to the specific toxicity of cetuximab, 55.5% of HNSCC patients presented acneiform rash, while dry skin was present in 45.5% of cases and pruritus in 20.9%, often in low grade. Despite a variation in the number of cetuximab cycles (10.54 ± 15.04), no statistically significant relationship was observed between accumulated doses and toxicity in the Mann-Whitney U test (dry skin p=0.116, pruritus p=0.787 and rash p=0.284).

Table 5: Polymorphisms analyzed in cetuximab-treated HNSCC patients.

Function	Gene	rs	ID	Change
Epidermal growth factor receptor	*EGFR*	2227983	C_16170352_20	Arg521Lys
		28384375	C_64062867_10	Val592Ala
		17336639	C_33816900_10	Pro266Arg
Antibody-dependent cell-mediated cytotoxicity (ADCC)	*FCGR2A*	1801274	C_9077561_20	His131Arg
	FCGR3A	396991	C_25815666_10	Val18Phe
EGFR downstream effectors	*KRAS*	61764370	PCR	3´-UTR
	CCND1	603965	PCR	Pro241Pro

EGFR polymorphisms rs28384375 and rs17336639 had only the major allele variant in our sample so they were excluded from the analysis. The remaining SNPs were analyzed according to the most common toxicity produced by monoclonal antibodies treatment: dry skin, pruritus and acneiform rash.

We showed a statistically significant association between dry skin and global toxicity in the *KRAS-LCS6* rs61764370 variant. These results showed that being a carrier of the G allele (genotypes TG+GG) of the *KRAS* rs61764370 polymorphism, in the dominant model, decreased the susceptibility to develop dry skin p= 0.006, OR=0.287 (0.119-0.695) and global toxicity (related to any presence of toxicity due to MAb treatment) p=0.002, OR=0.266 (0.114-0.622) after cetuximab treatment. *KRAS-LCS6* variant has a functional impact on let-7 miRNA joining to 3´-UTR of *KRAS* gene[54], causing less inhibition and increased *KRAS* expression[55]. Our results could be joined to this effect with less inhibition by cetuximab due to higher *KRAS* proliferation activity and low toxicity to anti-EGFR therapy. Moreover, as KRAS is an important effector on the pathway that maintains skin homeostasis[56], the increase of *KRAS* expression caused by rs61764370 variant could activate important transcription factors to keep skin homeostasis, reducing skin toxicity.

EGFR rs2227983 polymorphism showed an association with pruritus toxicity. Carriers of GA+AA genotypes were found to have a decreased risk of suffering from pruritus p=0.041, OR=0.345 (0.124-0.958), similar to reported association with lower incidence of skin rash in advanced HNSCC[57]. This SNP is in the region of extracellular domain and its change could produce structural changes that provoke a modification in EGF and cetuximab interaction, with a decrease binding, low effectiveness and less toxicity, related with a lower response[57].

FCGR2A rs1801274 CT+TT genotypes were close to being associated with a decreased risk of dry skin, p=0.051 OR=0.380 (0.144-1.003). This variant in the Fc gamma receptor gene *FCGR2A* of the ADCC pathway has been related with higher affinity to IgG2[58] and it is associated with longer progression-free survival in cetuximab monotherapy. Our result could be related with lower affinity to cetuximab (IgG1 antibody) in patients with the T allele resulting in less toxicity to MAb treatment, probably due to lowest antitumour cytotoxicity.

Our study suggests that *EGFR* rs2227983, *KRAS* rs61764370 and *FCGR2A* rs180127 variants are useful biomarkers for predicting reduced skin toxicity in HNSCC patients receiving cetuximab-based therapy. This could indicate that patients with these genetic variants could have less toxicity and a poor prognosis, being better scheduled in another therapeutic alternative.

5. Autophagy modulation in head and neck squamous cell carcinoma cell lines: a new target for cancer treatment.

Autophagy is one of the most conserved cellular degradation pathway[59]. Although molecular process of autophagy is well described[60], understanding its role in cancer, may allow us to develop novel therapeutic strategies to enhance the effects of chemotherapy and improve the clinical outcome of cancer patients[61]. Our purpose was to study the use of some autophagy modulators in commercial and new established molecularly characterized HNSCC tumour cell lines from all the main locations, to check their effect in viability, cell cycle and autophagy modification. This could define a promising novel cancer therapy, improving the poor response rates observed in HNSCC tumours.

We established three HNSCC derived cell lines from larynx (32860), vallecula (32661) and oropharynx (32816), while for oral cavity tumour we used the commercial cell line CAL33.

