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CAMPUS DE EXCELENCIA INTERNACIONAL

Tesis Doctoral

Estudio Clínico y Biológico de las Comutaciones en Contextos Específicos de los Síndromes Mielodisplásicos

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Que D^a. **Sofía María Toribio Castelló**, graduada en Biotecnología por la Universidad de Cádiz, ha realizado bajo nuestra dirección el trabajo de Tesis Doctoral titulado "*Estudio clínico y biológico de las comutaciones en contextos específicos de los síndromes mielodisplásicos*", y que éste reúne, a nuestro juicio, las condiciones de originalidad y calidad científica requeridas para su presentación y defensa ante el tribunal correspondiente para optar al grado de Doctor por la Universidad de Salamanca.

Y para que así conste a los efectos oportunos, firmamos el presente certificado en Salamanca a 26 de junio de 2024.

Fdo:

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*A mi hermano.
Por ser la persona más valiente.*

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LISTADO DE ABREVIATURAS

ADN	ácido desoxirribonucleico
AEE	agentes estimuladores de la eritropoyesis
AR	anemia refractaria
AraC	citarabina
AREB	anemia refractaria con exceso de blastos
AREB-1	anemia refractaria con exceso de blastos tipo 1
AREB-2	anemia refractaria con exceso de blastos tipo 2
AREB-t	anemia refractaria con exceso de blastos en transformación
ARN	ácido ribonucleico
ARNm	ARN mensajero
ARSA	anemia refractaria con sideroblastos en anillo
AZA	5-azacitidina
CAR-T	<i>Chimeric antigen receptor-T cell</i>
CHIP	hematopoyesis clonal de potencial indeterminado (<i>Clonal Hematopoiesis of Indeterminate Potential</i>)
CRDM	citopenia refractaria con displasia multilinea
CRDM-SA	citopenia refractaria con displasia multilinea y sideroblastos en anillo
CRDU	citopenia refractaria con displasia unilínea
CRISPR-Cas9	<i>Clustered Regularly Interspaced Short Palindromic Repeats - associated protein 9</i>
DT	dependencia transfusional
FAB	francés-americano-británico
GESMD	Grupo Español de Síndromes Mielodisplásicos
HMA	agentes hipometilantes (<i>hypomethylating agents</i>)
HR	hazard ratio
ICC	Consenso Internacional de Clasificación (<i>International Classification Consensus</i>)
ICUS	citopenia idiopática de significado incierto (<i>Idiopathic Cytopenia of Undertermined Significance</i>)

IPSS	Sistema de clasificación pronóstica internacional (<i>International Prognostic Scoring System</i>)
IPSS-M	Sistema de Clasificación Pronóstica Internacional molecular (<i>Molecular International Prognostic Scoring System</i>)
IPSS-R	Sistema de Clasificación Pronóstica Internacional revisado (<i>Revised-International Prognostic Scoring System</i>)
IT	independencia transfusional
Lena	lenalidomida
LMA	leucemia mieloide aguda
LMMC	leucemia mielomonocítica crónica
MO	médula ósea
NA	no aplica
NE	no estimado
NGS	secuenciación masiva (<i>Next-generation sequencing</i>)
NHEJ	recombinación no homóloga (<i>Non-Homologous End-Joining</i>)
OMS	Organización Mundial de la Salud
RH	recombinación homóloga
SG	supervivencia global
SLP	supervivencia libre de progresión
SMD	síndrome mielodisplásico
SMD-biTP53	síndrome mielodisplásico con inactivación bialélica de <i>TP53</i>
SMD-del(5q)	síndrome mielodisplásico con delección de 5q
SMD-DM	síndrome mielodisplásico con displasia multilínea
SMD-DM-SA	síndrome mielodisplásico con displasia multilínea y sideroblastos en anillo
SMD-DU	síndrome mielodisplásico con displasia unilínea
SMD-DU-SA	síndrome mielodisplásico con displasia unilínea y sideroblastos en anillo
SMD-EB-1	síndrome mielodisplásico con exceso de blastos tipo 1
SMD-EB-2	síndrome mielodisplásico con exceso de blastos tipo 2
SMD-f	síndrome mielodisplásico con fibrosis
SMD-h	síndrome mielodisplásico hipoplásico

SMD-I	síndrome mielodisplásico inclasificable
SMD-IB	síndrome mielodisplásico con incremento de blastos (<i>increased-blast</i>)
SMD-LB	síndrome mielodisplásico con bajo recuento de blastos (<i>low-blast</i>)
SMD-NOS	síndrome mielodisplásico no especificado de otra manera (<i>non-otherwise specified</i>)
SMD-SF3B1	síndrome mielodisplásico con bajo recuento de blastos y mutación en <i>SF3B1</i>
SP	sangre periférica
TPH	trasplante de progenitores hematopoyéticos
VAF	frecuencia de variación alélica (<i>variant allele frequency</i>)
VEN	venetoclax
WES	secuenciación de exoma completo (<i>Whole Exome Sequencing</i>)
WGS	secuenciación de genoma completo (<i>Whole Genome Sequencing</i>)

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INTRODUCCIÓN

1. Los síndromes mielodisplásicos

Los síndromes mielodisplásicos (SMD) son un conjunto heterogéneo de enfermedades clonales de la médula ósea (MO) que afectan principalmente a los precursores mieloides y se caracterizan por la producción anómala de células hematopoyéticas. Estos pacientes presentan hematopoyesis ineficaz, displasia morfológica y, como consecuencia, citopenias en sangre periférica (SP)¹⁻⁵.

La fisiopatología de la enfermedad, así como su diagnóstico y su pronóstico, son muy heterogéneos. Algunos pacientes tienen una supervivencia de varios años, mientras que otros, tan sólo de unos meses^{3,6}. Además, aproximadamente un 30% de los pacientes con SMD progresan a una leucemia mieloide aguda (LMA)^{2,3,7,8}.

1.1. Epidemiología, etiología y diagnóstico

Los SMD son enfermedades raras que afectan a 3-5 personas/año por cada 100.000 habitantes en la población adulta⁶. Es una enfermedad que se observa mayoritariamente en la población de edad avanzada, con una mediana de edad de los enfermos de 70 años y afecta en mayor proporción a varones^{6,9}. En personas mayores de 75 años, la incidencia puede superar los 50 pacientes/año por cada 100.000 habitantes en Europa.

Los SMD son, en su mayoría, idiopáticos o primarios (85-90%), resultantes de lesiones en células madre de la MO. Sin embargo, también pueden derivar de neoplasias previas o de la exposición a tratamientos considerándose, en estos casos, SMD secundarios¹⁰.

Estas neoplasias afectan principalmente a las células precursoras hematopoyéticas y dan lugar a una producción anómala del linaje mieloide¹¹⁻¹³. Al menos, en el 10% de las células de uno de los linajes mieloides en MO, debe haber una displasia inequívoca para considerar el diagnóstico de la enfermedad^{4,5,14-16}.

Los SMD requieren de un diagnóstico integrado y diferencial, teniendo en cuenta el hemograma, la morfología de las células de MO y la caracterización genética del paciente (Tabla 1)^{5,10,16-18}. En un paciente con características clínicas consistentes con un SMD, pero con rasgos morfológicos no concluyentes, el diagnóstico de la enfermedad puede resolverse en base a la presencia de alteraciones citogenéticas clonales recurrentes en los SMD^{5,18-20}.

Tabla 1: Estudios imprescindibles en SP y MO para el diagnóstico diferencial e integrado de los SMD. Adaptada de las Guías Españolas de SMD y LMMC, 2ª edición (2020) del GESMD¹⁶

Estudios imprescindibles en sangre periférica	
Hemograma completo	Recuento absoluto de leucocitos y plaquetas; recuento absoluto y relativo de neutrófilos, eosinófilos, basófilos y monocitos; hemoglobina, hematocrito; parámetros de serie eritroide (VCM, HCM, CHCM, ADE y recuento de reticulocitos).
Frotis de sangre periférica con tinción MGG y sin anticoagulantes	Recuento porcentual diferencial; valoración de rasgos mielodisplásicos (diseritropoyesis, disgranulopoyesis y distrombopoyesis).
Pruebas de diagnóstico diferencial de causas secundarias de citopenias/displasias	Test de Coombs (antiglobulina directa); niveles séricos de eritropoyetina, LDH, ácido fólico, B12; estudio del metabolismo del hierro; parámetros de función hepática, renal y tiroidea; serologías de VHB, VHC y VIH; perfil de autoinmunidad (FR, AAN).
Estudios imprescindibles en médula ósea	
Aspirado medular	Estudios morfológicos (tinciones MGG, Perls) para recuento de blastos y dismorfias; estudios genéticos (citogenética y mutaciones somáticas en <i>TP53</i> y <i>SF3B1</i>)
Biopsia medular*	*requerida en casos de aspirado medular hipoplásico, sospecha de mielofibrosis o ICUS.

VCM: volumen corpuscular medio; HCM: hemoglobina corpuscular media; CHCM: concentración de hemoglobina corpuscular media; ADE: amplitud de distribución eritrocitaria; LDH: lactato deshidrogenasa; VHB: virus de la hepatitis B; VHC: virus de la hepatitis C; VIH: virus de inmunodeficiencia humana; FR: factor reumatoideo; AAN: anticuerpos antinucleares; MGG: May-Grünwald Giemsa; ICUS: citopenia idiopática de significado incierto.

1.2. Clasificación de los SMD

A lo largo de los últimos 30 años se han publicado diversas clasificaciones diagnósticas de los SMD. La gran heterogeneidad de la enfermedad, así como los avances en el conocimiento, son la principal causa de las variaciones entre unas y otras clasificaciones.

La primera clasificación diagnóstica de los SMD en adultos fue propuesta por el grupo cooperativo francés-americano-británico (FAB) en 1982⁴. La organización mundial de la salud (OMS) publicó en 2001 su primer sistema de clasificación de neoplasias mieloides que, posteriormente, se actualizó en 2008 y 2017²¹⁻²³. Recientemente, en 2022, se ha publicado una nueva actualización de esta clasificación junto a la clasificación creada por un grupo cooperativo de expertos internacionales denominada Consenso Internacional de Clasificación de los SMD (ICC)^{5,24}.

Tradicionalmente, el porcentaje de blastos en MO y SP ha sido la principal característica clínica distintiva entre los distintos subtipos de SMD (FAB, OMS 2001-2017), seguido del número de displasias y citopenias y el porcentaje de sideroblastos en anillo^{4,21-23}. En las nuevas clasificaciones, sin embargo, la información genética y molecular ha adquirido una gran relevancia, siendo uno de los parámetros más importantes para la correcta clasificación diagnóstica de los SMD^{5,24}.

La revisión de la OMS de 2022 supone un cambio revolucionario en cuanto a la clasificación de los SMD, adquiriendo las alteraciones genéticas un papel protagonista. Tanto es así, que la primera división de los distintos tipos de SMD se basa en entidades con anormalidades genéticas definitorias y entidades morfológicamente definidas (Tabla 2). Si bien la delección del cromosoma 5, del(5q), y las mutaciones en *SF3B1* ya aparecían en clasificaciones anteriores, en la clasificación de la OMS de 2022 se describen, por primera vez, las mutaciones en *TP53* como definitorias de un subtipo de SMD. Las entidades morfológicamente definidas están basadas en el porcentaje de blastos⁵.

Tabla 2: Clasificación diagnóstica y eventos definitorios de los distintos subtipos de SMD según la revisión de 2022 de la OMS⁵.

CLASIFICACIÓN DIAGNÓSTICA DE LOS SMD (OMS 2022)		
	Porcentaje de blastos	Genética
SMD con anomalías genéticas definitorias		
SMD-5q	<5% MO y <2% SP	Del(5q) aislada o con otra alteración citogenética distinta de -7 y del(7q)
SMD-SF3B1	<5% MO y <2% SP	Mutación en SF3B1 y ausencia de del(5q), -7, del(7q) o cariotipo complejo
SMD-biTP53	>20% en MO y SP	dos o más alteraciones en TP53, generalmente asociado a cariotipos complejos
SMD morfológicamente definidos		
SMD-LB	<5% MO y <2% SP	n.a.
SMD-IB		
SMD-IB1	5-9% MO ó 2-4% SP	n.a.
SMD-IB2	10-19% MO ó 5-19% SP	n.a.
SMD-f	5-19% MO ó 2-19% SP	n.a.
SMD-h	<5% MO y <2% SP	n.a.

SMD-5q: SMD con bajo recuento de blastos y delección de 5q; SMD-SF3B1: SMD con bajo recuento de blastos y mutación en SF3B1; SMD-biTP53: SMD con inactivación bialélica de TP53; SMD-LB: SMD con bajo recuento de blastos; SMD-IB: SMD con incremento de blastos; SMD-IB1: SMD-IB tipo 1; SMD-IB2: SMD-IB tipo 2; SMD-f: SMD con fibrosis; SMD-h: SMD hipoplásico; n.a: no aplica.

Por otro lado, siguiendo el criterio de la versión de 2017 de la OMS, la ICC propone dos grandes grupos basados en el recuento de blastos (SMD sin exceso de blastos – bajo riesgo – y SMD con exceso de blastos) y añade dos nuevos grupos: SMD/LMA y neoplasias mieloides con mutación en TP53 (Tabla 3)²⁴.

Tabla 3: Clasificación Internacional Consensuada (ICC) de los SMD²⁴.

CLASIFICACIÓN INTERNACIONAL DE CONSENSO DE LOS SMD (ICC 2022)		
	Porcentaje de blastos	Genética
SMD sin exceso de blastos		
SMD-5q	<5% MO y <2% SP	Del(5q) aislada o con otra alteración citogenética distinta de -7 y del(7q); ausencia de <i>TP53</i> multi-hit
SMD- <i>SF3B1</i>	<5% MO y <2% SP	Mutación en <i>SF3B1</i> (VAF>10%); ausencia de del(5q), -7, del(7q), abn3q26, cariotipo complejo, <i>TP53</i> multi-hit o mutación en <i>RUNX1</i>
SMD-NOS sin displasia	<5% MO y <2% SP	Alteraciones -7, del(7q) o cariotipo complejo; ausencia de <i>SF3B1</i> (>10%) o <i>TP53</i> multi-hit
SMD-NOS con displasia - <i>SMD-NOS displasia unilínea</i> - <i>SMD-NOS displasia multilínea</i>	<5% MO y <2% SP	Cualquiera que no cumpla criterios de SMD-5q ni SMD- <i>SF3B1</i> ; ausencia de <i>TP53</i> multi-hit
SMD con exceso de blastos		
SMD-EB	5-9% MO, 2-9% SP	Cualquier alteración, excepto <i>TP53</i> multi-hit
SMD/LMA	10-19% MO/SP	Cualquier alteración, salvo definitorias de LMA; ausencia de <i>NPM1</i> , bZIP <i>CEBPA</i> o <i>TP53</i>
Neoplasias mieloides con mutación en <i>TP53</i>		
SMD- <i>TP53</i>	0-9% MO/SP	<i>TP53</i> multi-hit o mutación en <i>TP53</i> (VAF>10%) con pérdida de 17p
SMD/LMA- <i>TP53</i>	10-19% MO/SP	Mutación en <i>TP53</i> (VAF>10%)
LMA- <i>TP53</i>	>20% MO/SP	Mutación en <i>TP53</i> (VAF>10%)

SMD-5q: SMD con delección de 5q; SMD-*SF3B1*: SMD con mutación en *SF3B1*; SMD-NOS: SMD no especificado de otra manera (*no otherwise specified*); SMD-EB: SMD con exceso de blastos; SMD-*TP53*: SMD con mutación de *TP53*; VAF: frecuencia de variación alélica.

Hoy en día, la clasificación de la OMS 2017 sigue siendo la más extendida en la práctica clínica, dado que existen ciertas discrepancias a la hora de implementar las nuevas clasificaciones de la OMS e ICC.

1.3. Sistemas de estratificación pronóstica de los SMD

En el año 1997, Greenberg y colaboradores establecieron un Sistema de Clasificación Pronóstica conocido, por sus siglas en inglés, como IPSS (*International Prognostic Scoring System*). Este grupo de expertos internacionales combinó, en una única escala de riesgo, parámetros citogenéticos, morfológicos y clínicos de 7 grandes cohortes de pacientes, previamente analizadas con el mismo fin: generar un sistema pronóstico para los SMD. Con la reevaluación global de estos pacientes, se creó un consenso de pronóstico que incluía una clasificación citogenética refinada. Además del cariotipo, el impacto del porcentaje de blastos en MO y el número de las citopenias, fueron estadísticamente significativos en la supervivencia global (SG) y en la supervivencia libre de progresión (SLP)²⁵.

Años más tarde, se publicó una nueva escala pronóstica, teniendo en cuenta únicamente alteraciones citogenéticas, dando a cada una de las anormalidades cariotípicas más frecuentes en los SMD un pronóstico definido. Esta puntuación pronóstica citogenética fue el precedente a la publicación de un nuevo IPSS revisado, (IPSS-R, *Revised-International Prognostic Scoring System*). El número de pacientes estudiados para definir las bases del IPSS-R se incrementó notablemente con respecto a los pacientes incluidos en el IPSS (7012 en el IPSS-R frente a 816 en el IPSS), aumentando así la precisión de la escala pronóstica. Este nuevo modelo incluía la citogenética (con mejoría en su estratificación), el porcentaje de blastos en MO (definiendo diferentes rangos pronósticos) y los niveles de hemoglobina, plaquetas y neutrófilos como principales variables con impacto pronóstico²⁶.

Recientemente, se ha incorporado a este IPSS-R la información molecular de las mutaciones somáticas presentes en los pacientes con SMD²⁷ y en el año 2022 se publicó el nuevo IPSS-Molecular (IPSS-M). Este estudio incluyó 2.957 pacientes en los que se analizó el estado mutacional de 152 genes. De ellos, se identificaron 31 genes como predictores pronósticos. El estado *multihit* de *TP53*, así como las mutaciones en *FLT3* y *MLL* fueron reportados como los genes con pronóstico adverso más relevantes, mientras que *SF3B1* se asoció con un estado favorable de la enfermedad, sujeto a variación según los patrones de comutación. Así pues, las variables con impacto pronóstico en el IPSS-M, fueron: a) niveles

de hemoglobina; b) recuento de plaquetas; c) porcentaje de blastos en MO; d) categorías citogenéticas incluidas en el IPSS-R; e) estado mutacional (valor binario) de mutaciones en 16 genes pronósticos; y f) el número de genes mutados recogidos en una lista residual de 15 genes adicionales. Destaca la presencia de, al menos, una alteración genómica en el 94% de los pacientes, por lo que esta nueva clasificación, con 6 grupos de riesgo diferenciado, representa una herramienta de valor para la toma de decisiones clínicas según el pronóstico de la enfermedad (Tabla 4)²⁷.

Tabla 4: Supervivencia libre de progresión (SLP) y supervivencia global (SG) en años en los distintos grupos de riesgo de los SMD según el IPSS, IPSS-R e IPSS-M.

Riesgo	IPSS ²⁵	IPSS-R ²⁶	IPSS-M ²⁷
Muy bajo			
SLP ⁺	n.a.	n.e.	9,7
SG ⁺	n.a.	8,8	10,6
Bajo			
SLP	9,4	10,8	5,9
SG	5,7	5,3	6,0
Intermedio 1*			
SLP	3,3	3,2	4,5
SG	3,5	3,0	4,6
Intermedio 2*			
SLP	1,1	n.a.	2,3
SG	1,2	n.a.	2,8
Alto			
SLP	0,2	1,4	1,5
SG	0,4	1,6	1,7
Muy alto			
SLP	n.a.	0,73	0,76
SG	n.a.	0,8	1,0

⁺Los datos de SLP y SG están expresados como medianas (años); *Las categorías de riesgo intermedio se distribuyen de la siguiente manera (de menor a mayor riesgo): a) IPSS: intermedio-1 e intermedio-2; b) IPSS-R: un solo grupo de riesgo intermedio; c) IPSS-M: dos grupos diferenciados en riesgo moderado-bajo y moderado-alto.

