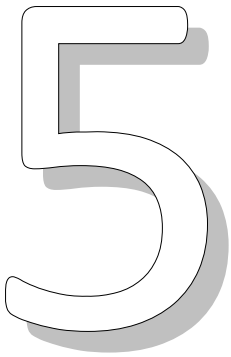
Mutational burden and prognostic factors in a cohort of homogenously treated Spanish HNSCC patients



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Oral Oncology

Sometido a publicación

Factor de impacto 2015: 4.286

-Journal Citation Reports Science Edition (Thomson Reuters, 2015)

Oncology: 52/213 Q1

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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Word count: 2970 words

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.

Acknowledgement

This study was supported by the health research program of the "Instituto de Salud Carlos III" (PI14/00071) co-financed with FEDER founds and for the Health Regional Management of the Junta de Castilla y León (GRS1385/A/16). J. Fernández-Mateos was partially supported by a predoctoral research grant from the Consejería de Educación—Junta de Castilla y León and the European Social Fund to CC-B (EDU/1084/2012). Authors would like to thank the individuals who consented to participate in this study and their relatives, and all their colleagues from the Spanish Group of Treatment of Head and Neck Cancer (TTCC) who have participated in this study and are not included in the list of authors. We would also like to thank the technician María del Carmen Rodríguez for its implication in the study, as well as the Institute of Biomedical Research of Salamanca (IBSAL).

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

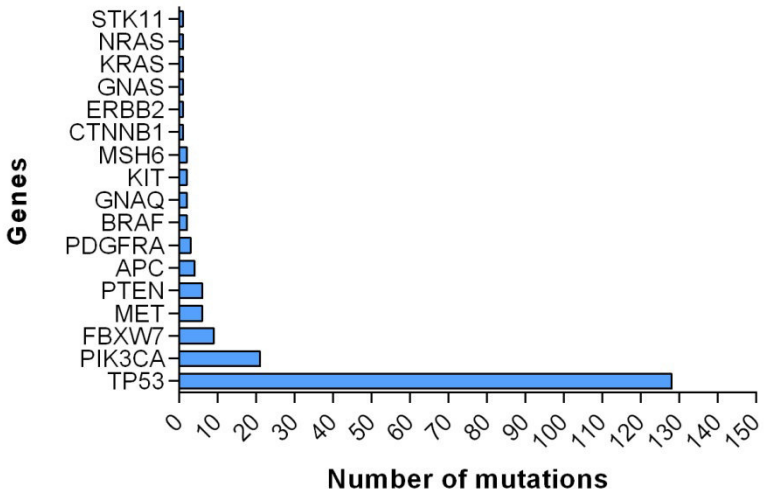

Figure 1: Number of mutations found in the sequencing of 150 HNSCC by TruSight Tumor 26 panel.