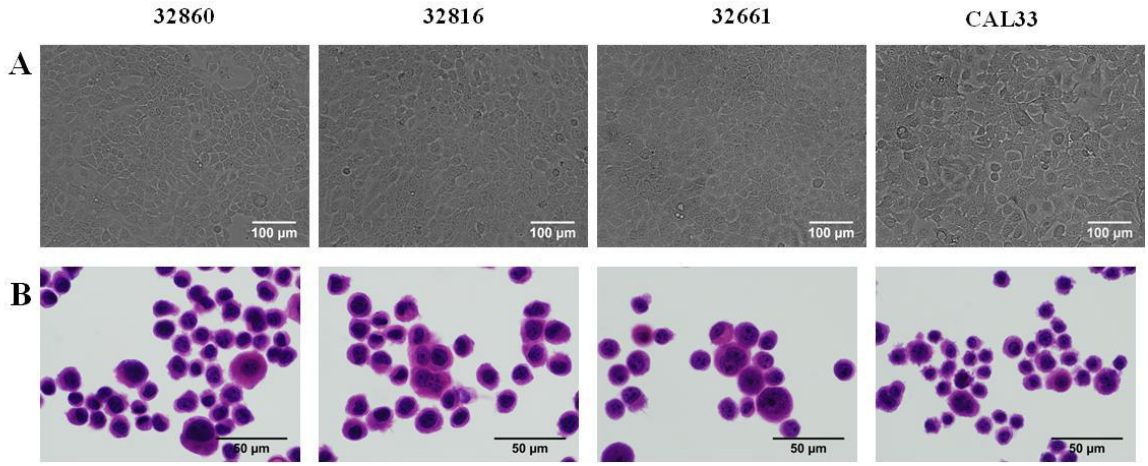

Figure 4: Phase contrast microscope photographs (A) and H&E (B) from the commercial cell line CAL33 and the established cell lines 32661, 32816 and 32860.

H&E staining confirmed tumour morphology and cell culture showed the typical cobblestone patterns (Figure 4). CNV and karyotyping analysis corroborated the different molecular aspects respect to the location origin, though the dissimilarities are more found in the karyotyping than in the CNV.

Cell viability assay by MTT showed similar results between cell lines except HPV+ oropharyngeal tumour-derived (32816) in which cells seem to have more viability than the rest in spite of the slower proliferation rate. Metformin, paclitaxel and panobinostat had a dose-dependent activity, whereas chloroquine treatment produced an important decrease at minimum concentration, and decitabine did not show any pronounced viability decrease maybe because it is normally used in combination with other chemotherapy agents. Concentrations are indicated in Table 2. After the treatment with the selected concentrations obtained by the MTT viability assay, cell cycle cytometry was performed (Figure 5).

Comparing with the untreated controls, chloroquine treatment, one the most specific autophagy inhibitor[62], produced a slight increase in G0/G1, a little decrease in G2/M and a reduced increment in cell death, same effect as in colon and hepatocellular carcinoma[63,64]. This data was more pronounced in 32816 HPV+ oropharyngeal cell line. Moreover, chloroquine treatment showed a decreased autophagy, with higher expression of p62 (except in 32816 where the expression was constant) and a slight decrease in beclin1 and LC3B, especially in LC3B-I.

Metformin, commonly used for type 2 diabetes mellitus treatment, has antitumour effects too. In our study, it produced a G0/G1 descent and consequently, an increase in S and G2/M phases, although in 32816 cell line these effects were not found. Different tumour cell lines treated with metformin proved a stop in G0/G1[65] and G2/M[66], but with higher concentrations and prolonged treatments. Anyway, metformin produced a strong inhibition of phospho-mTOR through AMPK induction[67], being an important autophagy activator, modifying LC3B expression, with a minor LC3B-II rise, with the exception of 32816 cell line.

Paclitaxel addition is the therapeutic option that showed the highest death rates, increasing respect the untreated control with an average of 35%, except in 32860 laryngeal cell line that seemed to be more resistant to taxol. This drug has a high antitumour potential, being accepted in the use of different tumours, among them in HNSCC[68] in the TPF combination. Apart from apoptosis induction, paclitaxel regulates other programmed cell response such as autophagy through two different mechanisms: inhibition of autophagosome formation and trafficking[69]. However, in our study autophagy protein expression did not show relevant modifications caused by paclitaxel, maybe because of the main effect in apoptosis.

During tumourigenesis, many of the changes in gene expression are due to an altered epigenetic

regulation such as DNA methylation and histone deacetylation[70]. The administration of the pan-inhibitor of histone deacetilase (HDACi), panobinostat, produced a diminished G0/G1 and an important increment in G2/M cell cycle phase, producing also a moderate cell death rise. This result has been reported in other *in vitro* studies like HNSCC[71]. Again, 32816 cell line showed different results. Autophagy induction by HDACi was also described[72] as it was observed in our cell lines with an important autophagy stimulation by up-regulation of mTOR, beclin1 and LC3B after treatment with panobinostat.

Decitabine, an inhibitor of DNA methylation by DNMTs, caused a G0/G1 decrease and a higher percentage in S and death phases in selected HNSCC, because it is a specific S phase agent. However, decitabine treatment caused an effect on autophagy, thought it did not alter phospho-mTOR and p62 expression, it showed increased beclin1 and LC3B-II levels with a slight activation of this process.