IPSS: *International Prognostic Scoring System*; IPSS-R: *Revised-IPSS*; IPSS-M: *IPSS-molecular*; n.a.: no aplica; n.e.: no estimado; SLP: supervivencia libre de progresión; SG: supervivencia global.

1.4. Transformación a LMA

Los pacientes con SMD presentan un alto riesgo de transformación leucémica. Se estima que en torno al 30% de los pacientes con SMD progresan a LMA secundaria. Por este motivo, el tiempo hasta la transformación de la enfermedad es uno de los factores principales en los sistemas de clasificación pronóstica en los SMD.

La LMA secundaria es una entidad distinta a la LMA *de novo*. Representa el 25-34% de todas las leucemias mieloblásticas y, en su mayoría, derivan de un SMD previo (60-80%)^{28,29}. De hecho, en la última actualización de la clasificación de neoplasias mieloides de la OMS, las LMA secundarias a un SMD pasaron a formar parte de las LMA relacionadas con mielodisplasia. Además, el ICC definió al subgrupo de SMD con 10-19% de blastos en MO como una transición entre ambas enfermedades (SMD/LMA), siendo estas predecesoras de las LMA secundarias a SMD. La LMA secundaria, por tanto, está definida por un porcentaje de blastos en MO mayor del 20%, con inmunofenotipo mieloide y, en algunos casos, por la presencia de alteraciones citogenéticas y moleculares propias de los SMD. Las mutaciones en genes como *STAG2*, *SRSF2*, *EZH2*, *ZRSR2* o *ASXL1*, entre otros, son un criterio diagnóstico de este tipo de leucemia^{5,30,31}.

La LMA secundaria a SMD se asocia con un peor pronóstico y tiempos de recaída inferiores, en comparación con las LMA *de novo*. Además, estos pacientes presentan bajas tasas de remisión de la enfermedad tras el tratamiento con quimioterapia intensiva, con una mediana de SG de tan solo 9-12 meses^{29,32}.

A pesar de que es un tema que suscita mucho interés, aún no se conocen en profundidad los mecanismos moleculares por los que se produce esta transformación leucémica. Sin embargo, estudios recientes han demostrado, mediante tecnologías de célula única, la existencia de patrones de evolución clonal de las alteraciones genéticas estrechamente relacionados con la transformación del SMD³³. Esto ha permitido que pacientes con SMD de alto riesgo puedan beneficiarse de tratamientos utilizados para las LMA, y viceversa, abriendo así nuevas opciones terapéuticas para los pacientes^{30,34-37}.

1.5. Tratamiento de los SMD

El tratamiento de los SMD difiere según la SG que presentan los pacientes, que puede verse afectada por el riesgo de transformación a LMA. De esta manera, algunos pacientes presentan una SLP de solo unos pocos meses, mientras que otros de varios años e, incluso, de décadas. En los pacientes de bajo riesgo, el objetivo principal del tratamiento es mejorar la calidad de vida y mantener a los pacientes en un buen estado de salud. Se realiza corrigiendo las citopenias sintomáticas mediante el tratamiento de soporte para retrasar o evitar la dependencia transfusional (DT) o, en caso de ser pacientes con DT, revertir esta situación en la medida de lo posible. Por el contrario, los tratamientos dirigidos a pacientes con SMD de alto riesgo tratan de aumentar la SG y retrasar o evitar la evolución a LMA³⁷. A continuación, se detallan los principales tratamientos utilizados en la práctica clínica actual, así como las nuevas terapias con un desarrollo prometedor en pacientes con SMD.

1.5.1. Pacientes de bajo riesgo: tratamiento de soporte y drogas inmunomoduladoras en los SMD-del(5q)

El principal objetivo de los tratamientos en estos pacientes está dirigido a la mejoría de calidad de vida en relación con la DT, así como con otros síntomas relacionados con la enfermedad, ya que estos pacientes tienen una esperanza de vida larga. Se trata, por tanto, de tratamientos de soporte y no existen tratamientos para los SMD de bajo riesgo que hayan demostrado un incremento en la SG de los pacientes^{37,38}. Por ello, ante la ausencia de sintomatología, los pacientes no necesariamente tienen que ser tratados.

Actualmente, la práctica clínica contempla tres tratamientos distintos para estos pacientes:

- *Agentes estimuladores de la eritropoyesis (AEE)*, que han resultado eficaces en el tratamiento de la anemia debido al aumento significativo de los niveles de hemoglobina en los pacientes. Supone la primera opción de tratamiento en los pacientes con SMD de bajo riesgo con anemia sintomática³⁹⁻⁴².
- *Lenalidomida (Lena)*, un agente inmunomodulador que se utiliza en pacientes con SMD y del(5q) aislada que presentan DT, reduciendo la necesidad de transfusiones e incluso revirtiendo a un estado de independencia transfusional (IT). El ensayo clínico SintraRev estudió, por primera vez, la administración precoz de Lena en pacientes con del(5q), anemia y sin DT para evaluar si su uso permitía retrasar la aparición de DT y LMA, demostrando que el tratamiento precoz permite prolongar significativamente la

IT en estos pacientes⁴³. Sin embargo, existen indicios de que las mutaciones en *TP53* podrían favorecer la expansión clonal y, con ello, la progresión a LMA, bajo tratamiento con Lena⁴⁴⁻⁴⁷. Además, se han observado tasas de respuesta a Lena inferiores en los pacientes con mutaciones en *TP53*⁴⁴. Por este motivo, y ante la falta de un conocimiento exhaustivo de los mecanismos que podrían favorecer la expansión clonal en pacientes con alteraciones en *TP53*, actualmente no se recomienda el tratamiento con Lena a los pacientes que presenten mutaciones en este gen^{48,49}.

- *Luspatercept*, un nuevo fármaco en SMD que ha mostrado resultados prometedores en el tratamiento de bajo riesgo, revirtiendo el estado de DT de los pacientes⁵⁰⁻⁵³. Su uso está aprobado en primera línea de tratamiento en pacientes con DT e IPSS-R muy bajo, bajo o intermedio, y en segunda línea para SMD con sideroblastos en anillo/mutación de *SF3B1* y respuesta insatisfactoria/no candidatos a AEEs.

La escasa variedad de tratamientos en estos pacientes, así como la eficacia limitada de los mismos, ha propiciado un desarrollo farmacológico en los últimos años que ha abierto una nueva ventana terapéutica para este subgrupo de pacientes de bajo riesgo. En este sentido, imetelstat o nuevos inhibidores de la vía del TFG-B, como KER-050, están en desarrollo clínico para este subgrupo de pacientes^{36,50-56}. Además, se está investigando el papel de los tratamientos modificadores de la enfermedad, utilizados en el alto riesgo, para el tratamiento de determinados pacientes con SMD de bajo riesgo, especialmente aquellos con características clínicas y biológicas adversas que puedan suponer un aumento del riesgo de la transformación de la enfermedad. En un futuro próximo, la utilización de hipometilantes orales (recientemente aprobados) podría considerarse como una opción óptima para el tratamiento del SMD de bajo riesgo^{57,58}.

1.5.2. Pacientes de riesgo intermedio y alto: trasplante de progenitores hematopoyéticos y agentes hipometilantes

El tratamiento de los pacientes con SMD de alto riesgo tiene como finalidad alargar la SLP y, con ello, la SG. La elección terapéutica para estos pacientes varía en función del estado del paciente y las características de la enfermedad, dada la alta heterogeneidad clínica observada en los SMD. En cualquier caso, se recomienda iniciar el tratamiento que se estime apropiado para cada paciente a la mayor brevedad posible, ya que estos pacientes presentan un alto riesgo de transformación leucémica^{59,60}.

Actualmente, los tratamientos utilizados para el tratamiento de los SMD de alto riesgo se resumen en:

- *Trasplante de progenitores hematopoyéticos (TPH)*, es la única opción con capacidad curativa que ha demostrado superioridad frente a otras estrategias terapéuticas, como la utilización de agentes hipometilantes⁶¹⁻⁶³. Sin embargo, la tasa de mortalidad y morbilidad es alta, lo que hace que no sea una estrategia adecuada para todos los pacientes^{61,64}. La opción de realizar un TPH a un paciente dependerá de factores como la edad, índice de comorbilidad, clasificación del SMD, porcentaje de blastos en MO, el grupo de riesgo citogenético y el pronóstico (IPSS-R), entre otros⁶⁵⁻⁶⁷. Asimismo, la presencia de mutaciones en *ASXL1*, *RUNX1*, *JAK2*, *NRAS* y, sobre todo, *TP53*, se han asociado con una menor supervivencia^{68,69}.
- *Quimioterapia intensiva*, similar a la utilizada en pacientes con LMA, se basa en combinaciones de antraciclinas y citarabina (Ara-C). Este tratamiento se utiliza en pacientes con LMA *de novo* o secundarias a SMD, pero debido a la agresividad del tratamiento, no todos los pacientes son candidatos a recibirla^{34,70}. De hecho, solo se recomienda para pacientes jóvenes, con cariotipo favorable y que puedan ser elegibles para TPH, lo que hace que no sea un tratamiento de elección.
- *Agentes hipometilantes (HMA, de sus siglas en inglés, hypomethylating agents)*, son el tratamiento de elección en los SMD de alto riesgo debido al aumento en la SG, retraso de la evolución a LMA y mejoría de las citopenias. Existen dos hipometilantes aprobados para su uso en la práctica clínica: 5-azacitidina (AZA) y decitabina^{37,64}.

Además de las escasas opciones terapéuticas que existen en la práctica clínica para el tratamiento de los SMD de alto riesgo, la resistencia a HMA resulta cada vez más preocupante, puesto que la SG de estos pacientes disminuye drásticamente hasta los 4-6 meses de vida cuando este tratamiento fracasa. En la actualidad, no existe ningún tratamiento que haya demostrado una mejora en los pacientes refractarios a hipometilantes, si bien se están investigando combinaciones con venetoclax (VEN) para cubrir esta necesidad médica no cubierta^{35,71,72}.

VEN es un inhibidor de BCL2 (proteína antiapoptótica) que ha sido aprobado recientemente en combinación con agentes hipometilantes (VEN+AZA) y bajas dosis de Ara-C (VEN+AraC) en LMA de nuevo diagnóstico o en recaída^{39,75}. Los pacientes tratados con estas combinaciones mejoran la SG frente a AZA, dado que alcanzan mayores tasas de remisión, así como respuestas moleculares duraderas, como sugieren los análisis de

enfermedad mínima residual⁷³⁻⁷⁶. Es por ello que se está testando la seguridad y la eficacia de estas combinaciones en los SMD de alto riesgo^{35,71,72,77-79}.

2. Genética de los SMD

Los SMD son un grupo heterogéneo de enfermedades clonales con un alto componente genético. Tanto es así, que se ha reportado en numerosas ocasiones que más del 90% de los pacientes presentan al menos 1 alteración genética^{13,27}. Estas alteraciones pueden ser anormalidades cromosómicas o citogenéticas, o mutaciones somáticas puntuales.

2.1. Citogenética

El primer estudio citogenético a gran escala en pacientes con SMD fue publicado hace más de 30 años. Desde entonces, la citogenética ha sido clave para conocer la diversidad fenotípica que existe en esta patología. Las técnicas citogenéticas convencionales, como el cariotipo y la FISH (de sus siglas en inglés, *Fluorescence In Situ Hybridization*) siguen siendo las técnicas de referencia para el estudio genético de los cromosomas en los SMD⁸⁰⁻⁸⁴.

El estudio de cariotipo se basa en la tinción de bandas cromosómicas de células en metafase que permite visualizar y analizar los cromosomas de un individuo. Identifica inestabilidades cromosómicas, como pérdidas y ganancias de cromosomas totales o parciales, y detecta desequilibrios estructurales, como traslocaciones e inversiones^{81,85}. Por otro lado, la técnica FISH permite identificar anomalías citogenéticas no sólo en células en metafase, también en interfase, mediante sondas marcadas con fluorescencia que se unen de manera específica a regiones localizadas de interés^{80,86}.

Las alteraciones en el cariotipo se encuentran en, aproximadamente, un 50% de los pacientes con SMD. Predomina la adquisición de cromosomas enteros o la pérdida total o parcial. Las alteraciones citogenéticas más frecuentemente observadas en los SMD se encuentran en los cromosomas 5, 8, 7 y 20, aunque también se han observado de manera recurrente alteraciones en los cromosomas 11, 12 o Y⁸⁷⁻⁹¹.

A pesar de la alta incidencia de estas anormalidades en los pacientes con SMD, estas alteraciones no son exclusivas de neoplasias mieloides y requieren de pruebas adicionales para garantizar un diagnóstico de SMD⁹². Además, la diversidad característica de los SMD también se refleja en las aberraciones de cariotipo de los enfermos.

En el año 2012, un estudio multicéntrico demostró la implicación que tienen estas alteraciones en el pronóstico de los enfermos con SMD y estableció un índice de puntuación pronóstica para cada una de estas anomalías del cariotipo (Tabla 5)⁹⁰.

Tabla 5: Supervivencia global (SG) y supervivencia libre de progresión (SLP) en los distintos grupos de riesgo citogenético. Adaptada de Schanz y colaboradores, 2012⁹⁰.

Subgrupo de riesgo	Anormalidades citogenéticas	SG		SLP	
		Mediana ⁺	HR	Mediana ⁺	HR
Muy bajo	del(11q) -Y	60,8 (50,3-NE)	0,5 (0,3-0,7)	NE (121,2-NE)	0,5 (0,2-1,2)
Bajo	Normal del(5q) del(5q) + otra del(12p) del(20q)	48,6 (44,6-54,3)	1,0 (0,9-1,1)	NE (189,0-NE)	1,0 (0,9-1,2)
Intermedio	del(7q) +8 i(17q) +19 otras	26,0 (22,1-31,0)	1,6 (1,4-1,8)	78,0 (42,6-NE)	2,2 (1,8-2,7)
Alto	inv(3)/t(3q)/del(3q) -7 -7 + otra 3 alteraciones	15,8 (12,0-18,0)	2,6 (2,1-3,2)	21,0 (13,4-42,2)	3,4 (2,5-4,6)
Muy alto	>3 alteraciones	5,9 (4,9-6,9)	4,2 (3,4-5,2)	8,2 (6,4-15,4)	4,9 (3,6-6,7)

⁺Los datos de SLP y SG están expresados en meses.

SG: supervivencia global; SLP: supervivencia libre de progresión; HR: hazard ratio.

2.1.1. Deleción del 5q

La deleción del brazo largo del cromosoma 5 (5q) es una de las alteraciones citogenéticas más frecuente en los SMD. Se observa en un 10% de los pacientes con SMD y anomalías en el cariotipo. Esta alteración representa la primera característica genética que se utilizó para definir a un subgrupo de pacientes en las clasificaciones de los SMD y que se mantiene en las últimas actualizaciones^{5,23,24}.

La del(5q) se asocia con un buen pronóstico de la enfermedad. De hecho, el subgrupo de pacientes con SMD-del(5q) presentan un bajo riesgo de progresión a LMA^{5,24}. Generalmente, cursan con anemia sintomática y adquieren DT poco tiempo después del diagnóstico⁹³. Aunque el estándar de tratamiento en los pacientes con anemia son los AEEs, los pacientes con SMD y del(5q) presentan una baja tasa de respuesta, por lo que la Lena se aprobó para el tratamiento en segunda línea en este subgrupo de pacientes^{40,94-96}. La administración de Lena inhibe selectivamente el clon con la del(5q) y reduce de manera efectiva la DT en los pacientes, aumentando así la calidad de vida de los enfermos, además de su SG y SLP^{97,98}. Sin embargo, se desconoce el efecto que tiene la Lena sobre las mutaciones adicionales observadas en el clon con la del(5q).

El perfil mutacional de los SMD con del(5q) está bien definido, y se caracteriza por un menor número de mutaciones por paciente que en el resto de SMD y una alta incidencia de mutaciones en *SF3B1* o en *TP53*⁹⁹. Esto último es paradójico en un subgrupo de SMD con un bajo riesgo de la enfermedad, pues las mutaciones en *TP53* se han asociado particularmente con mal pronóstico, siendo uno de los principales conductores de transformación leucémica^{100,101}. De hecho, los pacientes con SMD y del(5q) que presentan alteraciones en este gen, muestran respuestas deficientes a Lena y se ha planteado, incluso, la posibilidad de que los clones con *TP53* mutado puedan acelerar la progresión de la enfermedad durante el tratamiento^{46,102}.

Recientemente, las mutaciones en *SF3B1* en pacientes con SMD y del(5q) también se han asociado con un menor tiempo de transformación a LMA, así como de la SG, en comparación con los pacientes con del(5q) y *wild-type* para *SF3B1*^{103,104}. A pesar de ello, los pacientes con del(5q) y mutaciones en *SF3B1* muestran un pronóstico comparable, también favorable, al del resto de subtipos de SMD con *SF3B1* mutado¹⁰⁴. Cómo afectan los tratamientos a las alteraciones en *SF3B1* concomitantes con la del(5q) en los SMD, está aún por determinar.

2.1.2. Trisomía del cromosoma 8

La trisomía 8 (+8) es una de las alteraciones citogenéticas más frecuentes en los SMD, encontrándose hasta en un 10% de los pacientes con anomalías cromosómicas^{105,106}. Su incidencia es mayor en varones, población europea y no varía con la edad¹⁰⁶. La presencia de +8 se ha descrito de manera frecuente en otras patologías hematológicas, como la leucemia

mielomonocítica crónica, la LMA o neoplasias mieloproliferativas, no siendo exclusiva de los SMD^{105,106}.

La +8 se observa en combinación con otras anomalías citogenéticas en un 5% de los casos con SMD, siendo particularmente común la asociación con las trisomías de los cromosomas 19 o 21¹⁰⁶. Estudios citogenéticos en pacientes con SMD indican que la +8 no es un evento primario, sino que forma parte del clon maligno junto a otras alteraciones. Todo ello podría indicar que la +8 no es suficiente para iniciar, por sí sola, el desarrollo de la enfermedad, sino que requiere de eventos adicionales para que esto ocurra.

La +8, sin embargo, también se observa con frecuencia de manera aislada en los SMD. En este caso, se asocia con un riesgo citogenético intermedio y los pacientes muestran una gran variabilidad clínica. Tanto es así que algunos pacientes con +8 aislada progresan rápidamente a LMA, mientras que otros se mantienen estables durante años. De la misma manera, la SG puede variar entre unos pocos meses y varios años^{107,108}.

Ante el pronóstico heterogéneo de los SMD con +8 y en ausencia de alteraciones citogenéticas acompañantes, en 2018 Drevon y colaboradores analizaron en una cohorte reducida de SMD con +8 aislada y observaron un mayor número de mutaciones somáticas y la presencia de rasgos mieloproliferativos en un grupo con baja SG. Además, estos pacientes presentaban un menor tiempo hasta la progresión y una peor tasa de respuesta a tratamiento, en comparación con el grupo de pacientes con +8 que no mostraban características mieloproliferativas ni una alta incidencia de mutaciones somáticas¹⁰⁹. Asimismo, varios investigadores han descrito que la incidencia de mutaciones en genes como *ASXL1*, *EZH2*, *STAG2*, *SRSF2* o *U2AF1* es mayor en pacientes con +8 que en el resto de SMD¹⁰⁹⁻¹¹².

Estos datos sugieren que la presencia de mutaciones somáticas podría influir en el pronóstico de los pacientes con esta alteración citogenética. Sin embargo, se requiere de estudios con grandes cohortes de pacientes para determinar qué implicación pronóstica tienen las mutaciones somáticas en el contexto de SMD con +8 aislada.