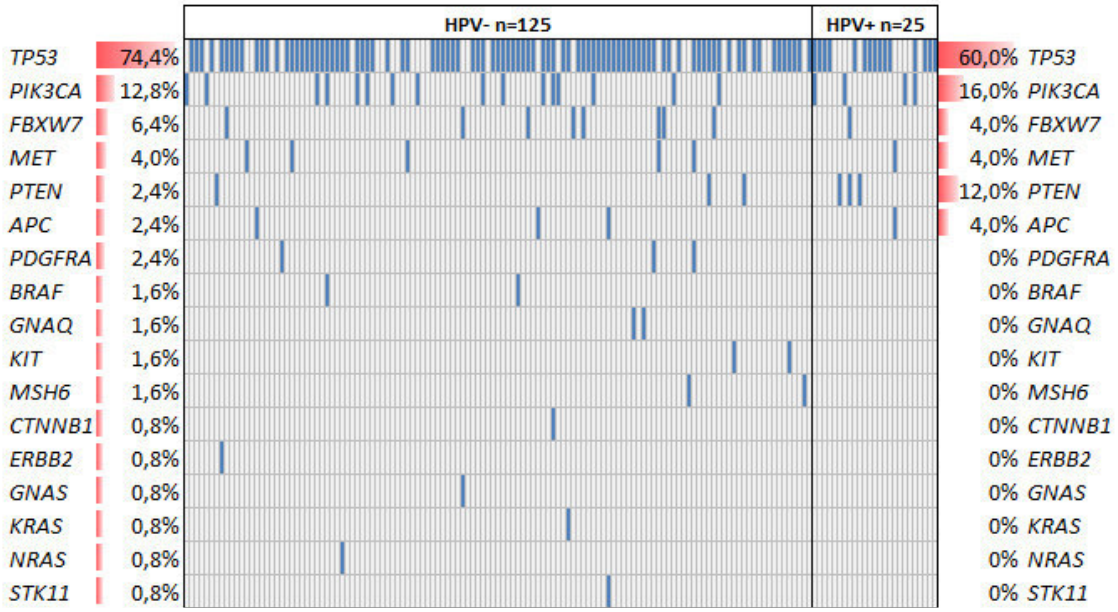

Figure 2: Mutational plot divided in HPV positive and negative HNSCC tumors. Blue rectangle indicates presence of mutations in each patient. Percentage of mutations in each gene divided per HPV group is indicated in the border of the table and red line represents its proportion.

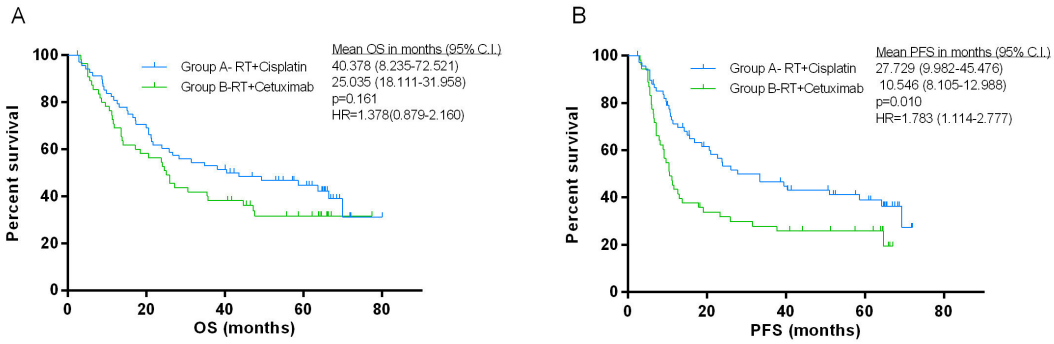

Figure 3: Kaplan-Meier survival curves in the 150 selected patients from the TTCC-2007-01 clinical trial. A) OS, B) PFS according to its treatment option. Median, log rank test p-values and hazard ratios are shown in each plot.