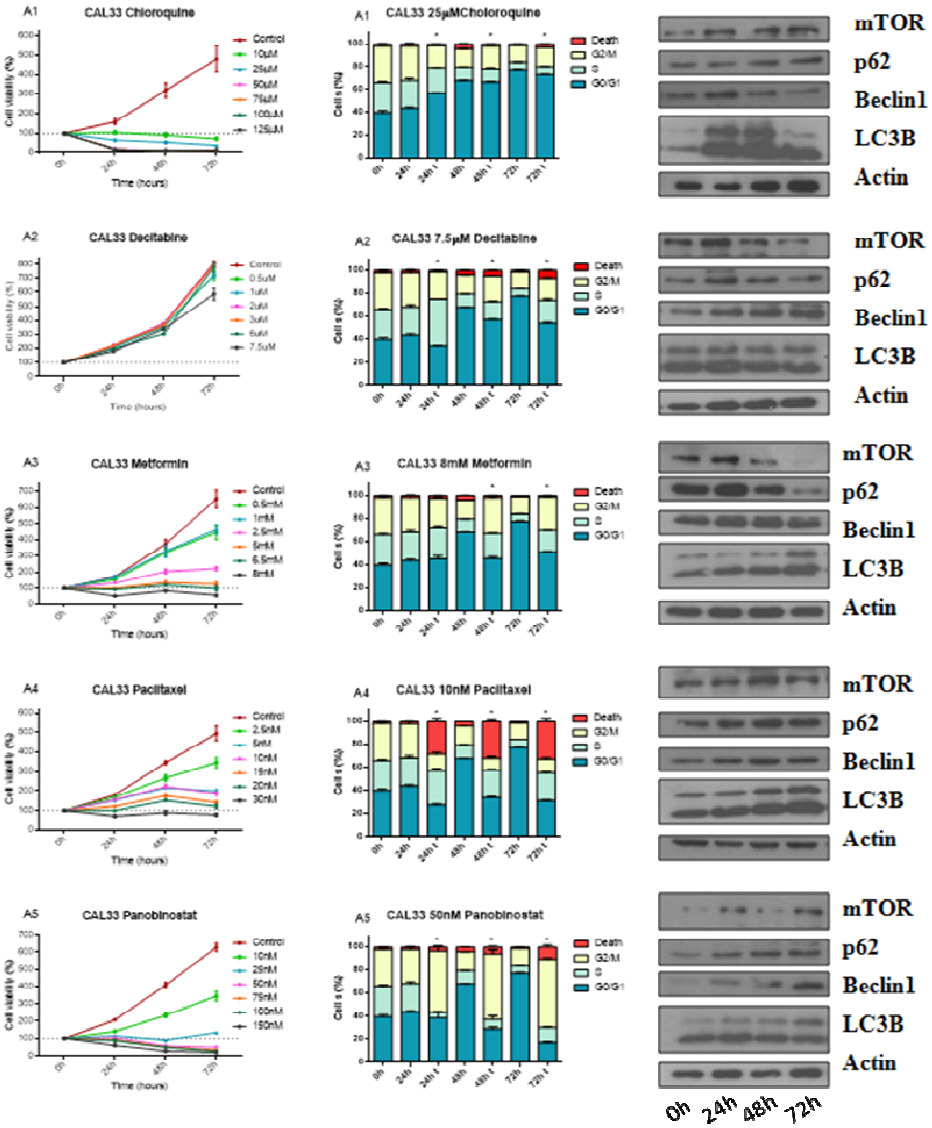

Figure 5: Example of the treatment experiment with the commercial cell line from tongue carcinoma CAL33. Cell viability assay by MTT (left), cell cycle analysis by flow cytometry (middle) and autophagy protein expression by western-blot (right).

Our results showed that the use of modulators of autophagy such as metformin, panobinostat, paclitaxel and chloroquine had an inhibitory activity on cellular proliferation. These drugs and decitabin produced changes in cell cycle and a deregulation of the autophagic process. According to our preclinical results, these agents could be used in HNSCC in monotherapy or in combination with the current therapies to modify the autophagy mechanisms. This synergy could be most appropriate in HPV+ tumours, due to the results from the oropharyngeal squamous carcinoma cell line.

CONCLUSIONS

First: epidemiological analysis confirmed the importance of tobacco and alcohol consumption as pathogenic factors in HNSCC in Spanish patients and the low incidence of HPV infection derived tumours. In this case it is usually accompanied by tobacco and alcohol intake, giving rise to a mix carcinogenesis model.

Second: gene candidate association study showed that variants in genes implicated in DNA repair, inflammation, autophagy, oxidation and apoptosis could play a role in the susceptibility to develop HNSCC in the Spanish population, changing its implication according to tumour location, confirming the diversity of these tumours.

Third: HPV negative tumours present a low percentage of mutations in relation with HPV positive ones. As HPV positive have better evolution, the study of the mutational burden could be used as a prognostic factor.

Fourth: our results of the *in vitro* effect of autophagy modulation show that the administration of these drugs could be a promising therapeutic option in the treatment of HNSCC. The fact that these drugs have been previously approved by the FDA or EMA, facilitate the development of clinical trial to check these effects in humans.

Fifth: our results confirm the heterogeneity in these tumours included under the term of HNSCC. The knowledge of the molecular characteristics of each location would allow to define a more individualized treatment.

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