2.2. Mutaciones somáticas

En la última década, la secuenciación de última generación (NGS, de sus siglas en inglés, *Next-Generation Sequencing*) se ha abierto paso en el estudio de las enfermedades hematológicas. Durante décadas, la secuenciación Sanger fue el método más utilizado para el estudio de alteraciones genéticas puntuales^{113,114}. Sin embargo, la aparición de la NGS supuso un cambio revolucionario en el estudio de las enfermedades clonales, permitiendo estudiar cientos de genes de manera simultánea e incluso el genoma completo^{27,69,111,115,116}.

Los estudios de NGS pueden llevarse a cabo mediante distintas metodologías, en función de la cantidad de genes analizados: secuenciación de genoma completo o WGS (*Whole Genome Sequencing*), secuenciación de exoma completo o WES (*Whole Exome Sequencing*) y paneles de genes personalizados (técnicas de captura o amplicones)^{115,117-119}.

Los paneles de genes personalizados permiten analizar una cantidad de genes determinada o regiones de interés relacionadas con la patología, por lo que los análisis requeridos son más sencillos que en el caso de secuenciación masiva del genoma o exoma completo. La reducción en el número de genes analizados supone un incremento de la sensibilidad y la cobertura de la secuenciación. La facilidad de interpretación de resultados, así como su costo-efectividad, son dos de las principales ventajas de esta técnica frente a otras metodologías de secuenciación¹¹⁹⁻¹²². Una de las estrategias utilizadas para la secuenciación dirigida es la secuenciación mediante captura, a través de la cual las regiones de interés se hibridan con sondas biotinizadas que permiten capturar estas regiones y enriquecerlas. Las ventajas que presentan los paneles dirigidos hacen que sea la tecnología de NGS más desarrollada en el estudio de los SMD y con mayor implementación en la práctica clínica.

Los SMD son enfermedades altamente heterogéneas a nivel genético. Probablemente, la gran diversidad de alteraciones moleculares que presentan estos pacientes es también responsable de la variabilidad clínica observada entre ellos¹³. Hoy en día, el estudio de las mutaciones somáticas en los SMD se ha reconocido tanto para la correcta clasificación diagnóstica de los pacientes, como para la estratificación y pronóstico del riesgo de la enfermedad^{5,27}.

2.2.1. Genes frecuentemente mutados en SMD

Desde el año 2013, son numerosas las publicaciones relacionadas con mutaciones somáticas en los SMD^{7,8,123}. Se estima que más del 90% de los pacientes con SMD presentan, al menos, una mutación somática puntual^{3,123}. El número de alteraciones difiere según el tipo de SMD, con una mediana de 2-3 mutaciones en los SMD de bajo riesgo y entre 9 y 13 alteraciones en los SMD de alto riesgo y LMA secundaria¹²⁴.

Se han descrito alteraciones en una gran cantidad de genes con funciones diversas (Figura 1)^{8,125}. Entre ellos, destacan distintas categorías:

- **Reguladores epigenéticos:** son genes implicados en la regulación de las marcas epigenéticas en la cromatina, regulando así la apertura de horquillas de replicación y, como consecuencia, que los genes se expresen. Los principales reguladores epigenéticos alterados en los SMD son *ASXL1*, *DNMT3A*, *TET2*, *IDH1*, *IDH2* y *EZH2*. Estos genes pueden estar implicados en la metilación del ADN (como *DNMT3A*, *TET2* e *IDH1/2*) o actuar como modificadores de cromatina (*ASXL1* y *EZH2*)^{7,8,123,126-128}. Este grupo de genes se encuentra alterado en un 60-70% de los pacientes con SMD¹²⁴.

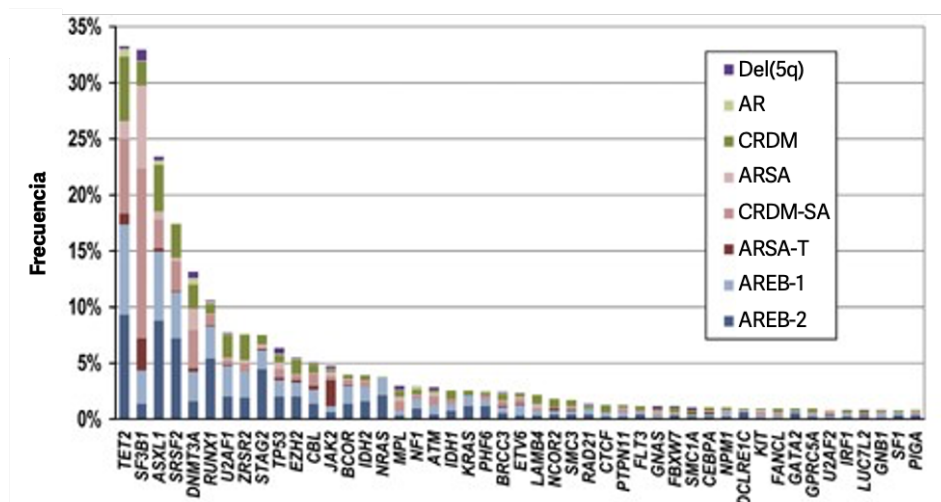
- **Factores de *splicing*:** estos genes regulan el mecanismo de *splicing*, que consiste en la eliminación de intrones del ARN mensajero (ARNm) para dar lugar a un ARNm maduro y asegurar la correcta síntesis de las diferentes isoformas de la proteína que codifican. En los SMD, se han descrito mutaciones en distintos componentes del complejo del *spliceosoma*, como son *SF3B1*, *SRSF2*, *U2AF1*, *U2AF2*, *ZRSR2* y *SF1*^{7,8,123,129-131}. En los últimos años, las mutaciones en el gen *SF3B1* han sido muy estudiadas debido a su alta frecuencia (20-30%), así como a la asociación con la formación de sideroblastos en anillo^{131,132}. Por este motivo, ya en la clasificación de la OMS en 2017 y en las posteriores clasificaciones de 2022, las mutaciones en *SF3B1* se consideran como definitorias del subgrupo específico de SMD que presenta sideroblastos en anillo y se asocian con un buen pronóstico de la enfermedad^{5,24,132,133}.

- **Complejo cohesinas:** este complejo multiproteico está involucrado en la organización del huso mitótico y en la segregación de las cromátidas hermanas cuando la célula se encuentra en división. Alteraciones en los genes *STAG1*, *STAG2*, *SMC1A*, *SMC3*, *RAD21* o *CTCF* se han descrito con frecuencia en los SMD^{7,8,123,134-137}. La incidencia de mutaciones en genes del complejo cohesinas en SMD y otras patologías mieloides varía entre un 10 y un 15%¹²⁴.

- **Transducción de señales:** se han descrito alteraciones en componentes de importantes rutas de señalización, como la vía de RAS (*NRAS*, *KRAS*, *CBL*, *PTPN11*, *NF1*, *KIT*) o tirosin-quinasa (*JAK2*, *FLT3*), implicados en proliferación y diferenciación celular. Alteraciones en la vía de RAS se han asociado con un mayor riesgo de transformación a LMA y, por consiguiente, con un peor pronóstico de la enfermedad. Su incidencia se sitúa en torno al 10-15% de los pacientes con SMD^{7,8,123,137,138}.

- **Factores de transcripción:** están implicados en la transcripción de genes con distintas funciones celulares relacionadas con la hematopoyesis, diferenciación, proliferación, regulación del ciclo celular y respuestas inmunológicas e inflamación. Entre los factores de transcripción en los que se han descrito mutaciones en los SMD, se encuentran *RUNX1*, *GATA2*, *ETV6*, *BCOR/BCORL*, *IRF1*, *NPM1*, *PHF6* o *CEBPA*. Estas alteraciones se encuentran en un menos del 15% de los SMD^{7,8,123,127,139}.

- **Reparación del daño celular:** el gen supresor de tumores *TP53* es uno de los principales genes mutados en los SMD. Está implicado en la reparación del ADN. La presencia de mutaciones en este gen, es uno de los principales factores pronósticos en los SMD, teniendo un papel muy importante en la clasificación pronóstica de los enfermos. Las mutaciones en *TP53* se observan en, aproximadamente, el 20% de los pacientes con SMD, presentando una alta prevalencia en los SMD con del(5q)^{7,8,100,103,123,127}.



Del(5q): SMD con delección de 5q; *AR*: anemia refractaria; *CRDM*: citopenia refractaria con displasia multilinea; *ARSA*: anemia refractaria con sideroblastos en anillo; *CRDM-SA*: CRDM con sideroblastos en anillo; *ARSA-T*: ARSA en transformación; *AREB-1*: anemia refractaria con exceso de blastos tipo 1; *AREB-2*: anemia refractaria con exceso de blastos tipo 2.

Figura 1: Genes más frecuentemente mutados en los distintos subgrupos de SMD según la clasificación de la OMS de 2008. Imagen adaptada de Haferlach et al. 2014⁸

A pesar de la gran heterogeneidad de genes alterados en los SMD, la combinación de mutaciones entre ellos no es arbitraria, existiendo patrones de co-mutación frecuentes en los pacientes. Así, las mutaciones en los genes de metilación del ADN con frecuencia se observan junto a alteraciones en factores reguladores de *splicing* y pueden estar presentes en estadios tempranos de la enfermedad, considerándose mutaciones “fundadoras” en más del 50% de los SMD^{3,7,8,140}. Por el contrario, las alteraciones en los genes reguladores de *splicing* o del complejo cohesinas, son mutuamente excluyentes^{3,8,124,129,135}.

Por lo tanto, a nivel genético, los SMD se caracterizan por la adquisición de mutaciones conductoras o en genes “driver” que inician la patología, acompañadas de complejas combinaciones de mutaciones. Mediante un proceso de evolución clonal, dan lugar a una gran heterogeneidad, no sólo entre pacientes, sino también intratumoral.

2.2.2. Evolución clonal y transformación leucémica

Con frecuencia, algunas de las mutaciones adquiridas en las células madre pueden estar asociadas a hematopoyesis clonal y conferir ventajas proliferativas a las células^{12,13}. Se han descrito mutaciones en individuos sanos que afectan a genes frecuentemente mutados en los SMD, las cuales aumentan el riesgo de padecer este tipo de enfermedades. Este estado en el que una persona sana presenta mutaciones en genes relacionados con patología mieloide sin ser indicativo de enfermedad, se conoce como CHIP (de sus siglas en inglés, *Clonal Hematopoiesis of Indeterminate Potential*)¹⁴¹⁻¹⁴³. Las mutaciones asociadas a CHIP se observan con mayor incidencia en personas de edad avanzada (10% en >65 años; Figura 2) y, además, se relacionan con una predisposición aumentada (0,5% cada año) de desarrollar una neoplasia mieloide¹⁴¹⁻¹⁴³.

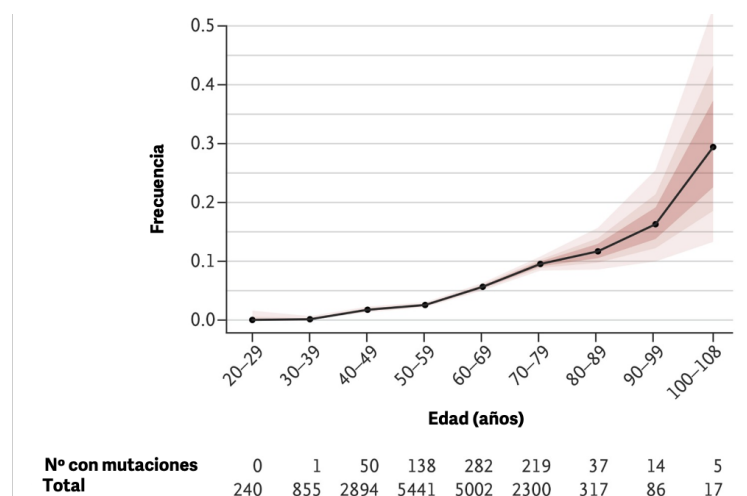


Figura 2: Incidencia de mutaciones asociadas a CHIP en individuos sanos por grupo de edad.

Imagen adaptada de Jaiswal et al. 2014¹⁴².

El descubrimiento de las mutaciones CHIP demostró que el desarrollo de la enfermedad con frecuencia involucra procesos dinámicos que empiezan años o décadas antes de la manifestación clínica^{141–143}. Estos procesos conllevan la combinación de mutaciones somáticas que se adquieren en distintos momentos, de manera que algunas alteraciones están relacionadas con estadios tempranos previos a la enfermedad, liderando la expansión clonal, mientras que otras mutaciones se relacionan con procesos más tardíos que favorecen la transformación leucémica (Figura 3)^{12,33,141–148}.

En este sentido, las alteraciones más frecuentes que se observaron en individuos sanos con CHIP se encontraban principalmente en 3 genes: *TET2*, *DNMT3A* y *ASXL1*^{141–143}. Estos genes se han asociado en numerosas ocasiones con estadios iniciales de los SMD.

Estudios de evolución clonal en pacientes con SMD han mostrado que mutaciones en genes reguladores de *splicing* (*SF3B1*, *SRSF2*) y componentes del complejo cohesinas (*STAG2*) son comunes en las etapas tempranas de los SMD, mientras que mutaciones en factores transcripcionales (*RUNX1*, *GATA2*, *CEBPA*) y genes implicados en la transducción de señales (*FLT3*, *NRAS*) se adquieren en estadios avanzados de la enfermedad y, por tanto, se asocian en mayor medida con la progresión del SMD a LMA^{12,13,33,137,138,140,148–151}.

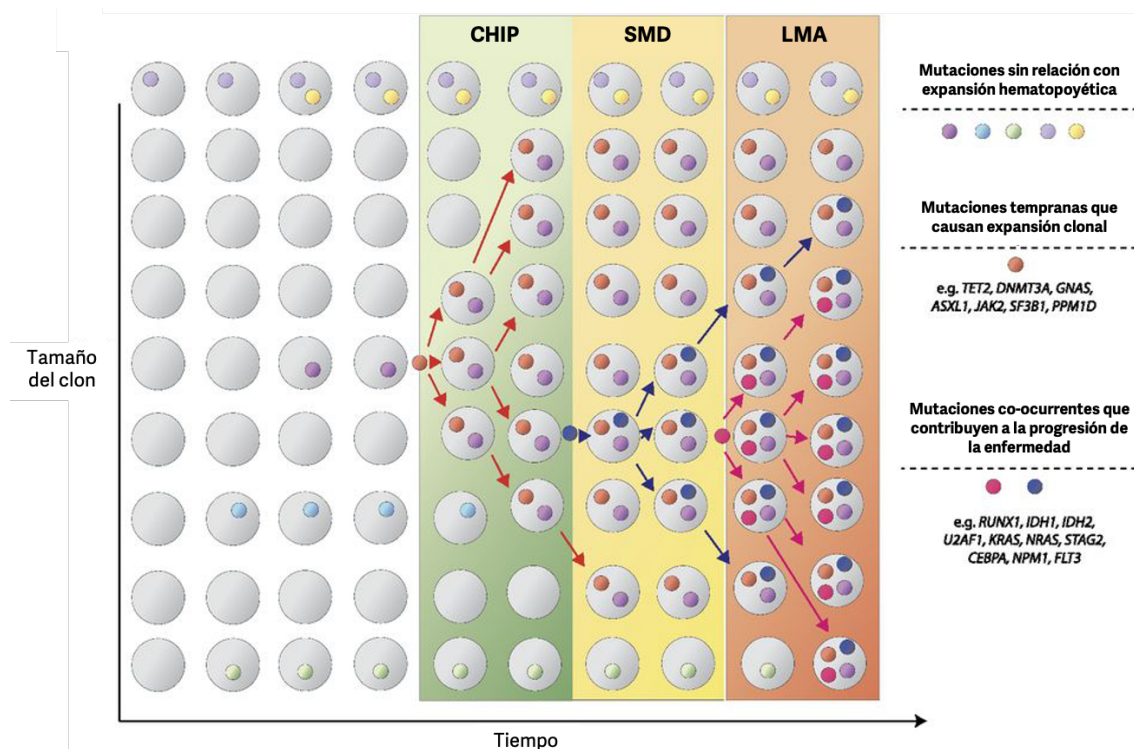


Figura 3: Adquisición secuencial de mutaciones somáticas a nivel celular desde un estadio sano hasta la progresión a LMA. Adaptada de Steensma y colaboradores, 2015¹⁴⁸

La presencia de mutaciones asociadas a mielodisplasia en LMA demuestra que esta enfermedad puede, en ocasiones, considerarse como un estado continuado del SMD en el que distintas mutaciones emergen y sufren procesos dinámicos de evolución clonal^{13,31,33,140,151–154}. Estas enfermedades representan, por tanto, un modelo de jerarquía clonal que comienza con la adquisición de mutaciones asociadas a CHIP, continúa con la evolución a SMD y tiene como desenlace la transformación leucémica en los pacientes.

La LMA secundaria es un proceso dinámico en el que la aparición de un clon fundador del SMD favorece la adquisición de alteraciones adicionales. Este proceso da lugar a subclones con diferentes combinaciones de mutaciones y distinta capacidad proliferativa. Mediante un proceso de selección clonal, algunos clones predominarán en la MO, lo que requiere de una adaptación dinámica de las mutaciones^{140,152}.

En este contexto de expansión clonal, la co-ocurrencia de mutaciones en distintos genes, así como el orden de adquisición de estas, son críticos para la patogénesis de la enfermedad^{33,140,152,153,155}. En este sentido se ha propuesto la existencia de dos subgrupos de genes asociados con la transformación leucémica del SMD. Por un lado, se definieron “mutaciones de tipo 1” aquellas que se encontraban enriquecidas en pacientes con LMA secundaria, en comparación con SMD de alto riesgo. Entre los genes mutados, se encontraban *FLT3*, *PTPN11*, *WT1*, *IDH1*, *IDH2*, *NPM1* y *NRAS*. Por otro lado, las “mutaciones de tipo 2” fueron aquellas que se encontraban en mayor proporción en pacientes con SMD de alto riesgo, comparado con los SMD de bajo riesgo, como mutaciones en *TP53*, *GATA2*, *KRAS*, *RUNX1*, *STAG2*, *ASXL1*, *ZRSR2* o *TET2*¹⁴⁰.

Asimismo, otros estudios con muestras pareadas de la transformación del SMD en LMA, han confirmado que existe un orden de adquisición de mutaciones somáticas en este proceso de evolución clonal. La presencia de alteraciones en genes de regulación epigenética o factores de *splicing*, con frecuencias de variación alélica (VAF) elevadas en SMD desde estados tempranos de la enfermedad, podría favorecer la hematopoyesis clonal^{140,151,153}.

Las mutaciones en genes de señalización, por el contrario, no son frecuentes en SMD de bajo riesgo, estando asociados al proceso de transformación leucémica. En concreto, mutaciones de pérdida de función en factores de transcripción, como *RUNX1*, *CEBPA* o *RARA*, alteran la diferenciación hematopoyética y llevan a la acumulación de células madre en la MO, no siendo suficientes por sí solas para iniciar el proceso de transformación leucémica.

Son las alteraciones en genes de transducción de señales (por ejemplo, *FLT3*, *RAS* o *KIT*) las que favorecen estadios malignos de la enfermedad y la consecuente progresión a LMA^{138–140}.

Además de la importancia de la organización jerárquica de las mutaciones, la cooperación entre genes también tiene un papel relevante en la transformación leucémica. Un estudio reciente donde se estudiaron muestras pareadas de SMD y su posterior transformación a LMA mostró que, aproximadamente, un 15-20% de pacientes presentaban concurrencia de mutaciones en genes del complejo cohesinas y de la vía de RAS. Específicamente, alteraciones de pérdida de función en *STAG2* precedían a mutaciones en *NRAS*¹³⁷. La alta incidencia de co-mutaciones en estos genes en LMA secundaria sugiere que la interacción entre ellos podría tener efectos funcionales que lideren la progresión de la enfermedad.