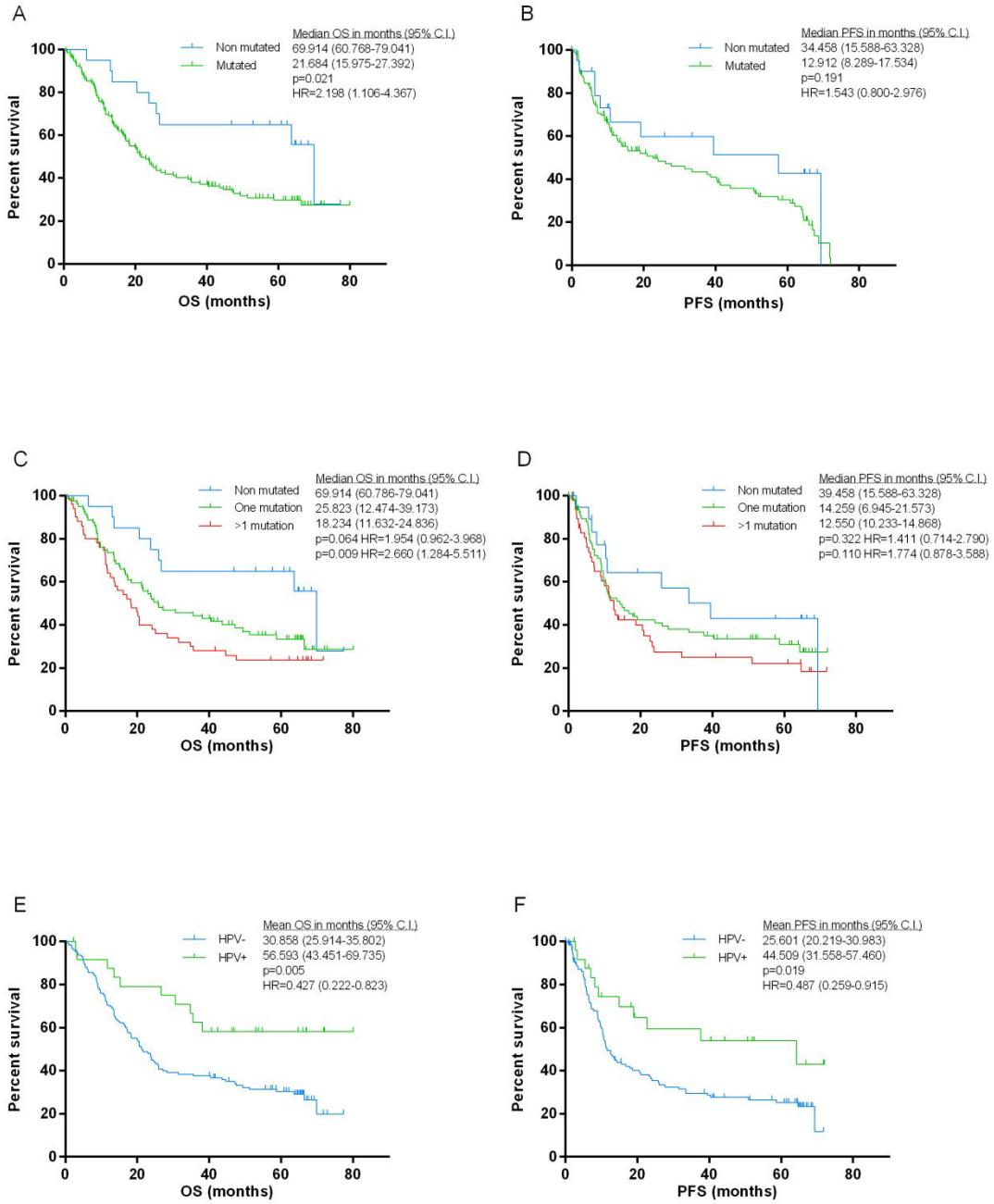

Figure 4: Kaplan-Meier survival curves. Mutational status and overall survival (A) and progression-free survival (B), number of mutations and their overall survival (C) and PFS (D). Lastly, HPV status and OS (E) and PFS (D). Median (mean in E and F), log rank test p-values and hazard ratios are shown 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
	splice_donor_variant	NM_000546.5:c.919+1G>A	
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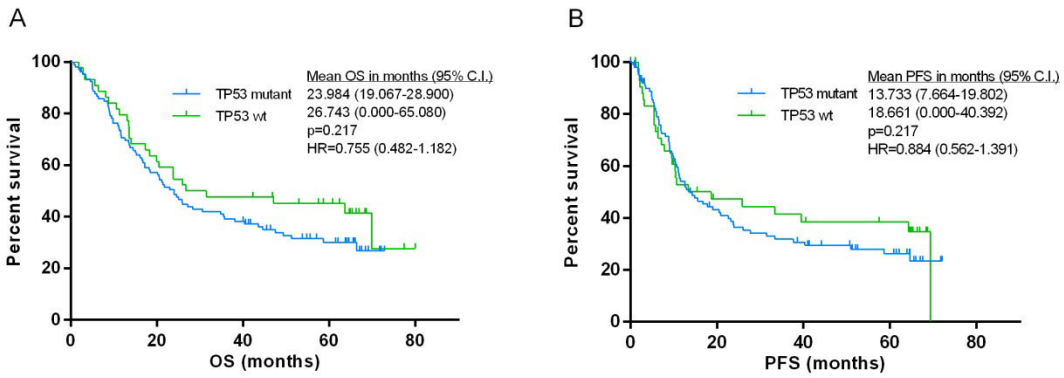
	missense_variant	NM_000546.5:c.578A>G	NP_000537.3:p.His193Arg
	missense_variant	NM_000546.5:c.578A>T	NP_000537.3:p.His193Leu
	missense_variant	NM_000546.5:c.526T>A	NP_000537.3:p.Cys176Ser
	missense_variant, feature_truncation	NM_000546.5:c.465_479delCCGCGTCCGCGCCAT	NP_000537.3:p.Arg156_Met160del
	stop_gained	NM_000546.5:c.687T>A	NP_000537.3:p.Cys229Ter
	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
	missense_variant	NM_000546.5:c.517G>T	NP_000537.3:p.Val173Leu
	missense_variant	NM_000546.5:c.763A>T	NP_000537.3:p.Ile255Phe
	missense_variant	NM_000546.5:c.578A>G	NP_000537.3:p.His193Arg
	frameshift_variant, feature_truncation	NM_000546.5:c.880delG	NP_000537.3:p.Glu294SerfsTer51
	missense_variant	NM_000546.5:c.434T>A	NP_000537.3:p.Leu145Gln
	missense_variant	NM_000546.5:c.742C>T	NP_000537.3:p.Arg248Trp
	stop_gained	NM_000546.5:c.949C>T	NP_000537.3:p.Gln317Ter
	frameshift_variant, feature_truncation	NM_000546.5:c.1146delA	NP_000537.3:p.Lys382AsnfsTer40
	inframe_deletion, splice_region_variant	NM_000546.5:c.784_786delGGT	NP_000537.3:p.Gly262del
	missense_variant	NM_000546.5:c.583A>T	NP_000537.3:p.Ile195Phe
	missense_variant, splice_region_	NM_000546.5:c.840A>T	NP_000537.3:p.Arg280Ser