A pesar de los avances en el conocimiento de la transformación genética del SMD, aún se desconocen los mecanismos moleculares por los que se produce este proceso patológico. La traslación de los hallazgos genéticos a estudios biológicos podría ayudar a dilucidar el papel que juegan las mutaciones en la transformación leucémica del SMD.

2.3. Edición genética y terapias celulares

Los sistemas de edición genética que se han desarrollado en los últimos años han permitido profundizar en el conocimiento de la fisiopatología de numerosas enfermedades mediante la recreación de modelos *in vitro*. En concreto, la tecnología CRISPR-Cas9 (de sus siglas en inglés, *Clustered Regularly Interspaced Short Palindromic Repeats – associated protein 9*) ha revolucionado el mundo de la edición genética. Este sistema se basa en la rotura de doble cadena del ADN dirigida mediante nucleasas y la posterior activación de los sistemas de reparación del ADN intrínsecos en la célula^{156–158}.

La reparación del ADN constituye un proceso fundamental para la supervivencia celular. Existen dos mecanismos principales de reparación del ADN: por recombinación homóloga (RH) y no homóloga (NHEJ, de sus siglas en inglés, *non-homologous end-joining*) (Figura 4). Por un lado, la RH es un proceso complejo que requiere de una cadena de ADN guía o molde – homóloga a la hebra en la que se ha producido la rotura – que se utiliza de referencia para reparar el ADN. Por el contrario, la NHEJ es un mecanismo rápido, con alta capacidad para unir dos extremos de ADN sin necesidad de que exista una secuencia de referencia^{159–161}.

Debido a su mecanismo de acción, la RH permite introducir mutaciones específicas gracias a la sustitución de uno o varios nucleótidos por una secuencia determinada en el ADN guía, siendo útil en la generación de mutaciones de ganancias de función. Sin embargo, la NHEJ permite generar mutaciones de pérdida de función a través de roturas inespecíficas en una zona de interés, llevando así a la inactivación y consecuente pérdida de función de uno o varios genes¹⁵⁸.

En el estudio de los SMD, son numerosas las publicaciones que han utilizado esta metodología para generar modelos *in vitro* que permiten analizar la implicación funcional de las mutaciones genéticas observadas en estas neoplasias mieloides. Se han generado modelos de SMD mediante CRISPR-Cas9 tanto en líneas celulares como en células de pacientes reprogramadas¹⁶²⁻¹⁶⁶. Además, esta tecnología ha permitido la generación de modelos murinos de SMD y LMA, lo que ha sido crucial para el estudio *in vivo* del desarrollo de la enfermedad, la invasión leucémica asociada a las mutaciones y la sensibilidad a fármacos¹⁶⁷.

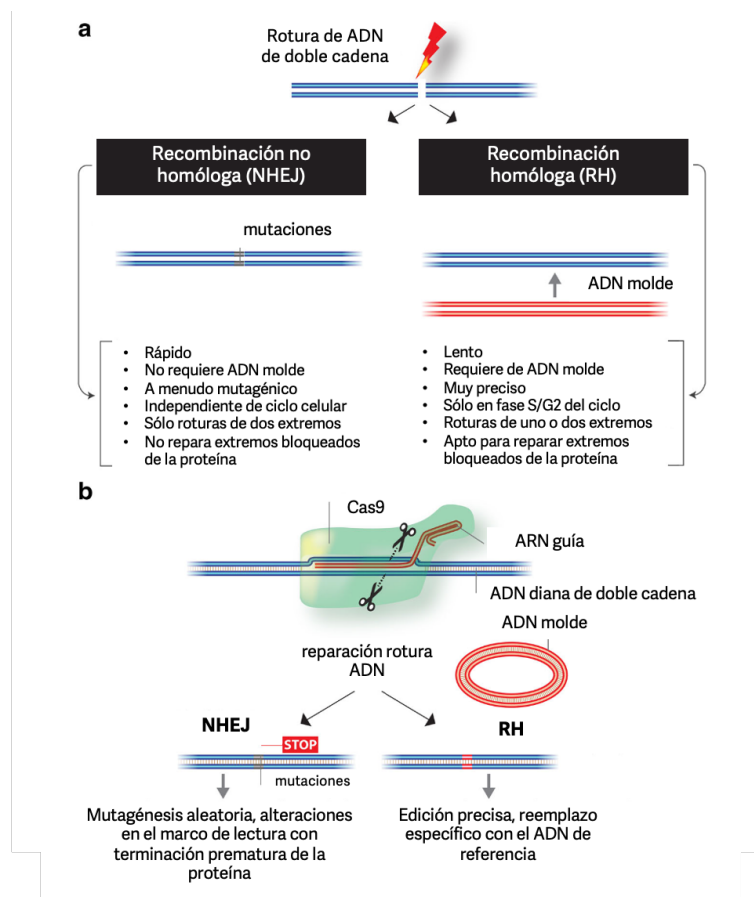


Figura 4: Mecanismos fisiológicos de reparación del ADN: RH y NHEJ. Figura modificada de Ranjha y colaboradores, 2018¹⁶⁰

En cuanto a la aplicabilidad clínica de la edición genética, la utilización de la tecnología CRISPR-Cas9 ha permitido el desarrollo de terapias génicas, como la generación de las células CAR-T (de sus siglas en inglés, *Chimeric Antigen Receptor-T cells*), para el tratamiento de enfermedades hematológicas. Si bien han sido una revolución terapéutica en las leucemias linfoblásticas agudas, los linfomas y en el mieloma múltiple, aún no han llegado a la clínica en SMD ni LMA¹⁶⁸⁻¹⁷⁰. No son las limitaciones de la técnica lo que ha impedido su utilización en el tratamiento de las neoplasias mieloides, sino la falta de biomarcadores específicos que se encuentren sobreexpresados en las células tumorales mieloides¹⁷⁰⁻¹⁷². Actualmente, solo hay 2 ensayos clínicos en fase 1 que utilizan CAR-T frente a CD33 en SMD de alto riesgo, en los que el número de pacientes incluidos es limitado y no se conocen aún resultados de seguridad ni eficacia¹⁷⁰. Es por ello que la aplicación de la edición genética al tratamiento de las neoplasias mieloides está en fases muy tempranas.

HIPÓTESIS

La heterogeneidad genética de los SMD, así como la diversidad de manifestaciones clínicas y biológicas que presentan estos pacientes, dificulta el estudio de esta patología. Clasificar a los pacientes en base a la presencia de características comunes podría ayudar a identificar qué factores genéticos condicionan el pronóstico de los pacientes.

Han sido muchos los esfuerzos que ha hecho la comunidad científica para establecer escalas de riesgo en los SMD. El IPSS-M es la primera propuesta que incorpora información molecular para determinar el riesgo de la enfermedad y, sin embargo, aún es difícil establecer con precisión el pronóstico de determinados pacientes. En este sentido, el IPSS-M no ha sido capaz de estratificar a ciertos subgrupos de pacientes de riesgo intermedio, como los que presentan +8 aislada. Probablemente, los genes establecidos como factores pronósticos en el IPSS-M no necesariamente son útiles para identificar el riesgo de cualquier SMD, sino que es preciso estudiar el perfil mutacional de los pacientes en el contexto específico de su enfermedad. La presencia de alteraciones citogenéticas concomitantes con mutaciones somáticas podría modificar el valor pronóstico de estas anomalías cuando se encuentran de forma aislada. La primera hipótesis de este trabajo de tesis doctoral es que el pronóstico heterogéneo asociado a los pacientes con SMD y +8 aislada, podría redefinirse en subgrupos de alto o bajo riesgo en función de la presencia de mutaciones somáticas en estos enfermos.

Las mutaciones somáticas también han demostrado tener una implicación importante en la respuesta al tratamiento. Monitorizar el perfil mutacional en los ensayos clínicos podría ser una estrategia útil para identificar pacientes resistentes a las terapias de manera anticipada, así como para evaluar la seguridad de los tratamientos a nivel molecular y conocer la repercusión clínica que tiene la dinámica de las mutaciones. Este tipo de estudios podrían ser de gran ayuda para conocer qué pacientes podrían beneficiarse de estrategias terapéuticas tempranas en base a las mutaciones que presenten al diagnóstico. La segunda hipótesis de este trabajo doctoral es, por tanto, que el estudio de la evolución clonal permitiría mejorar la predicción de respuesta a tratamiento y la seguridad en el uso precoz de lenalidomida en pacientes con SMD, del(5q), anemia y sin dependencia transfusional.

Las terapias actualmente disponibles para los SMD siguen siendo muy limitadas y no consiguen evitar, en muchas ocasiones, que se produzca una transformación leucémica. La aparición de clones aberrantes debido a la adquisición de mutaciones somáticas desempeña un papel fundamental en la transformación a LMA. Sin embargo, no está clara cuál es la implicación biológica de las mutaciones en la progresión de la enfermedad, por lo que llevar a cabo estudios funcionales mediante la generación de modelos de evolución clonal permitiría

profundizar en el conocimiento de la patología. Asimismo, evaluar la respuesta a tratamientos en estos modelos permitiría incorporar agentes terapéuticos con dianas específicas que reviertan el efecto de estas mutaciones en la evolución de los SMD. La tercera hipótesis de esta tesis es que la generación de un modelo de transformación leucémica basada en la adquisición secuencial de mutaciones en *STAG2* y *NRAS* en líneas celulares, podría ayudar a profundizar en el conocimiento de la progresión de la enfermedad.

Debido a todo lo expuesto anteriormente, esta tesis doctoral tiene como hipótesis principal que existe una relación directa entre las alteraciones genéticas y su implicación clínica y ésta varía en función del contexto específico de la enfermedad. Por lo tanto, el estudio genético de los SMD mediante la utilización de técnicas de última generación, como la secuenciación masiva y la edición genética, sería fundamental para desarrollar nuevas estrategias dirigidas a subgrupos específicos que permitan el manejo clínico óptimo para cada paciente.

OBJETIVOS

Objetivo general:

Evaluar el impacto clínico y biológico de las mutaciones somáticas en grupos citogenéticos específicos de pacientes con SMD (5q- y +8) y en modelos celulares, combinando tecnologías de secuenciación masiva y de edición genómica.

Objetivos específicos:

1. Analizar el perfil mutacional de los pacientes con SMD y +8 aislada para determinar si las mutaciones somáticas permiten establecer una clasificación pronóstica más precisa en este grupo específico de pacientes.
2. Estudiar la evolución clonal de las mutaciones somáticas que presentan los pacientes en Ensayo Clínico SintraRev, que analiza en SMD de bajo riesgo y del(5q), con anemia sin dependencia transfusional, el tratamiento precoz con lenalidomida a dosis bajas frente a un grupo control con placebo.
3. Evaluar, mediante un modelo de transformación *in vitro*, los mecanismos moleculares por los que las mutaciones en *STAG2* y *NRAS* cooperan en el proceso de progresión de un SMD a una LMA, así como determinar si la presencia de estas alteraciones confiere ventajas de supervivencia celular y resistencia a fármacos.

RESULTADOS

Capítulo 1

El papel de la NGS en la clasificación pronóstica de los SMD de riesgo intermedio

*Estudio del perfil mutacional en pacientes con SMD y trisomía 8 aislada
e implicaciones pronósticas.*

EL PERFIL MUTACIONAL PERMITE IDENTIFICAR UN SUBGRUPO DE ALTO RIESGO EN LOS SÍNDROMES MIELODISPLÁSICOS CON TRISOMÍA 8 AISLADA

Introducción

La trisomía 8 (+8) es una de las anomalías de cariotipo más frecuentes observadas en los SMD presente en, aproximadamente, un 10% de los casos con alteraciones citogenéticas. Cuando se encuentra de forma aislada se asocia con un riesgo citogenético intermedio y una gran heterogeneidad clínica, ya que algunos pacientes progresan a LMA en varias semanas, mientras que otros varios años después del diagnóstico del SMD. Las mutaciones somáticas son, en muchos casos, factores pronósticos en los SMD, por lo que estudiar el perfil mutacional en pacientes de riesgo intermedio podría identificar distintos subgrupos de pacientes con riesgo variable de la enfermedad. Por ello, el objetivo de este trabajo fue analizar el perfil mutacional de pacientes con +8 aislada y determinar si la presencia de mutaciones específicas puede ayudar a identificar pacientes con un pronóstico adverso y alto riesgo de progresión a LMA.

Pacientes incluidos y metodología del estudio

Se incluyeron 2.602 pacientes con SMD al momento del diagnóstico y se analizó la incidencia de +8. De ellos, 94 pacientes (3,6%) presentaban +8 como única alteración citogenética. Los pacientes con +8 aislada se compararon con controles sin +8 pareados según el IPSS-R en una proporción 3:1 (282 pacientes). Se analizaron datos de NGS mediante un panel de captura personalizado de 92 genes relacionados con patología mieloide en un total de 187 pacientes: 79 con SMD y +8 aislada y 108 controles con SMD y sin +8.

Resultados

Un total de 289 mutaciones somáticas fueron identificadas en 43 genes distintos en los pacientes con SMD y +8 aislada. *TET2* fue el gen más frecuentemente mutado (38% de los pacientes), seguido de *STAG2* (34,2%), *SRSF2* (29,1%) y *RUNX1* (26,6%). La incidencia de mutaciones en *STAG2*, *SRSF2* y *RUNX1*, entre otros genes, fue significativamente mayor en los SMD con +8 aislada en comparación con los pacientes control ($p < 0,01$). Además, la mediana de mutaciones por paciente fue superior en los pacientes con +8 aislada, con respecto al grupo control (4 vs 3; $p < 0,001$).

La presencia de mutaciones en *STAG2*, *SRSF2* y/o *RUNX1* en los SMD con +8 aislada se asoció con un menor tiempo hasta la progresión de la enfermedad y una menor supervivencia global (SG), en comparación con los pacientes que no mostraban estas mutaciones. Además, el tiempo hasta la transformación a LMA y la SG de los pacientes con

alteraciones en *STAG2*, *SRSF2* y/o *RUNX1* y +8 aislada se asemejó a lo observado en los pacientes control con SMD sin +8 de alto riesgo según el IPSS-R y presentaron características clínicas similares.

El análisis multivariante reveló que la presencia de mutaciones en *STAG2*, *SRSF2* y/o *RUNX1* es un factor pronóstico independiente en los pacientes con SMD y +8 aislada tanto para la progresión a LMA (HR: 15,4; $p<0,01$), como para la SG de estos pacientes (HR: 3,1; $p<0,05$). Asimismo, la presencia de estas alteraciones permitió reclasificar a un 39,5% y 15,4% de los pacientes con +8 aislada estratificados como bajo-intermedio riesgo según el IPSS-R e IPSS-M, respectivamente. Todos estos resultados fueron replicados en una cohorte externa de 2.494 pacientes con SMD.

Conclusiones

La identificación de mutaciones somáticas ha demostrado ser útil para distinguir pacientes con SMD y +8 aislada con distinto riesgo de la enfermedad. De hecho, la presencia de mutaciones en *STAG2*, *SRSF2* y/o *RUNX1* permite distinguir a un subgrupo de pacientes de alto riesgo en términos de progresión a LMA y SG. Este estudio valida el valor pronóstico del IPSS-M en el subgrupo de pacientes con SMD y +8 aislada, añadiendo valor pronóstico a *STAG2*. Estos resultados podrían generar nuevas oportunidades en el tratamiento de los SMD de alto riesgo en el contexto específico de +8 aislada.

Article

Mutational Profile Enables the Identification of a High-Risk Subgroup in Myelodysplastic Syndromes with Isolated Trisomy 8

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Simple Summary: Trisomy 8 (+8) is one of the most frequent cytogenetic alterations found in myelodysplastic syndromes (MDS). MDS patients harboring isolated +8 show clinical heterogeneity, and life expectancy varies between several months and several years after diagnosis. We aimed to investigate whether the mutational profile of isolated +8 MDS patients could help to clarify the heterogeneous prognosis of these patients. In fact, we found that mutations in *STAG2*, *SRSF2* and *RUNX1* are independent prognostic factors, enough to define the course of the disease in terms of overall survival and leukemic transformation in isolated +8 MDS. Therefore, these findings might help to identify patients at a high risk of evolving disease and open new horizons by changes in the management of a high subset of patients within MDS with isolated +8.

Abstract: Trisomy 8 (+8) is the most frequent trisomy in myelodysplastic syndromes (MDS) and is associated with clinical heterogeneity and intermediate cytogenetic risk when found in isolation. The presence of gene mutations in this group of patients and the prognostic significance has not been extensively analyzed. Targeted deep sequencing was performed in a cohort of 79 MDS patients showing isolated +8. The most frequently mutated genes were: *TET2* (38%), *STAG2* (34.2%), *SRSF2* (29.1%) and *RUNX1* (26.6%). The mutational profile identified a high-risk subgroup with mutations in *STAG2*, *SRSF2* and/or *RUNX1*, resulting in shorter time to acute myeloid leukemia progression (14 months while not reached in patients without these mutations, $p < 0.0001$) and shorter overall survival (23.7 vs. 46.3 months, $p = 0.001$). Multivariate analyses revealed the presence of mutations in these genes as an independent prognostic factor in MDS showing +8 isolated (HR: 3.1; $p < 0.01$). Moreover, 39.5% and 15.4% of patients classified as low/intermediate risk by the IPSS-R and IPSS-M, respectively, were re-stratified as a high-risk subgroup based on the mutational status of *STAG2*, *SRSF2* and *RUNX1*. Results were validated in an external cohort ($n = 2494$). In summary, this study validates the prognosis significance of somatic mutations shown in IPSS-M and adds *STAG2* as an important mutated gene to consider in this specific subgroup of patients. The mutational profile in isolated +8 MDS patients could, therefore, offer new insights for the correct management of patients with a higher risk of leukemic transformation.

Keywords: myelodysplastic syndromes; NGS; isolated trisomy 8; prognostic stratification; AML progression

1. Introduction

Myelodysplastic syndromes (MDSs) are a group of clonal hematological disorders characterized by clinical heterogeneity and genetic diversity [1,2]. MDSs display peripheral cytopenias due to ineffective hematopoiesis, morphologic dysplasia in one or more cell lineages and an increased risk of leukemia transformation. In fact, more than 30% of MDS patients progress to acute myeloid leukemia (AML). Approximately, 50% of patients show cytogenetic alterations and more than 90% have at least one genetic lesion at diagnosis [2–5].

In the last decade, the identification of genetic alterations by next-generation sequencing (NGS) has changed the understanding of MDS pathogenesis [1,6]. In fact, in 2017 the presence of somatic mutations was incorporated for the first time as a new parameter for a better diagnosis of morphological subtypes [3]. Moreover, the recently published update of the diagnosis classification by both the World Health Organization (WHO) and the International Consensus Classification (ICC), defines the presence of genetic abnormalities as an important feature to consider in diagnosis of myeloid malignancies [7,8].

Until now, the Revised International Prognostic Scoring System (IPSS-R) was the most commonly used tool for prognostic assessment of primary untreated MDSs in adults. Cytogenetic alterations are one of the most important features that have an impact on risk-stratification [9]. In fact, a cytogenetic IPSS scoring system was implemented to determine the risk of each karyotypic alteration in order to incorporate this molecular information into the IPSS-R [10]. In addition, a role for somatic mutations in the outcome of MDS patients has been described and genetic variants have been incorporated into the recently published International Molecular Prognostic Scoring System (IPSS-M). The classification of MDS patients by the IPSS-M shows improved discrimination between risk subgroups by including information from alterations in 31 recurrently mutated genes in MDSs [11].

One of the most common cytogenetic alterations found in MDSs is trisomy 8 (+8), which is present in 10% of patients with chromosomal abnormalities [12,13]. Patients with isolated +8 have an intermediate cytogenetic risk and are also characterized by clinical heterogeneity: life expectancy is different among patients, varying between several months and several years after diagnosis. Two subgroups of MDS patients with isolated +8 have been described, showing differences in overall survival (OS) based on myeloproliferative features [14]. Some of these patients showed a higher number of mutations. In that way, some studies have described the presence of mutated genes in MDS displaying +8, such as *ASXL1*, *EZH2*, *STAG2*, *SRSF2* or *U2AF1* [14–17]. Furthermore, only one study described a group of genes worsening the prognosis of MDS patients, but those cases were associated with myeloproliferative features [14]. Thus, the role of somatic mutations in isolated +8 MDS is still undefined, as there is no evidence of its impact in the outcome of isolated +8 in large cohorts of MDS patients without features such as myeloproliferation.

Given the clinical heterogeneity of MDS patients with isolated +8, accurate survival prediction and risk stratification are critical for the proper management of and treatment decision-making for these patients. Therefore, the aim of the study herein was to evaluate the mutational landscape of isolated +8 and to determine whether specific somatic mutations might help to identify patients with adverse outcomes in this heterogeneous group of MDS.

2. Patients and Methods

2.1. Patients Cohort and Validation Cohort

A total of 2602 patients with de novo MDS, diagnosed between 1997 and 2022, were analyzed for incidence of trisomy 8. There were 94 patients (3.6%) who showed isolated +8, while 30 cases (1.1%) showed +8 in combination with additional cytogenetic alterations. Most of these combinations (80%) were complex karyotypes (CKs) and, consequently, were

not included in the study. Clinical and biological data are shown in Table 1. Patients with isolated +8 were compared to IPSS-R-based matched MDS controls without +8 in a 3:1 ratio (282 patients). NGS data were available in a total of 187 patients: 79 out of 94 MDS patients with isolated +8 and in 108 out of the 282 control patients. The procedures followed were in accordance with the ethical standards of the Institutional Committee on Human Experimentation and with the Helsinki Declaration of 1975. The research was approved by the Local Ethics Committee from the University Hospital of Salamanca, Spain.

Table 1. Clinical and biological features of isolated +8 MDS cohort.

	MDS with Isolated +8 (n = 94)
Demographics	
Age at diagnosis, years (range)	75 (33–94)
Gender, n (%)	
Female	30 (31.9%)
Male	64 (68.1%)
MDS diagnosis and risk stratification	
2017 WHO classification, n (%)	
MDS-SLD	3 (3.3%)
MDS-MLD	29 (31.5%)
MDS-RS	11 (12%)
MDS-del(5q)	0 (0%)
MDS-EB-1	27 (29.3%)
MDS-EB-2	19 (20.6%)
Hypoplastic MDS	3 (3.3%)
Cytogenetic risk, n (%)	
Intermediate	94 (100%)
IPSS-R category, n (%)	
Low	24 (32.9%)
Intermediate 1–2	21 (28.8%)
High	21 (28.8%)
Very high	7 (9.6%)
Blood and bone marrow counts at diagnosis	
Hemoglobin, g/dL (range)	9.9 (5.4–14.6)
Platelets	95 (2–520)
Neutrophils	1.7 (0.2–11.8)
Bone marrow blasts, %	4 (0–16)
Circulating blasts, %	0 (0–13)
Ring sideroblasts, %	0 (0–72)
NGS data, n (%)	79 (84%)

MDS-SLD: MDS with single lineage dysplasia; MDS-MLD: MDS with multilineage dysplasia; MDS-RS: MDS with ring sideroblasts; MDS-del(5q): MDS with deletion of 5q; MDS-EB-1: MDS with excess of blasts type 1; MDS-EB-2: MDS with excess of blasts type 2.

In addition, an external cohort comprising 2494 MDS patients from the International Working Group for the Prognosis of MDS was also analyzed to validate the results [11]. A total of 94 patients showing isolated +8 were compared to 2311 MDS cases as controls without +8, while 89 cases were excluded due to the presence of additional cytogenetic alterations together with +8 ($n = 81$) or because the status of chromosome 8 could not be ascertained ($n = 8$).

2.2. Sample Procedure and DNA Extraction

Bone marrow (BM) and peripheral blood (PB) samples were obtained from patients at diagnosis. Mononuclear cells were isolated by gradient density using Ficoll.

Genomic DNA was obtained using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's standard protocol. The concentration of the extracted DNA was assessed by Qubit 2.0 Fluorometer system (Life Technologies, Carlsbad, CA, USA) and the integrity was analyzed using a TapeStation 4200 (Agilent Technologies, Santa Clara, CA,

USA) and a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) by measuring absorbance ratio at 260/230 and 260/280 nm.

2.3. Cytogenetics and FISH

Conventional G-banding analysis (CBA) data were available for all patients, and karyotypes were described in accordance with the International System for Human Cytogenetic Nomenclature [18].

Karyotype information was confirmed in all patients by interphase fluorescence in situ hybridization (FISH).

2.4. Next-Generation Sequencing

Samples from 187 patients underwent targeted deep sequencing using an update of a previously validated in-house custom capture-enrichment panel [19] (SureSelect XT HS Target Enrichment System, Agilent Technologies, Santa Clara, CA, USA) of 92 genes related to the pathogenesis of myeloid malignancies (Table S1). Sequencing libraries were prepared according to manufacturer's instructions and sequenced on Illumina MiSeq or NexSeq 500 sequencers.

The mean coverage of TDS was $665 \times$ (range 251–1198) and 99.5% of target regions were captured at a level higher than $100 \times$.

For true oncogenic somatic variant calling, a severe criterion for variant filtering was applied. Variants were considered candidate somatic mutations based on the following criteria: (i) variants with ≥ 10 mutated reads; (ii) described in COSMIC and/or ClinVar as being cancer-associated and known hotspot mutations; and (iii) classified as deleterious and/or probably damaging by PolyPhen-2 and SIFT web-based platforms, as previously described [19].

2.5. Statistical Analysis

Dichotomous variables were compared between different groups using the χ^2 test and continuous variables by the Student's *t*-test, Mann–Whitney and Kruskal–Wallis tests. Results were considered significant at $p < 0.05$.

Multivariate Cox regression analysis was applied to compare clinical and molecular characteristics of patients. Survival and disease progression analyses were performed with Kaplan–Meier method and groups were compared using two-sided log-rank test. OS was defined as the period from the date of initial diagnosis to the date of death regardless of the cause. Data were censored at the last follow-up in (a) patients with loss of follow-up and (b) patients undergoing hematopoietic stem cell transplantation (HSCT), with date of HSCT as the last point of time considered. Time to AML progression were considered as the time from the MDS to the secondary AML diagnoses and censored in patients without transformation at last follow-up or date of death.

Statistical analyses were performed using SPSS Statistics version 25 (IBM Corporation, Armonk, NY, USA), Graphpad prism version 5.03 for Windows, (Graphpad software, San Diego, CA, USA) and R version 4.0.2 with biostatistical packages (<https://cran.r-project.org/> accessed on 25 May 2022).

3. Results

3.1. MDS with Isolated Trisomy 8 Have a Differential Mutational Pattern

A total of 289 oncogenic mutations were identified in 43 genes in isolated +8 MDS patients. *TET2* was the most frequently mutated gene in isolated +8 MDS patients (38%). Interestingly, *STAG2* (34.2%), *SRSF2* (29.1%) and *RUNX1* (26.6%) were also frequently mutated in MDS patients displaying isolated +8. Nine genes were mutated in more than 10% of patients, with an additional ten genes carrying mutations in 5 to 10% of patients (Figure 1a; Table S2). The mean number of mutations per patient was 3.7 (0–10). Seventy-one (89.8%) patients had at least one mutated gene: nine patients (11.4%) had only one

mutated gene while nine patients (11.4%) showed two mutated genes, and the remaining fifty-three cases (67.1%) had three or more mutated genes.

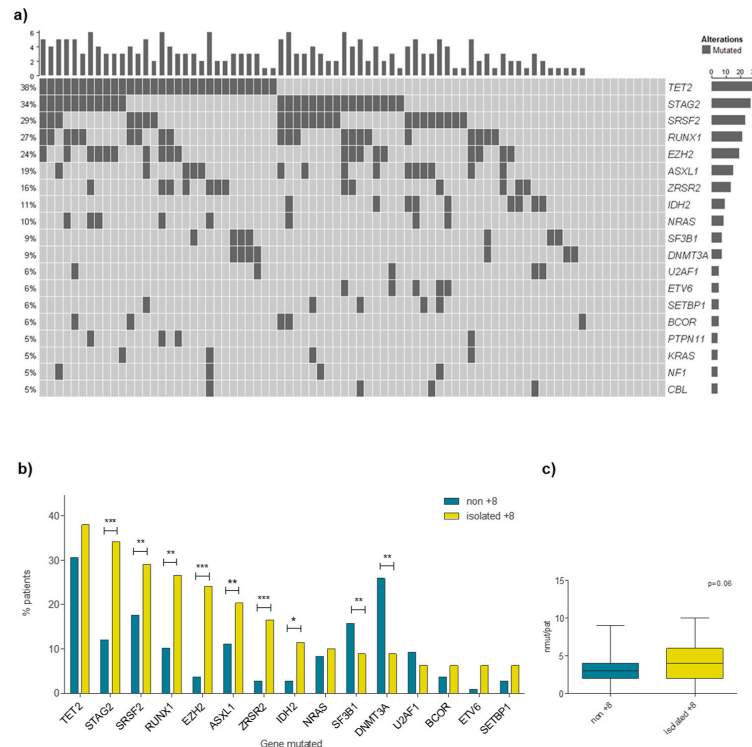


Figure 1. Mutational pattern of patients with MDS and isolated +8. (a) Mutational profile of patients with MDS and isolated +8 MDS of most frequently mutated genes (>5%); (b) comparison of top 15 most frequently mutated genes in isolated +8 and the control cohort (non-trisomy 8 MDS); and (c) mean number of mutations per patient in isolated +8 compared to the non-trisomy 8 MDS group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To investigate whether the pattern of recurrently mutated genes was different in this set of patients, mutational profiles were compared between isolated +8 MDS patients and the 282 IPSS-R-matched MDS patients without +8, defined as the control group. A differential mutational pattern on isolated +8 patients was found compared to the controls (Figure 1b). Thus, the incidence of mutations in *STAG2*, *SRSF2*, *RUNX1*, *EZH2*, *ASXL1*, *ZRSR2* and *IDH2* was higher in the isolated +8 MDS group than in the control cohort ($p < 0.05$). By contrast, mutations in *SF3B1* and *DNMT3A* were more frequent in non-trisomy 8 MDS group ($p < 0.001$). In addition, the median number of mutations per patient was higher in isolated +8 patients compared to those MDS patients without +8 (4 vs. 3, $p < 0.001$; Figure 1c).

3.2. Mutational Profile Allows the Distinction of a Worse Prognosis High-Risk-Like Subgroup in MDS with Isolated Trisomy 8

The presence of mutations in *STAG2*, *SRSF2* and *RUNX1* in isolated +8 MDS patients was significantly associated with shorter time to AML ($p < 0.05$; Figure 2a, Table S3). Mutations in either *STAG2*, *SRSF2* and/or *RUNX1* were, therefore, considered as an entire group of alterations worsening the prognosis among patients with MDS and isolated +8 (Table S4). In fact, isolated +8 MDS with mutations in at least one of these three genes, showed a median time to AML progression and OS similar to high/very high-risk patients from the control group without +8 stratified by the IPSS-R (AML: 14 vs. 11.4 months; OS: 23.7 vs. 10.1 months, respectively; $p > 0.05$; Figure 2b,c). By contrast, isolated +8 MDS

patients without mutations in *STAG2*, *SRSF2* and *RUNX1* showed a longer time to AML progression (comparable to very low/low-risk MDS patients without +8 classified by IPSS-R) and remained as MDS patients with a low–intermediate IPSS-R risk in terms of OS (Figure 2b,c).

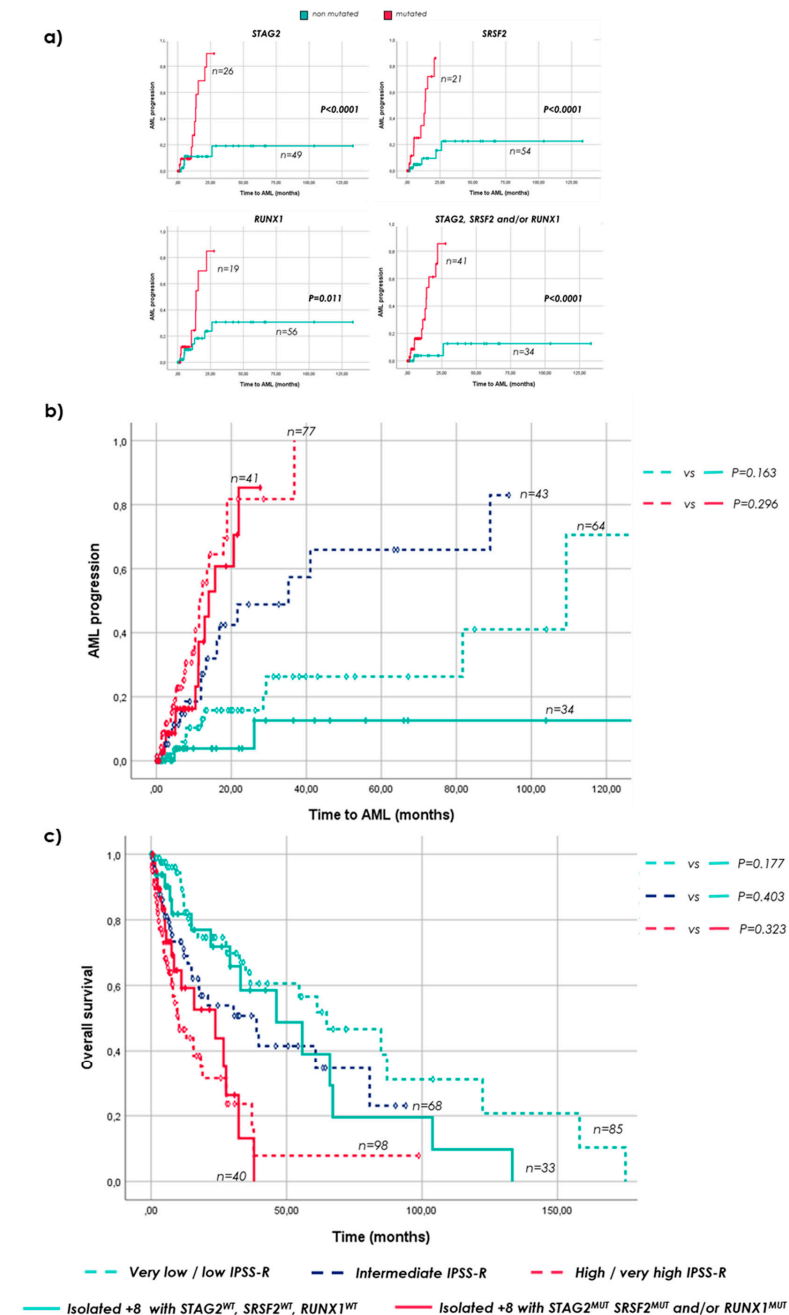


Figure 2. Time to AML progression and OS of isolated +8 MDS patients regarding mutational status of *STAG2*, *SRSF2* and/or *RUNX1*. (a) Time to AML progression in patients with MDS and isolated +8 according to mutational status of *STAG2*, *SRSF2* and *RUNX1*; (b) time to AML progression in newly described isolated +8 categories and the MDS control group stratified by IPSS-R; and (c) OS in newly defined isolated +8 risk categories and the MDS control cohort stratified by IPSS-R.

Moreover, time to AML progression in isolated +8 patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1* was similar to CK (14 vs. 11.7 months; $p > 0.05$; Figure S1a) and tended to have a comparable OS to those patients (Figure S1b). In addition, time to AML progression in isolated +8 with no mutations in these genes was even longer than in MDS control patients with good cytogenetic risk (median not reached vs. 29.3 months; $p < 0.05$; Figure S1a) and a similar OS was observed between them (46.3 vs. 34.9 months, respectively; $p > 0.05$; Figure S1b).

Clinical features of patients with isolated +8 and the presence of mutations in *STAG2*, *SRSF2* or *RUNX1* differed from MDS patients without +8 classified as high/very high risk by the IPSS-R in levels of hemoglobin and percentage of BM blasts (Table S5). Interestingly, despite having higher hemoglobin levels and lower counts of blast in BM, no differences were found in patient's outcome in terms of both AML progression and OS (Figure 2b,c). Otherwise, patients with isolated +8 and without mutations in neither *STAG2*, *SRSF2* nor *RUNX1*, showed a similar age, hemoglobin level, absolute neutrophils count and outcome to MDS patients without +8, classified as low risk by the IPSS-R, but a higher percentage of BM blasts and lower levels of platelets and ring sideroblasts (Figure 2b,c, Table S6).

3.3. Mutational Status of *STAG2*, *SRSF2* and/or *RUNX1* Is an Independent Prognostic Factor Associated with Shorter Time to Progression to AML and Overall Survival in MDS with Isolated Trisomy 8

In a multivariate Cox regression analysis, clinical parameters included in the IPSS-R were separately evaluated. In this regard, age (≥ 75 years), percentage of BM blasts ($\geq 5\%$), number of cytopenias (> 2) and the mutational status of *STAG2*, *SRSF2* or *RUNX1* were considered. Alterations in these genes were associated with shorter time to AML ($p < 0.01$; Table 2). Interestingly, the number of mutations per patient was not associated with time to AML ($p > 0.05$; Figure S2a), indicating that mutations in either *STAG2*, *SRSF2* and/or *RUNX1* are enough to predict early disease progression in isolated +8 MDS patients.

Table 2. Multivariate analysis of time to AML progression and OS in isolated +8 MDS patients including *STAG2*, *SRSF2* and *RUNX1* mutations.

AML Progression		
Variable	Hazard Ratio (Range)	p Value
Presence of mutations (<i>STAG2</i> , <i>SRSF2</i> and/or <i>RUNX1</i>)	15.46 (2.7–88.7)	0.002 **
Age (≥ 75 years)	0.28 (0.07–1.1)	0.075
BM blasts ($\geq 5\%$)	0.75 (0.2–3)	0.685
Number of cytopenias (> 2)	1.72 (0.4–7.3)	0.462
OS		
Variable	Hazard ratio (range)	p value
Presence of mutations (<i>STAG2</i> , <i>SRSF2</i> and/or <i>RUNX1</i>)	3.1 (1.3–7.7)	0.012 *
Age (≥ 75 years)	1.6 (0.7–3.6)	0.243
BM blasts ($\geq 5\%$)	1.3 (0.5–3.6)	0.586
Number of cytopenias (> 2)	1.2 (0.4–3.1)	0.743

* $p < 0.05$; ** $p < 0.01$.

OS was also evaluated in a similar multivariate analysis considering age (≥ 75 years), percentage of BM blasts ($\geq 5\%$), number of cytopenias (> 2) and the mutational status of *STAG2*, *SRSF2* and *RUNX1*. Only mutations in these genes remained as independent predictors of worse outcomes ($p = 0.01$; Table 2). By contrast, the number of mutations per patient (> 4) was not associated with shorter OS (Figure S2b). Furthermore, multivariate analyses also revealed that when considering a percentage of BM blasts higher than 10%, the mutational status of *STAG2*, *SRSF2* and *RUNX1* was still an independent prognostic factor of shorter OS, together with $> 10\%$ blast counts and age (Figure S2c,d).