	variant		
	inframe_deletion, splice_region_variant	NM_000546.5:c.772_780delGAAGACTCC	NP_000537.3:p.Glu258_Ser260delinsdel
	frameshift_variant, feature_truncation	NM_000546.5:c.768_769delAC	NP_000537.3:p.Leu257GlyfsTer6
	missense_variant	NM_000546.5:c.734G>T	NP_000537.3:p.Gly245Val
	frameshift_variant, feature_truncation	NM_000546.5:c.697delC	NP_000537.3:p.His233ThrfsTer14
	missense_variant	NM_000546.5:c.833C>A	NP_000537.3:p.Pro278His
	missense_variant	NM_000546.5:c.733G>A	NP_000537.3:p.Gly245Ser
	frameshift_variant, feature_truncation	NM_000546.5:c.686_687delGT	NP_000537.3:p.Cys229TyrfsTer10
	splice_acceptor_variant	NM_000546.5:c.920-1G>A	
	missense_variant	NM_000546.5:c.742C>T	NP_000537.3:p.Arg248Trp
	splice_donor_variant, coding_sequence_variant, intron_variant, feature_truncation	NM_000546.5:c.548_559+3delCAGATAGCGATGGTG	
	missense_variant	NM_000546.5:c.524G>T	NP_000537.3:p.Arg175Leu
	missense_variant	NM_000546.5:c.725G>A	NP_000537.3:p.Cys242Tyr
	missense_variant	NM_000546.5:c.722C>A	NP_000537.3:p.Ser241Tyr
	splice_donor_variant	NM_000546.5:c.782+1G>A	
	missense_variant	NM_000546.5:c.644G>A	NP_000537.3:p.Ser215Asn
	missense_variant	NM_000546.5:c.535C>T	NP_000537.3:p.His179Tyr
	missense_variant	NM_000546.5:c.734G>A	NP_000537.3:p.Gly245Asp
	missense_variant	NM_000546.5:c.818G>T	NP_000537.3:p.Arg273Leu
	missense_variant	NM_000546.5:c.524G>A	NP_000537.3:p.Arg175His
	missense_variant	NM_000546.5:c.434T>A	NP_000537.3:p.Leu145Gln
	missense_variant	NM_000546.5:c.473G>T	NP_000537.3:p.Arg158Leu
	missense_variant	NM_000546.5:c.707A>G	NP_000537.3:p.Tyr236Cys
	stop_gained	NM_000546.5:c.853G>T	NP_000537.3:p.Glu285Ter
	stop_gained	NM_000546.5:c.708C>A	NP_000537.3:p.Tyr236Ter
	frameshift_variant, feature_truncation	NM_000546.5:c.695_701delTCCACTA	NP_000537.3:p.Ile232ThrfsTer13
	missense_variant	NM_000546.5:c.658T>A	NP_000537.3:p.Tyr220Asn
	stop_gained	NM_000546.5:c.859G>T	NP_000537.3:p.Glu287Ter
	missense_variant	NM_000546.5:c.724T>G	NP_000537.3:p.Cys242Gly
	frameshift_variant, feature_truncation	NM_000546.5:c.356_360delCCAAG	NP_000537.3:p.Ala119ValfsTer28
	missense_variant	NM_000546.5:c.725G>T	NP_000537.3:p.Cys242Phe
	missense_variant	NM_000546.5:c.329G>C	NP_000537.3:p.Arg110Pro
	stop_gained	NM_000546.5:c.574C>T	NP_000537.3:p.Gln192Ter
	splice_acceptor_variant	NM_000546.5:c.376-2A>T	
	stop_gained	NM_000546.5:c.159G>A	NP_000537.3:p.Trp53Ter
	frameshift_variant, feature_elong	NM_000546.5:c.823dupT	NP_000537.3:p.Cys275LeufsTer31