3.4. The Newly Defined Prognostic Mutations Could Refine Prognosis of Patients with Isolated Trisomy 8 within the IPSS-R and the IPSS-M Systems

Patients with MDS and isolated +8 were classified within both IPSS-R and IPSS-M. A comparison between our defined mutational prognostic signature and both the IPSS-R and IPSS-M was assessed to evaluate whether the mutational status of *STAG2*, *SRSF2* and *RUNX1* could add value to these prognostic scoring systems in isolated +8 MDS. Remarkably, 39.5% of patients categorized as low /intermediate risk by the IPSS-R showed a high-risk mutational profile (this is, mutations in either *STAG2*, *SRSF2* or *RUNX1*) (Figure 3).

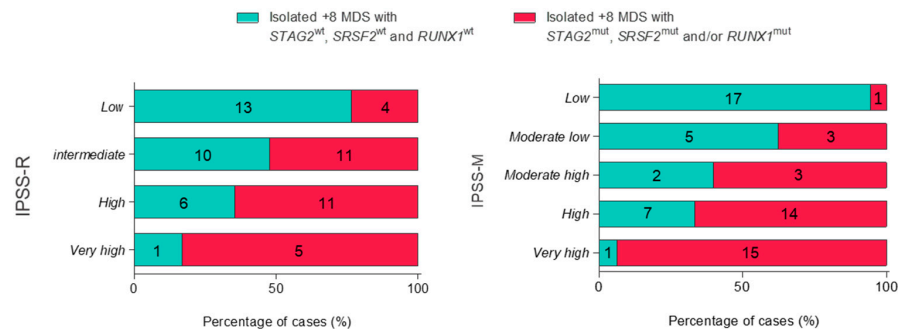


Figure 3. Stacked bar plots of IPSS-R and IPSS-M reclassification of isolated +8 MDS patients regarding *STAG2*, *SRSF2* and/or *RUNX1* mutational status.

Otherwise, 15.4% of low/moderate-low-risk patients as categorized by the IPSS-M were classified as high risk by the mutational profile (Figure 3).

As a result, most of the patients showing mutations in *STAG2*, *SRSF2* and/or *RUNX1* were categorized as moderate-high, high or very high risk by the IPSS-M (88.9%) while only 51.6% were categorized as high or very high by the IPSS-R. Consequently, the risk of isolated +8 patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1* was refined in a total of 11.1% of patients by the IPSS-M and in 48.4% of cases by the IPSS-R (Figure S3).

3.5. *STAG2*, *SRSF2* and *RUNX1* Differentiate a Subgroup of Isolated Trisomy 8 MDS with Worse Outcome in an Independent Validation Cohort

Finally, to further characterize the mutational landscape associated with isolated +8 in MDS, additional analyses were carried out in an external validation cohort. Thus, a previously published MDS population comprising 2494 patients from the International Working Group for the Prognosis of MDS was evaluated [11].

A total of 94 MDS patients presented isolated +8 (3.8%) and clinical characteristics were similar to those of the discovery cohort (Table S7). In this set of patients, the most frequently mutated genes were *ASXL1* (44.7%), *TET2* (39.4%) and *STAG2* (36.2%). The mutational incidence observed in most of the genes were similar to those of our cohort of MDS with isolated +8 patients (Figure S4). Twenty-three (24.5%) and twenty-one (22.3%) patients showed mutations in *SRSF2* and *RUNX1*, respectively. In addition, the median number of mutations per patient was higher in isolated +8 patients compared to MDS without +8 (four vs. three, respectively; $p < 0.0001$), as in our discovery cohort.

As in our own cohort, the presence of mutations in *STAG2*, *SRSF2* and/or *RUNX1* was able to distinguish a worse prognosis subgroup of patients. In fact, patients with mutations in these genes showed a similar time to AML and OS to high/very high-risk IPSS-R MDS patients (2.2 years of AML transformation in the high-risk patients without +8 and 1.6 and 1.2 years of OS, respectively; $p > 0.05$; Figure S5a,b). Multivariate analysis also revealed an independent prognostic value for these mutations in isolated +8 MDS ($p < 0.05$; Figures S5c and S6).

4. Discussion

MDS patients are characterized by clinical and genetic heterogeneity that generally translate into varied outcomes. In fact, some cases with isolated +8 progress quickly to AML while others remain stable for years. Some studies have analyzed the clinical variability in patients with MDS and isolated +8, precluding any clear-cut conclusions [20,21]. However, most of this research was carried out before the implementation of NGS into clinics and evidence a lack in understanding of the pathogenesis of isolated +8 patients. In view of the limited evidence on the specific impact of mutational landscape in the isolated +8 MDS setting, the present study was undertaken to determine whether the mutational profile might help to stratify patients with isolated +8. The deeply targeted gene sequencing reported in this work provides an unprecedented overview of the genomic landscape of MDS in a context of isolated +8 and provides evidence on how genetic lesions play a role in the differential outcome of MDS patients with isolated +8.

The comparison of mutational landscape between isolated +8 and non-trisomy 8 MDS showed that patients with isolated +8 displayed a differential mutational profile characterized by a slight increase in the mean number of mutations per patient (Figure 1c). In addition, mutations in *STAG2*, *RUNX1*, *EZH2*, *ZRSR2* and *IDH2* were more frequent in the isolated +8 MDS than in the control group (Figure 1b). Remarkably, the proportion of patients with isolated +8 MDS and mutations in *STAG2* was nearly three times more than in the control MDS group as previously reported [14]. As expected, the incidence of mutations in *SF3B1* was lower in the isolated +8 cohort compared to the control MDS group due to these mutations being extensively associated with MDS with ring sideroblasts [22–24] or normal karyotypes [25], which is concordant to the low incidence of these alterations in our cohort. By contrast, our results did not show *TP53* as frequently mutated in MDS with isolated +8, which was consistent with the high association of mutations in this gene to both complex karyotype and 5q deletion previously described in MDS [26–28]. All this data together could indicate a specific spectrum of genetic lesions associated with isolated +8.

The presence of *STAG2*, *SRSF2* and *RUNX1* mutations, alone or in combination, shortening both the time to leukemia transformation and the OS within MDS patients with isolated +8. Consequently, the presence of mutations in *STAG2*, *SRSF2* and/or *RUNX1* distinguishes between two different prognostic subgroups displaying specific clinical characteristics and outcomes similar to high-risk (with at least one mutation in these genes) and low-risk (without any of these genes altered) MDS patients without +8. Despite the well-known key prognostic significance that percentage of BM blasts plays in MDS, patients with isolated +8 and mutations in *STAG2*, *SRSF2* and/or *RUNX1* showed similar outcomes to high/very high-risk control patients, even with significant lower levels of blasts in BM (Table S5). By contrast, higher levels of BM blasts were observed in isolated +8 MDS without these mutations, when compared to low-risk MDS controls. In addition, the low frequency of *SF3B1* mutations in isolated +8 patients might explain the lower levels of ring sideroblasts and platelets (of which high levels have been associated with *SF3B1* mutants [20]) observed in these patients (Table S6). All these data suggest that not clinical, but genetic information might have an essential role in the prognosis of patients with isolated +8.

In fact, it is not the number of mutations but the number of alterations in these genes that has become an independent prognostic factor within isolated +8 MDS patients with a low percentage of blasts, as shown in the multivariate analysis in both the discovery and the validation cohorts (Table 2, Figure S5c). Otherwise, when patients showed a higher percentage of blasts (>10%), as described, this clinical feature also plays a role in the prognosis of MDS [21], together with mutational status of *STAG2*, *SRSF2* and/or *RUNX1* in the specific context of isolated +8 (Figure S2c).

When IPSS-R was applied to our cohort of MDS with isolated +8, 23.5% of patients included in the low-risk category and 52.4% of intermediate-risk cases categorized by the IPSS-R, showed worse outcomes and were described as high-risk isolated +8 patients due to the presence of mutations in *STAG2*, *SRSF2* and/or *RUNX1* (Figure 3a). In addition, 30.4%

of patients categorized as high and very high risk by the IPSS-R were considered as low risk by the mutational profile. Therefore, our results suggest that comprehensive genomic analyses might refine the risk classification in a subset of MDS patients with isolated +8.

Otherwise, these isolated +8 differentiated subgroups were validated by the new IPSS-M classification, as a high number of patients (88.9%) with mutations in *STAG2*, *SRSF2* and/or *RUNX1* were classified as moderate–high, high and very high risk by the IPSS-M (Figure 3b). Of note, 37.5% of patients with moderate–low risk based on the IPSS-M showed a high risk based on the status of *STAG2*, *SRSF2* and *RUNX1*. In this regard, while *SRSF2* and *RUNX1* are genes considered as relevant factors increasing the score, *STAG2* shows less relevance in the IPSS-M [11]. However, *STAG2* is the second most frequent mutated gene in isolated +8 MDS patients and plays a role in disease progression and OS [19,29], as has been demonstrated in this work. Therefore, the score associated with each somatic mutation might vary depending on the subset of MDS patients studied. Specifically, analysis of mutations in *STAG2*, *SRSF2* and/or *RUNX1*, in addition to the IPSS-M, could refine prognostic information of patients presenting isolated +8. In addition, our results could be of special interest in patients classified as moderate–low risk by the IPSS-M, where *STAG2* mutations might be worsening the prognosis.

It is well known that the heterogeneity of hematological diseases such as MDS makes the management of patients difficult. Indeed, only high-risk MDS patients are eligible to receive HSCT or be treated with HMA agents due to the increasing risk of leukemia transformation [30]. The identification of patients likely to be treated in the earliest stages of the disease would enable the anticipation of clinical deterioration in patients and increase the options for stabilizing the disease when the patient's health is in a good state. In fact, both a shorter time to initiating HMA treatment and immediate HSCT at the time of diagnosis maximized OS and complete responses in high-risk MDS patients [31–33], while delays during the first cycles of therapy adversely affect OS, independently of the IPSS-R status of the patient [34]. Consequently, identifying a subgroup of isolated +8 MDS with higher risk could transform the therapeutic approach of these patients.

In summary, the identification of somatic mutations has shown to be useful in distinguishing different risks within the isolated +8 MDS patients. In fact, the presence of mutations in three genes (*STAG2*, *SRSF2* and *RUNX1*) would be enough to define the course of the disease in terms of OS and AML progression. This study validates the prognostic value of the IPSS-M in the specific isolated +8 MDS subgroup, adding prognostic value to *STAG2*. Overall, these data could lead to new opportunities in the management of a high-risk subset of patients with isolated +8 MDS.

5. Conclusions

The mutational profile may help to correctly stratify patients with MDS and isolated +8 when clinical features identify them as intermediate risk. Indeed, mutations in *STAG2*, *SRSF2* and/or *RUNX1* distinguish a high-risk subgroup of patients with isolated +8 in terms of OS and AML transformation.

In view of these results and given the high heterogeneity of MDS, an independent mutational evaluation of each distinct biological subgroup may be helpful for a better understanding of the outcome of the disease.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers15153822/s1>, Table S1: Genes included in the in-house custom capture-enrichment panel used in the study. Table S2: Somatic mutations found in patients with MDS and isolated trisomy 8 (+8). Table S3: Univariate analyses of mutation status for AML progression of patients with isolated +8. Table S4: Clinical characteristic of patients with low-risk-like and high-risk-like MDS and isolated trisomy 8. Table S5: Clinical features of isolated +8 MDS patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1* compared to high-risk IPSS-R and high-risk IPSS-R MDS controls. Table S6: Clinical characteristic of patients with isolated +8 MDS without mutations in *STAG2*, *SRSF2* and *RUNX1* compared to low-risk IPSS-R control patients. Table S7: Clinical and biological features of isolated +8 MDS cohorts. Figure S1: (a) Time to AML progression in

newly described isolated +8 MDS subgroups compared to non-trisomy 8 MDS patients with normal and complex karyotypes; (b) overall survival in newly defined risk subgroups in isolated +8 MDS patients compared to non-trisomy 8 MDS patients with normal and complex karyotypes. Figure S2: (a) Multivariate analysis of patients with isolated +8 depending on number of mutations per patient (≥ 4) in AML progression; (b) multivariate analysis of OS in isolated +8 MDS patients depending on the number of mutations per patient; (c) multivariate analysis of OS in isolated +8 MDS considering 10% of BM blasts in patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1*; (d) multivariate analysis of patients with isolated +8 MDS regarding percentage of BM blasts ($>10\%$) and the number of mutations per patient (≥ 4). Figure S3: (a) Percentage of patients with isolated +8 MDS with and without mutations in *STAG2*, *SRSF2* and/or *RUNX1* stratified by the IPSS-R; (b) percentage of patients with MDS and isolated +8 with and without mutations in *STAG2*, *SRSF2* and/or *RUNX1* stratified by the IPSS-M. Figure S4: Mutational pattern of mutated genes in more than 5% of isolated +8 MDS patients of the International Working Group for the Prognosis of MDS. Figure S5: (a) Time to AML progression in newly defined prognostic subgroups in isolated +8 compared to the control MDS without +8 stratified by IPSS-R risk subgroups; (b) OS of isolated +8 MDS patients reclassified in prognostic groups according to mutational profile within IPSS-R MDS control groups; (c) multivariate analysis for OS in isolated +8 patients of the validation cohort. Figure S6: (a) Percentage of patients from the validation cohort stratified within IPSS-R and IPSS-M categories reconsidered as low risk and high risk based on mutational profile; (b) percentage of patients classified by the IPSS-R and IPSS-M in patients with isolated +8 MDS depending on mutational status of *STAG2*, *SRSF2* and *RUNX1*.

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Capítulo 2

Las mutaciones somáticas en la monitorización del tratamiento precoz en los SMD de bajo riesgo

Estrategia terapéutica precoz para el tratamiento de SMD y del(5q) para mejorar la calidad de vida del paciente y prolongar el tiempo hasta la progresión de la enfermedad: seguridad molecular en el ensayo clínico SintraRev.

DINÁMICA MUTACIONAL EN PACIENTES CON SMD Y DEL(5Q) TRATADOS CON LENALIDOMIDA PREVIO A LA DEPENDENCIA TRANSFUSIONAL – RESULTADOS MOLECULARES DEL ENSAYO CLÍNICO SINTRAREV

Introducción

La lenalidomida (lena) es un fármaco inmunomodulador que actúa selectivamente sobre las células con del(5q) y es el estándar de tratamiento para los pacientes con SMD que presentan esta alteración citogenética cuando son dependientes de transfusiones (DT). El ensayo clínico de fase III SintraRev confirmó la eficacia del tratamiento precoz con lena en pacientes con SMD, del(5q), anemia y sin DT. Sin embargo, no se conoce qué efecto produce la lena en la evolución clonal de estos pacientes. Por este motivo, el presente estudio evaluó los cambios en el perfil mutacional observados en pacientes con SMD, del(5q) sin DT que fueron tratados con dosis bajas de lena para determinar si la administración precoz del tratamiento reduce la frecuencia de variación alélica (VAF) de las mutaciones somáticas observadas en los pacientes o, por el contrario, podría expandir clones preexistentes y favorecer la adquisición de nuevas mutaciones.

Metodología

Los pacientes incluidos en el estudio fueron aleatorizados en una proporción 2:1 en un grupo de tratamiento con bajas dosis de lena (5mg/día en ciclos de 28 días) y un grupo placebo, durante una fase de tratamiento de 2 años y un seguimiento posterior de, al menos, 4 meses, hasta DT o toxicidad inaceptable. La mediana de seguimiento fue de 60,6 meses. Se llevaron a cabo estudios de NGS en muestras de médula ósea de 51 pacientes, obtenidas en tres momentos distintos: al inicio del ensayo (previo a la administración de tratamiento), en la semana 12 y cada 24 semanas hasta el último seguimiento. La NGS se realizó mediante paneles de captura dirigidos a 65 genes relacionados con patología mieloide.

Resultados

Los genes más frecuentemente mutados fueron *SF3B1* (25,5%) y *TP53* (21,3%). La evolución clonal de los pacientes varió entre los grupos de tratamiento con Lena y placebo, con una reducción del 41,2% de los clones predominantes en los pacientes tratados, mientras que permanecían estables o aumentaban en el 100% de los casos en el grupo control tras la fase de tratamiento de 2 años. Los análisis de VAF mostraron que las mutaciones en *SF3B1* y *TP53* permanecían estables o disminuían durante el tratamiento con lena. Por el contrario, se observó un aumento del VAF en alteraciones en *DNMT3A* en pacientes con respuesta citogenética, sugiriendo que podría tratarse de mutaciones asociadas a hematopoyesis clonal

de significado incierto. En relación con la eficacia del tratamiento, el 100% de los pacientes con mutaciones en *SF3B1* presentaron respuesta a lena. Por el contrario, las tasas de respuesta fueron inferiores en los casos con alteración de *TP53*, con un 60% y 40% de pacientes alcanzando respuestas citogenética y eritroide, respectivamente. La incidencia de progresión a LMA fue inferior en los pacientes con mutaciones en *SF3B1* (25%, n=2/8) tratados con lena, en comparación con los del grupo placebo que presentaban alteraciones en este gen (50%, n=2/4), así como un tiempo hasta la progresión más prolongado en los pacientes con *SF3B1* mutado (24,1 vs 16 meses). Por otro lado, sólo un 20% de los pacientes tratados con lena que presentaban mutaciones en *TP53* progresaron a LMA, una proporción similar a la observada en el grupo placebo. Se observó la aparición de clones transitorios con *TP53* en dos pacientes tratados con lena, pero ninguno de ellos progresó a LMA en 64,8 y 96,6 meses de seguimiento.

Conclusiones

Estos resultados sugieren que el tratamiento precoz con bajas dosis de lena en pacientes con SMD y del(5q), anemia y sin DT, reduce la carga mutacional de la mayoría de los genes y no promueve la expansión de clones preexistentes ni la transformación a LMA. En consecuencia, la administración precoz de lena en este subgrupo de pacientes podría ser un tratamiento eficaz y con un perfil de seguridad manejable en relación con la evolución clonal de los pacientes.

Original article

MUTATIONAL DYNAMICS IN PATIENTS WITH DEL(5Q) MDS TREATED WITH LENALIDOMIDE PRIOR TO TRANSFUSION DEPENDENCY – MOLECULAR RESULTS FROM THE SINTRAREV CLINICAL TRIAL

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SUMMARY

Lenalidomide (Len) is the standard of care for patients with myelodysplastic syndromes (MDS) and del(5q) when they become transfusion dependent (TD). SintraRev is the first phase 3 clinical trial confirming efficacy and safety of an early therapeutic approach with Len in anemic del(5q) MDS patients without TD. However, clonal evolution in these patients, remains unexplored. Here, we evaluated the changes in the mutational profile of transfusion independent 5q- MDS patients treated with Len to assess whether the early administration of Len could reduce the variant allele frequency (VAF) of somatic mutations or might be responsible for the expansion and acquisition of additional alterations.

Molecular profile of patients included in the SintraRev trial was analyzed. Median follow-up of patients was 60.6 months (32.3-73.9). NGS was available in a total of 51 patients: Thirty-two in the Len group and nineteen in the placebo (Pcb) arm. In addition to 5q-, *SF3B1* (25.5%) and *TP53* (21.3%) were the most frequently mutated genes. Clonal evolution during treatment among patients receiving Len or Pcb was markedly different, with a 41.2% reduction in predominating clones in Len arm, while remaining stable or increase in 100% of the Pcb group after the 2-year treatment period. VAF analysis showed that mutations in both *SF3B1* and *TP53* decreased or remained stable in the Len cohort, while only *DNMT3A* mutations increased in patients treated with Len under cytogenetic response, suggesting a CHIP-associated behavior. Regarding disease progression, only 20% of patients treated with Len and carrying *TP53* mutations developed AML, a proportion similar to the Pcb group (20%), indicating that Len did not induce AML evolution nor *TP53* mutation acquisition.

In summary, treatment with low-dose Len in transfusion independent del(5q)-MDS reduced the mutational burden of most genes and did not promote the expansion of preexisting clones or AML progression, regardless of the mutations involved. Consequently, the early administration of Len in del(5q)-MDS patients without TD might be an effective therapeutic approach with a manageable safety profile regarding clonal evolution.

INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal bone marrow disorders characterized by ineffective hematopoiesis leading to blood cytopenias and a variable risk of progression to acute myeloid leukemia (AML) through genetic instability^{1,2}. Cytogenetic alterations are found in approximately 50% of patients with MDS^{3,4}. Deletion of the long arm of chromosome 5 [del(5q)], when isolated and in the absence of excess marrow blasts, defines a distinct clinical and pathological MDS entity in the 2022 World Health Organization (WHO) and the International Consensus Classification (ICC)^{5,6}, and is associated to about 20% risk of AML progression after 4 years^{7,8}.

Somatic point mutations, found in 80-90% of MDS, generally have a poor prognostic impact⁹. *TP53* mutation is particularly associated with poorer outcome, especially in MDS with isolated del(5q)^{10,11}. These mutations occur in 20-25% of the cases, often during evolution, and are a main driver of progression to AML through genetic instability, leading to complex karyotype¹². *SF3B1*, generally associated with favorable prognosis in MDS, is paradoxically also associated with higher risk of AML progression and shorter overall survival (OS) in del(5q) MDS¹³⁻¹⁸.

Patients with MDS and del(5q) generally become red blood cells (RBC) transfusion-dependent (TD) shortly after diagnosis^{19,20}. Erythropoietic stimulating agents (ESA) are less effective in MDS with isolated del(5q) than in other low risk MDS, while Lenalidomide (Len), that selectively inhibits the 5q deletion clone, is the standard of care in del(5q) MDS when they become RBC-TD²¹. It leads to RBC transfusion independence (TI) in about 2/3 of the patients, with a median duration of about 22 months, and to complete cytogenetic response in about 50% of the patients^{22,23}. However, it is unclear if Len has a positive effect on disease progression and OS^{19,24,25}. In addition, the effect of Len on the mutational profile in MDS with isolated del(5q) is not known. In particular, response to Len is lower and shorter in patients with *TP53* mutations²⁶⁻²⁸. In those patients, it is hypothesized that Len, by positively selecting the *TP53* mutated subclones, could potentially accelerate disease progression, although it is not demonstrated.

Our cooperative groups completed SintraRev, a phase III clinical trial comparing low-dose Len treatment and placebo (Pcb) in anemic, non-TD patients with del(5q) MDS, which showed a high rate of RBC-TI, cytogenetic response and longer time to RBC-TD in treated patients, potentially suggesting a role for early Len treatment in delaying disease progression²⁹.

We took advantage of this study to evaluate the molecular changes of the early administration of Len and assess if this early therapeutic approach could drive molecular responses or might be related to the expansion and acquisition of somatic mutations.

METHODOLOGY

Patients included in the SintraRev clinical trial

Inclusion criteria have been described previously²⁹. In brief, they included 1) age ≥ 18 years old; 2) MDS with del(5q) (isolated or accompanied by another additional cytogenetic abnormality not involving chromosome 7) with low or intermediate-1 risk categorized by the revised-international prognostic scoring system (IPSS-R)⁸; 3) baseline Hb ≤ 12 g/dL, but documented confirmation of no prior packed red blood cell (RBC) transfusions due to the MDS; 4) Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 ; and 5) signed written informed consent. Full eligibility and exclusion criteria are detailed in López-Cadenas et al²⁹.

Study design

SintraRev was a phase III multicenter, randomized, double-blind, placebo-controlled trial. Patients were enrolled between February 2010 and February 2018 in 22 different locations from three European countries (Spain, France and Germany). Briefly, patients were randomized 2:1 to receive Len (5 mg/day in a 28-day cycle) or placebo (Pcb) for 104 weeks (treatment phase of 2 years) followed by at least 4 months of follow-up, until disease RBC-TD or unacceptable toxicity. Responses were assessed following criteria on the International Working Group³⁰. Patients were monitored for two additional years of follow-up after the end of the treatment period. Data on RBC-TD occurrence, AML transformation and death were collected. Additionally, an extended long-term follow-up after the end of the study was reported to update information about transfusion dependence and patient's outcome²⁹.

Sample procedure and genetic analyses

Bone marrow (BM) samples were obtained from patients at inclusion prior to treatment, at week 12 and then every 24 weeks until last follow-up. Additional samples were obtained to confirm clinical indications of disease progression.

Conventional G-banding analysis was performed in all patients and karyotypes were described in accordance with the International System for Human Cytogenetic Nomenclature³¹. Deletion of the long arm of chromosome 5 was confirmed by interphase fluorescence in situ hybridization (FISH).

Next-generation sequencing (NGS) was performed in available samples using an in-house custom capture-enrichment panel (Twist Bioscience) targeting 65 myeloid genes.

Libraries were prepared according to manufacturer's instructions and sequenced on Illumina platforms³².

For true oncogenic somatic variant calling, synonymous, noncoding variants and polymorphisms, present at a population frequency (MAF) $\geq 1\%$ in SNP databases, were excluded. In addition, variants recurrently observed suspected of being sequencing errors were removed. The remaining variants were considered candidate somatic mutations when: i) the sequencing depth at the genomic position of the mutation was higher than 2000 reads; ii) variant allele frequency (VAF) was higher than 5% in at least one of the sequenced samples; iii) VAF was higher than 2%; iv) described in COSMIC and/or ClinVar as being cancer-associated and known hotspot mutations with clinical pathogenic significance^{33,34}.

Statistical analysis

Dichotomous variables were compared between different groups using the χ^2 test and continuous variables by the Kolmogorov, Student's t-test, Mann-Whitney and Kruskal-Wallis tests. Results were considered significant at $p < 0.05$.

Paired-sample tests (Wilcoxon and Friedman tests) were applied to compare each arm of the study within the different points of time under analysis.

Time to RBC-TD, AML progression, OS or time of responses to treatment were estimated by Kaplan-Meier method, and groups were compared by two-sided log-rank test. Time to AML progression were considered as the time from the inclusion in the study to the AML diagnosis and censored in patients without AML transformation at last follow-up or date of death.

Statistical analyses were performed using SPSS Statistics version 25 (IBM Corporation, Amonk, NY, USA) and R version 4.0.2 with biostatistical packages (<https://cran.r-project.org/>).

RESULTS

***SF3B1*, *TP53* and *DNMT3A* were the most frequently mutated genes in patients included in RBC-TI patients with del(5q) included in the SintraRev clinical trial**

Sixty-one patients were included in the SintraRev clinical trial. Median follow up of patients was 60.6 months (32.3-73.9). Clinical and biological data are shown in Table 1.

A total of 51 patients (32 at the treatment group and 19 in the placebo arm) had NGS available in at least one-time point. Molecular analysis at baseline revealed the presence of 41 somatic mutations (24 in the Len arm and 17 in the Pcb group). Median number of mutations per patient was 1 (0-3) in both Len and Pcb groups.

At baseline, no somatic mutation was identified in 36.2%, while 44.7% and 19.1% had 1 and 2 or more mutations per patient, respectively. The most frequently mutated genes were *SF3B1* (25.5%) and *TP53* (21.3%), followed by *DNMT3A* (19.1%). Distribution of *SF3B1*, *TP53* and *DNMT3A* gene mutations were well-balanced in the treatment and the placebo arms. Eight patients treated with Len showed *SF3B1* mutations while five patients had *TP53* mutations at baseline. Mutations in these genes were present in four and five cases in the Pcb group, respectively (Table 1, Fig. 1). Median VAF of mutations at baseline was 18% (2-40%) in the Pcb arm and 14.7% (0-87%) in the Len group. All these data confirmed that patients included in the Pcb and Len arms were comparable at baseline regarding both clinical and molecular features.

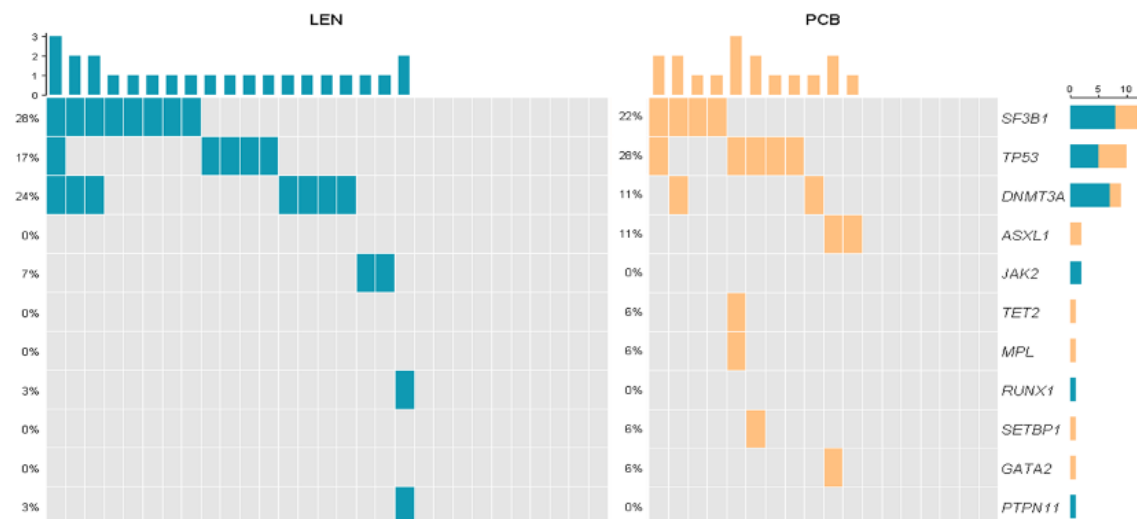


Figure 1: Mutational profile of patients included in the SintraRev within the Len and Pcb arms at baseline.

Table 1: Clinical and molecular features of patients included in the SintraRev clinical trial.

	Placebo (n=21)	Lenalidomide (n=40)	P value
Gender, n (%)			0.733
Male	3 (14.3%)	8 (20%)	
Female	18 (85.7%)	32 (80%)	
Age (years), n (range)	71.9 (64.7-80.7)	72.2 (65-82.4)	>0.99
Hb, g/dL	9.8 (8.9-10.2)	9.8 (9.3-10.7)	0.096
Platelets, x10⁹/L	272 (200-327)	238 (171-312)	0.379
Neutrophils, x10⁹/L	2.2 (1.5-2.7)	2.1 (1.5-3.2)	0.633
Bone marrow blasts, %	2 (0-3)	1.5 (0.6-3)	0.951
WHO 2008, n (%)			0.718
RCUD	0 (0%)	2 (5%)	
RARS	1 (4.8%)	0 (0%)	
RCMD	5 (23.8%)	10 (25%)	
RAEB-1	1 (4.8%)	2 (5%)	
MDS with del(5q)	14 (66.6%)	26 (65%)	
WHO 2017, n (%)			n.s
MDS-EB-1	1 (4.8%)	2 (4.9%)	
MDS del(5q)	20 (95.2%)	38 (95.1%)	
IPSS			0.912
Low	14 (66.7%)	29 (72.5%)	
Intermediate 1	7 (33.3%)	11 (27.5%)	
IPSS-M			n.s
Very low	3 (16.7%)	5 (17.2%)	
Low	13 (72.2%)	20 (69%)	
Moderate low	2 (11.1%)	2 (6.9%)	
Moderate high	0 (0%)	2 (6.9%)	
Cytogenetic abnormalities, n (%)			n.s
Isolated del(5q)	19 (90.4%)	35 (87.5%)	
Del(5q) plus additional abnormalities	2 (9.6%)	5 (12.5%)	
Time from diagnosis to inclusion in SintraRev, months	4 (1.7-13.6)	2.6 (0.9-11-3)	
NGS data			n.s
NGS at baseline	18 (85.7%)	29 (72.5%)	
Median mutation per patient, n (range)	1 (0-3)	1 (0-3)	
patients without mutations, n (%)	7 (38.9%)	10 (34.5%)	
patients with 1 mutation, n (%)	6 (33.3%)	15 (51.7%)	
patients with 2 or more mutations, n (%)	5 (27.8%)	4 (13.8%)	
TP53 mutated patients, n (%)	5 (27.8%)	5 (17.2%)	0.48
SF3B1 mutated patients, n (%)	4 (22.2%)	8 (27.6%)	0.74
DNMT3A mutated patients, n (%)	2 (11.1%)	7 (24.1%)	

Cytogenetic response in patients treated with Len was accompanied by a reduction in VAF mutations at week 12 remaining stable during treatment

To evaluate the effect of Len in early treated patients with del(5q) MDS, we analyzed clonal architecture in sequential samples of twenty-one patients treated with Len in the short-term (baseline vs week 12) and during the entire treatment phase (baseline vs end of Len administration). In addition, 13 paired-samples in the Pcb group were analyzed at baseline, week 12 and after 2 years.

In the short-term, the mean percentage of cells with the 5q deletion by FISH decreased from 37.8% to 3.7% in treated patients ($p < 0.0001$), while in the Pcb group, it slightly increased (50.8% at baseline and 56.2% at week 12, $p = 0.063$). In addition, in patients treated with Len, a total of 17 somatic mutations were analyzed, of which seven (41.2%) showed a VAF reduction (Fig. 2a). The mean VAF reduction was 26.3%. Point mutations in *SF3B1* ($n=2$; baseline VAF of 20% and 15.3%) and *JAK2* ($n=1$, with 87% initial VAF) became undetectable under Len treatment, and no evidence of two of these mutations was found at the end of the therapeutic intervention (Fig. 2a). Seven mutations (41.2%) remained stable after 12 weeks of treatment. Moreover, 8 patients did not show somatic mutations at baseline and all of them remained without detectable mutation at week 12 of treatment. Only one patient showed an increase in the VAF of a *DNMT3A* mutation at this time. This patient achieved cytogenetic response and harbored a VAF reduction in additional mutations (Fig. 2a; Suppl. Fig. 1, $p < 0.0001$). Of note, one patient showed two *TP53* mutations at week 12 for the first time, both with VAF below 2%. These mutations were not detected in the baseline sample, but no disease progression was observed after >20 months of follow-up and, specifically, 25.8 months after Len treatment discontinuation.

Altogether, median VAF of mutations harbored in patients treated with Len showed a significant reduction at week 12 [from 14.7% (0-87%) to 7% (0-36%), $p = 0.013$; Fig. 2b,d). Conversely, the VAF of all mutations found in patients from the Pcb group ($n=9$) remained stable in the short-term (18% median VAF at baseline and week 12, from 1 to 40%, $p = 0.594$, Fig 2c,d).

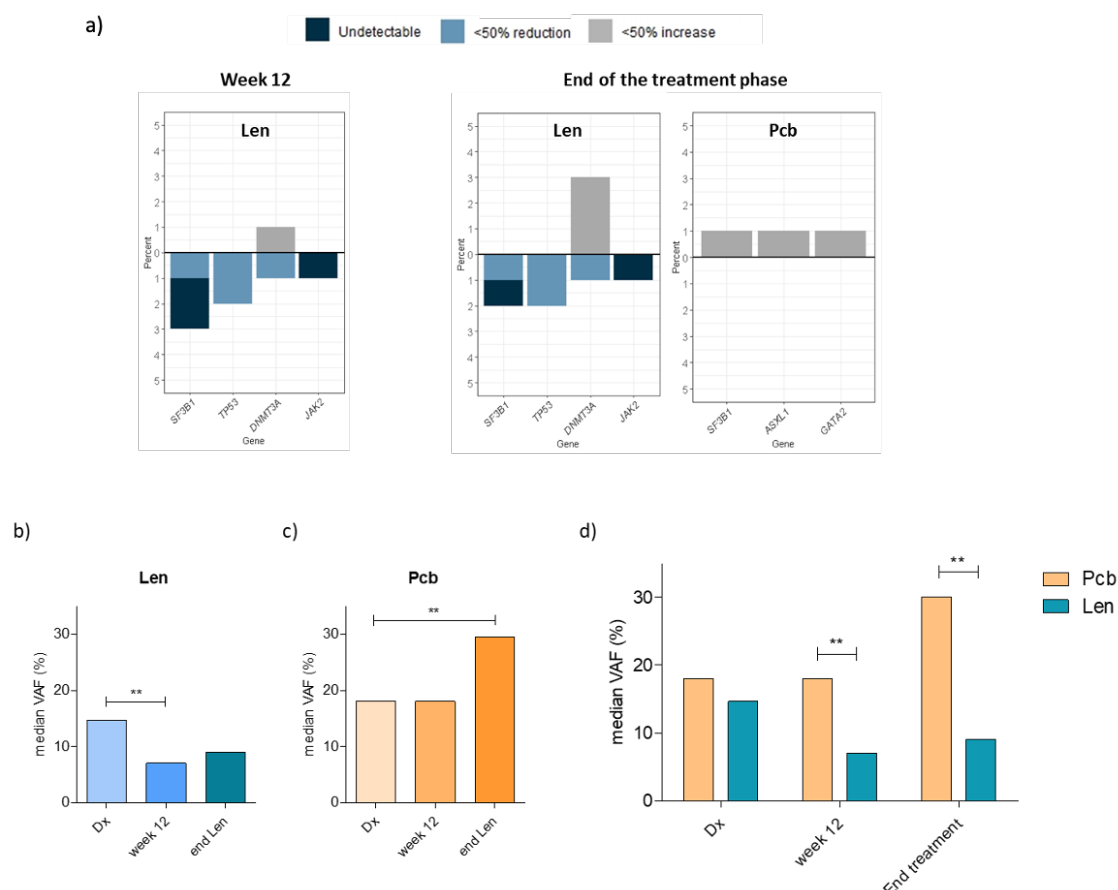


Figure 2: Changes in the VAF of the more frequently mutated genes in non-dependent MDS with del(5q) including in the SintraRev trial; a) clonal evolution of mutations found at baseline in Len (at week 12) and Len and Pcb (at the end of treatment phase); b) mutational changes in median VAF of treated-patients during Len administration; c) median VAF variation in Pcb patients during the treatment phase; 4) comparison of median VAF (%) of mutations between Pcb and Len patients at baseline, week 12 and end of treatment. ** $p < 0.01$

Interestingly, at the end of Len administration, most mutations found at baseline remained stable or decreased VAF/disappeared under treatment (82.3%). Only three *DNMT3A* mutations showed an increment of their VAF, all of them in patients achieving cytogenetic response (CyR) to Len. Furthermore, 2 patients with *SF3B1* mutations at baseline showed additional *TP53* mutations after 2 years of treatment, in both cases after losing response to treatment and with VAF below 5% (Suppl. Fig. 2). It should be noted that neither of these 2 patients experienced AML transformation. Overall, mean VAF of mutations in patients treated with Len was 9% (0-41%) at the end of treatment administration, remaining inferior to baseline VAF (Fig. 2b,d). By contrast, the long-term monitoring of somatic mutations in Pcb patients revealed a clonal expansion in four mutations, while the remaining alterations were stable (74.9%). A significant increase in median VAF was observed in Pcb patients compared to baseline (29.5% vs 18%, respectively, $p = 0.007$; Fig. 2c,d).

Mutations in *SF3B1*, *TP53* and *DNMT3A* exhibited a different behavior under Len administration

Clonal dynamics of *SF3B1*, *TP53* and *DNMT3A* mutations under Len administration were independently analyzed in treated patients. Incidence of these mutations in both the Len and the Pcb groups is shown in Table 1.