	ation		
	missense_variant	NM_000546.5:c.752T>A	NP_000537.3:p.Ile251Asn
	missense_variant	NM_000546.5:c.653T>G	NP_000537.3:p.Val218Gly
	missense_variant	NM_000546.5:c.652_653delGTinsTG	NP_000537.3:p.Val218Trp
	missense_variant	NM_000546.5:c.652G>T	NP_000537.3:p.Val218Leu
	stop_gained	NM_000546.5:c.492_493delGCinsTT	NP_000537.3:p.LysGln164AsnTer
	stop_gained	NM_000546.5:c.493C>T	NP_000537.3:p.Gln165Ter
	missense_variant	NM_000546.5:c.492G>T	NP_000537.3:p.Lys164Asn
	missense_variant	NM_000546.5:c.733G>A	NP_000537.3:p.Gly245Ser
	frameshift_variant, feature_truncation	NM_000546.5:c.365_366delTG	NP_000537.3:p.Val122AspfsTer26
	frameshift_variant, feature_truncation	NM_000546.5:c.632delC	NP_000537.3:p.Thr211IlefsTer36
	missense_variant	NM_000546.5:c.614A>G	NP_000537.3:p.Tyr205Cys
	missense_variant	NM_000546.5:c.638G>T	NP_000537.3:p.Arg213Leu
	stop_gained	NM_000546.5:c.661G>T	NP_000537.3:p.Glu221Ter
	missense_variant	NM_000546.5:c.659A>G	NP_000537.3:p.Tyr220Cys
	stop_gained	NM_000546.5:c.961A>T	NP_000537.3:p.Lys321Ter
	stop_gained	NM_000546.5:c.892G>T	NP_000537.3:p.Glu298Ter
	frameshift_variant, feature_truncation	NM_000546.5:c.716_723delACAGTTCC	NP_000537.3:p.Asn239MetfsTer22
	missense_variant	NM_000546.5:c.833C>T	NP_000537.3:p.Pro278Leu
	stop_gained	NM_000546.5:c.916C>T	NP_000537.3:p.Arg306Ter
	missense_variant	NM_000546.5:c.734G>A	NP_000537.3:p.Gly245Asp
	frameshift_variant, feature_truncation	NM_000546.5:c.532delC	NP_000537.3:p.His178ThrfsTer69
	missense_variant	NM_000546.5:c.529C>T	NP_000537.3:p.Pro177Ser
	stop_gained	NM_000546.5:c.511G>T	NP_000537.3:p.Glu171Ter
	splice_region_variant, synonymous_variant	NM_000546.5:c.375G>T	NM_000546.5:c.375G>T(p.=)
	missense_variant	NM_000546.5:c.527G>A	NP_000537.3:p.Cys176Tyr
	missense_variant	NM_000546.5:c.734G>A	NP_000537.3:p.Gly245Asp
	splice_acceptor_variant	NM_000546.5:c.994-1G>A	
	missense_variant	NM_000546.5:c.701A>G	NP_000537.3:p.Tyr234Cys
	stop_gained	NM_000546.5:c.661G>T	NP_000537.3:p.Glu221Ter
	missense_variant	NM_000546.5:c.729_730delGGinsTT	NP_000537.3:p.MetGly243IleCys
	stop_gained	NM_000546.5:c.372C>A	NP_000537.3:p.Cys124Ter
	stop_gained	NM_000546.5:c.949C>T	NP_000537.3:p.Gln317Ter
	missense_variant	NM_000546.5:c.794T>C	NP_000537.3:p.Leu265Pro
	splice_donor_variant	NM_000546.5:c.672+1G>T	
	missense_variant	NM_000546.5:c.434T>C	NP_000537.3:p.Leu145Pro
	stop_gained	NM_000546.5:c.499C>T	NP_000537.3:p.Gln167Ter
	frameshift_variant, feature_truncation	NM_000546.5:c.569delC	NP_000537.3:p.Pro190LeufsTer57
	missense_variant	NM_000546.5:c.451C>G	NP_000537.3:p.Pro151Ala

	missense_variant	NM_000546.5:c.578A>T	NP_000537.3:p.His193Leu
	missense_variant	NM_000546.5:c.659A>G	NP_000537.3:p.Tyr220Cys
	frameshift_variant, feature_truncation	NM_000546.5:c.448_460delACACCCCCGCCCG	NP_000537.3:p.Thr150AlafsTer16
	frameshift_variant, feature_truncation	NM_000546.5:c.472delC	NP_000537.3:p.Arg158AlafsTer12
	missense_variant	NM_000546.5:c.614A>G	NP_000537.3:p.Tyr205Cys



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.