Dynamics of *SF3B1* mutations under Len treatment was heterogeneous. In fact, 37.5% of treated patients showed a significant reduction in *SF3B1* mutant allele burden, while it remained stable in 62.8% of cases. All patients achieved CyR and erythroid response (ER) with Len, but most of them lost responses at the end of the treatment. Indeed, median duration of CyR was shorter in patients with *SF3B1* mutations than in those without *SF3B1* alteration [12.0 (5.57-21.97) vs 24.6 (3.53-82.80) months, $p=0.047$]. Regarding disease progression, a lower percentage of patients with *SF3B1* mutations became TD in the Len (37.5%, $n=3$) versus the Pcb group (100%, $n=4$) ($p=0.08$). Median time until TD in patients with *SF3B1* mutations was longer in the Len arm than in the Pcb arm [21.8 (16.3-27.3) vs 6.3 (2.9-9.6) months, $p=0.006$]. In addition, incidence of AML progression was lower in *SF3B1*-mutated patients in the Len (25%, two out of eight), as compared to the Pcb group (50%, two out of four) (Table 2). In the subset of patients harboring *SF3B1* mutations, median time to AML was 24.1 (12.1-50.4) and 16 (10.9-50.4) months in Len and Pcb groups, respectively (HR: 0.48, $p=0.41$).

Clonal evolution of *TP53*-mutated patients treated with Len revealed a substantial reduction in VAF of all mutations (median VAF reduction of 10%, from 3-19%) under treatment. Conversely, VAF of *TP53* alterations observed in Pcb patients remained stable and one even increased. Among five patients with *TP53* mutations, three (60%) and two (40%) achieved CyR and ER with Len, respectively, while higher percentage of patients without these alterations achieved responses to treatment (CyR: 91.7%, $n=22$; ER: 83.3%, $n=20$). In the long-term follow-up, all patients with *TP53* mutations were considered, regardless of the *TP53* status at baseline (see Table 3 for more details). A similar percentage of *TP53*-mutated patients became TD (80% and 60%, $p=0.56$) and underwent AML transformation (20%: three out of fifteen and one out of five within the Len and Pcb arms, respectively). Of note, transformation to AML occurred in a total of eleven patients in this study, and 72.7% of them did not harbored mutations in *TP53* during all the follow-up. Two *TP53* mutations appeared after loss of response to Len, without evidence that these mutations were present at baseline. VAF were lower than 5% and neither of these two patients progressed to TD, nor develop AML in 64.8 and 96.6 months of follow-up (40 and 71 months after Len discontinuation, respectively). Moreover, a patient with <2% VAF mutation in *TP53* first observed at week 12, did not exhibit AML transformation after 35.1 months of follow-up (*exitus* due to infection).

Table 2: AML transformation and molecular information of VAF at diagnosis and at the end of treatment of patients with *SF3B1* mutations included in the study.

Patient ID	Arm	ER	Loss ER	CyR	Loss CyR	TD	AML TF	LFS	Follow-up	VAF at dx	VAF variation study	Additional mutations
1 (P7-01)	Len	Yes	No	Yes	Yes	No	Yes*	59.4	59.4	0.2	0.02	<i>TP53</i> , <i>DNMT3A</i>
2 (P11-04)	Len	Yes	Yes	Yes	No	Yes*	No	69.8	30.3	0.32	na	<i>DNMT3A</i>
3 (P11-06)	Len	Yes	No	Yes	No	Yes*	No	60.9	14.8	0.2	0	No
4 (P13-03)	Len	Yes	No	Yes	Yes	Yes*	No	96.6	57.9	0.07	0.12	<i>TP53</i>
5 (P13-10)	Len	Yes	Yes	Yes	Yes	Yes**	No	65.7	62.1	0.38	0.37	No
6 (P31-02)	Len	Yes	Yes	Yes	Yes	Yes*	Yes	11.9	35.1	0.15	0.06	No
7 (P34-01)	Len	Yes	No	Yes	Yes	Yes**	No	20.2	108.2	0.1	0.03	<i>DNMT3A</i>
8 (P46-01)	Len	Yes	Yes	Yes	No	Yes*	No	64.8	54.5	0.41	0.41	<i>TP53</i>
9 (P2-01)	Len	Yes	Yes	Yes	Yes	No	No	129.9	96.6	na	0.15	<i>TP53</i>
12 (P13-06)	Pcb	na	na	na	na	No	No	81.7	64.8	0.24	0.33	No
13 (P17-02)	Pcb	na	na	na	na	No	Yes*	50.4	65	0.15	0.39	No
14 (P21-01)	Pcb	na	na	na	na	No	No	14.7	69	0.13	0.2	<i>DNMT3A</i>
15 (P46-02)	Pcb	na	na	na	na	Yes	Yes*	46.2	92.6	0.27	na	<i>TP53</i>

*After the end of the study; TF: transformation; LFS: leukemia free survival; dx: diagnosis; LFS and follow-up are expressed in months

Regarding clonal evolution of *DNMT3A* mutations, four out of seven patients (57.1%) harbored mutations only in *DNMT3A*, while three cases showed mutational concurrence with *SF3B1* (3) and *TP53* (1) (Suppl. Fig. 1). Most *DNMT3A* mutations (85.7%) were not affected by Len, with VAF either remaining stable or even increasing under treatment. Interestingly, in all cases with comutations, Len revealed either substantial reduction or stability in the additional coexisting mutations. All patients with *DNMT3A* mutations achieved response to treatment and maintained it during all the trial, suggesting that these mutations might have an independent role from the 5q- clone and its persistence did not seem to influence responses to Len.

Table 3: Response to treatment and long-term follow-up of patients with TP53 mutations included in the study.

Patient ID	Arm	ER	Loss ER	CyR	Loss CyR	TD	AML TF	LFS	Follow-up	VAF at dx	VAF variation study	Additional mutations at diagnosis
1 (P7-01)	Len	Yes	No	Yes	Yes*	No	Yes**	59.4	59.4	0.14	decrease long-term	SF3B1, DNMT3A
2 (P13-02)	Len	Yes	na	Yes	Yes*	Yes*	Yes**	21.9	30.3	0.23	decrease week 12	No
3 (P1-09)	Len	No	na	Yes	No	Yes*	No	14.8	14.8	0.2	decrease week 12	No
4 (P36-02)	Len	No	na	No	na	Yes*	No	57.9	57.9	0.13	decrease long-term	No
5 (P15-04)	Len	No	na	No	na	Yes**	No	62.1	62.1	0.13	no molecular follow-up	No
6 (P1-05)	Len	No	na	Yes	No	Yes*	No	35.1	35.1	0	stable <10% long-term	Biallelic TP53
7 (P2-01)	Len	Yes	Yes*	Yes	Yes*	Yes**	No	108.2	108.2	na	no molecular follow-up	SF3B1
8 (P12-01)	Len	Yes	Yes*	No	na	Yes*	No	54.5	54.5	na	no molecular follow-up	No
9 (P13-03)	Len	Yes	No	Yes	Yes*	No	No	96.6	96.6	0	5% at loss of response	SF3B1
10 (P46-1)	Len	Yes	Yes*	Yes	No	No	No	64.8	64.8	0	5% at loss of response	SF3B1
11 (P7-02)	Pcb	No	na	No	na	No	No	65	65	0.29	increase long-term	No
12 (P13-8)	Pcb	No	na	No	na	No	No	69	69	0.4	stable	SETBP1
13 (P1-08)	Pcb	No	na	No	na	Yes	No	92.6	92.6	0.05	stable	TET2, MPL (acq JAK2)
14 (P13-5)	Pcb	No	na	No	na	Yes	No	85.6	85.6	0.36	stable	No
15 (P46-2)	Pcb	No	na	No	na	Yes	Yes**	46.2	63.2	0.23	no molecular follow-up	SF3B1

*Under Len treatment; **After the end of the study. ER: erythroid response; CyR: cytogeneic response; TD: transfusion dependency; TF: transformation; LFS: leukemia free survival; dx: diagnosis; LFS and follow-up are expressed in months

Long-term follow-up revealed clonal stability in del(5q) patients treated with Len

A total of 23 BM samples from patients with del(5q) (17 in the Len arm and 6 in the Pcb group) were sequenced after the end of the SintraRev trial monitoring. Disease progression and patient follow-up was available up to 104 months after the end of the trial, with a median of 43.6 months of extended follow-up. Expansion of preexisting mutations, as well as acquisition of additional mutations, were found in 4 (17.4%) patients after the end of the SintraRev trial monitoring, which was less frequent in the Len arm (11.8%) than in the Pcb arm (33.3%).

In the Len group, 15 patients (88.2%) did not show clonal expansion or acquisition of new mutations after Len administration with a median follow-up of 43.7 months. Patients without baseline mutations, remained stable and did not acquire somatic alterations after the end of the trial. By contrast, a *DNMT3A* mutated clone that was decreased under Len treatment, expanded in the long-term, consistent with a relapse of the malignant clone after Len discontinuation (patient 04-001, Suppl. Fig. 1).

Regarding *TP53* mutations, two out of three patients remained stable after the end of the trial. Only in one *TP53*-mutated patient, treatment failure was observed, leading to premature study exit. Interestingly, during Len treatment, the VAF of this *TP53* mutation dropped from 23 to 4%. Seventeen months after leaving the trial, the *TP53* mutant clone re-expanded (40% VAF) and the disease transformed into AML, ultimately causing death of the patient. Of note, no clinical data was available from the end of the study regarding additional treatments received and clinical parameters that might have influenced the expansion of this *TP53* mutant clone.

In the Pcb group, four patients (66.7%) analyzed in the long-term remained stable, while two cases showed clonal expansion. Expansion of a *TP53* mutant clone was observed (with a VAF increase from 29% at baseline to 42% in the last follow-up). Moreover, the acquisition of a *JAK2* mutation, as well as the expansion of a preexisting mutation in *MPL*, were observed in a Pcb patient after the end of the study.

DISCUSSION

In this study, we showed that low doses of Len during only 2y of treatment in non-TD del(5q) MDS patients, reduced the mutational burden of *TP53* and *SF3B1*, and did not increase the risk of AML transformation.

In our cohort, *SF3B1* and *TP53* were the most frequently mutated genes, with a similar incidence for *TP53* (21.3%) and slightly higher in *SF3B1* (25.5%), as compared to previous cohorts of del(5q) MDS^{12,17,18}. Both mutations have been reported as poor prognostic factors in del(5q) MDS, but scarce data are available on their effect on response to Len and their evolution in patients treated with Len. We found that similar molecular responses were achieved in the Len group, regardless of the presence of mutations in *TP53* or *SF3B1*. Specifically, a significant reduction of mutation VAF was observed in the Len group, but not in the Pcb. This finding is in accordance with the decrease of major mutations after Len treatment reported in both del(5q) and non-del(5q) MDS patients^{35,36}. In non-del(5q) MDS patients, Chesnais et al. found that some additional mutations remained stable or displayed a relative increase under treatment in responders³⁵, similar to what we observed in *DNMT3A* mutations in patients who achieved CyR (Supplementary Fig. 2). This observation could potentially be explained by a selective effect on Len on del(5q) clones, versus its effect on non-clonal hematopoietic cells³⁷ or CHIP-associated alterations (like *DNMT3A* mutations)^{38,39}.

The long-term follow-up data confirmed that Len administration was safe in our cohort, as near 90% of the treated patients remained stable or even decreased the mutated allele burden, confirming molecular responses induced by Len treatment, conversely to what occurred in Pcb patients. Remarkably, no mutations were found with the 65-gene panel used in eight patients in the Len group at baseline, and all of them remained unmutated until the last follow-up, several years after the end of the trial. Furthermore, only a 10% of patients showed an increased VAF in *DNMT3A* mutations, which, in most cases, could be related to a CHIP process. In fact, only one *DNMT3A* mutated patient showed a reduction in this mutation VAF after Len treatment. By contrast, approximately a 30% of patients in the Pcb arm showed clonal expansion, with acquisition of a *JAK2* mutation and expansion of *MPL* and *TP53* mutations, all these alterations related to poor prognosis within the MDS and AML patients^{40,41}.

The presence of a *TP53* mutation was associated with a lower response rate to Len, as only 40% of *TP53* mutated patients achieved ER and 60% CyR, in agreement with previously published data²⁷. The administration of Len in patients with del(5q) MDS carrying *TP53* mutations is still controversial. An expansion of *TP53* mutations under Len treatment has been described. Indeed, there is a concern about Len promoting exacerbated AML progression in patients with these mutations^{27,28,42} and, for this reason, *TP53* must be

carefully studied in patients with del(5q) MDS before being treated with Len⁴³. To date, scarce data regarding sequential samples before and after Len treatment have been evaluated (only in 2 patients)²⁷. Here, we evaluated the mutational dynamics of *TP53* in several time points under Len treatment. In our cohort, the mutational burden of *TP53* decrease with Len, and only a small proportion of patients showed disease progression years after treatment. In fact, 20% of patients treated with Len and showing *TP53* mutations developed AML, an incidence similar to that of the Pcb group, and similar to the 23.2% observed in the MDS-004 clinical trial²² (Supplementary Table 1). Moreover, not only patients with *TP53* mutations at baseline were evaluated regarding disease progression, but also patients showing these alterations at any time in the trial. Interestingly, two patients showed newly-acquired *TP53* mutations (with 5% VAF) several months after Len failure, both of them maintaining RBC-TI and with leukemia free survival times beyond 40 months after treatment discontinuation (Supplementary Fig. 1). According to this finding, small *TP53* clones are known to appear frequently transiently in del(5q) MDS²⁷.

Few data regarding long-term follow-up of del(5q) MDS patients treated with Len and carrying *TP53* mutations are available, and the expansion of a clone with an acquired *TP53* mutation after losing response to Len is infrequent³⁶. It should be noted that the poor prognosis traditionally associated with *TP53* mutations in MDS has recently being linked to “multiple hits” or “biallelic alterations” in *TP53*, which may occur by either the presence of two mutations on distinct alleles or loss of heterozygosity, mainly due to del(17p)^{5,6,10}. In that sense, none of the patients in our cohort showed biallelic *TP53* affection, which may explain the relatively favorable prognosis observed in our *TP53* mutated cases. Therefore, this study opens a new possibility of making Len the standard treatment in all del(5q) MDS patients without RBC-TD. Additional studies are needed to evaluate the safety of Len administration in larger cohorts of del(5q) MDS patients with *TP53* mutations after being treated with Len.

In conclusion, the clonal dynamics of mutations generally decrease or remained stable after treatment with Len in non-TD MDS with del(5q). Moreover, treatment with Len does not appear to promote expansion of preexisting clones or accelerate AML progression, especially in *TP53*-mutated cases. Our study provides evidence that the early administration of low doses of Len in del(5q) MDS patients without RBC-TD is an effective and safe therapeutic approach that could improve disease symptoms by prolonging time until RBC transfusion requirements without accelerating disease progression. It remains to be demonstrated whether early reduction of the del(5q) clone, with or without other mutations, can delay disease progression.

SUPPLEMENTARY MATERIALS

The following supporting information can be found in the **Appendix 3** of the Doctoral Dissertation; **Supplementary Table S1**: Response ratios and AML progression comparison in patients from the MDS-004 and SintraRev clinical trials; **Supplementary Figure S1**: Clonal dynamics of patients with mutations in *DNMT3A* in the Len arm; **Supplementary Figure S2**: Acquisition of *TP53* mutations >10 months after the start of Len administration in 2 patients from the treatment (Len) arm.

AUTHOR CONTRIBUTIONS

MDC conceptualized the study and design research. STC and AVR performed the research and analyzed the data. STC and CP undertook the methodology. FLC, LF, BX, AR, JSG, RC, BS, JAHR, ST, TB, AGB, KG, PF, MDC provided patient samples and clinical data. STC, MDC, JMHR, PF wrote the paper. MDC, JMHR contributed to the scientific discussion, data interpretation and paper revision. MDC supervised the study and acquired the funding. All authors have read and agreed to the current version of the manuscript.

DECLARATION OF INTERESTS

MDC reports receiving consulting fees from BMS, NOVARTIS, BLUEPRINT MEDICINES, GSK, AGIOS, HEMAVAN, SYROS, KEROS and CURIS; honoraria for lectures, presentations, speaking bureaus, manuscript writing or educational events from BMS, NOVARTIS and KEROS. RC reports receiving support for attending meetings/travel from NOVARTIS, ABBVIE and ASTRAZENECA; and her participation on Data Safety Monitoring Board or Advisory Board from ASTRAZENECA and NOVARTIS. JAHR reports grants or contracts from JANSSEN and SANOFI; consulting fees from JANSSEN, ABBVIE, ASTRAZENECA, BEIGENE, LILLY and MENARINI STEMLINE; Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from JANSSEN, ROCHE, ABBVIE, BEIGENE, BMS/CELGENE, AMGEN, TAKEDA, ASTRAZENECA, SANOFI and LILLY; support for attending meetings and/or travel from JANSSEN, ABBVIE, AMGEN, ASTRAZENECA, BEIGENE and NOVARTIS, support for attending meetings and/or travel from JANSSEN, ABBVIE, AMGEN, ASTRAZENECA, BEIGENE, NOVARTIS; participating on a Data Safety Monitoring Board or Advisory Board from: JANSSEN, ROCHE, ABBVIE, BEIGENE, BMS/CELGENE, AMGEN, TAKEDA, ASTRAZENECA, SANOFI and LILLY. KG reports Grants or contracts from BMS; Payment or honoraria for lectures, presentations, speakers' bureaus, manuscript writing or educational events from BMS and ABBVIE, participation on a Data Safety Monitoring Board or Advisory Board from BMS, ABBVIE and JAZZ. All other authors declare no competing interests.

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Capítulo 3

Implicaciones biológicas de la genética en los SMD con alto riesgo de transformación leucémica y en la guía de decisiones clínicas

*Evaluación de los eventos biológicos causados por la adquisición de
mutaciones somáticas en un modelo de transformación leucémica in
vitro: de SMD a LMA.*

MUTACIONES EN *STAG2* Y *NRAS* COOPERAN EN LA TRANSFORMACIÓN DEL SMD A LMA REDUCIENDO LA VIABILIDAD CELULAR E INDUCIENDO RESISTENCIA A LAS TERAPIAS BASADAS EN VENETOCLAX

Introducción

Los SMD son enfermedades clonales heterogéneas que presentan un riesgo variable de progresión a LMA. La adquisición secuencial de mutaciones somáticas se ha identificado como un factor crucial para la transformación leucémica de los SMD. En este sentido, las mutaciones en genes del complejo cohesinas se han relacionado con estadios tempranos de la enfermedad. Por el contrario, alteraciones en genes implicados en vías de señalización se han descrito en etapas de alto riesgo y progresión del SMD. Sin embargo, no está claro cuál es el mecanismo por el que estas mutaciones son necesarias para la progresión a LMA. El objetivo de este trabajo fue evaluar qué eventos biológicos causados por la adquisición secuencial de mutaciones somáticas, son críticos para la transformación de un SMD en una LMA.

Metodología

Se generó un modelo de transformación in vitro con células SKM-1, derivadas de un paciente con LMA con mutaciones de pérdida de función en *STAG2*. Se restauró la expresión proteica de *STAG2* mediante la utilización de vectores lentivirales. Además, se introdujo la mutación G12D en el gen *NRAS* mediante recombinación homóloga a través de la tecnología CRISPR-Cas9. Se generaron cuatro líneas diferentes: sin mutaciones en *STAG2* ni *NRAS* (doble wild-type), con alteración de *STAG2*, con mutaciones en *NRAS* y con *STAG2* y *NRAS* mutados (doble mutante). Se llevaron a cabo estudios de proliferación, ciclo celular y apoptosis en las cuatro líneas generadas. También, se evaluó el perfil mutacional y de expresión mediante NGS.

Resultados

Las células con mutaciones en *STAG2* mostraron un incremento de la apoptosis y una proliferación reducida, en comparación con las líneas sin alteraciones en *STAG2*. Acorde a estos resultados, se observó una expresión reducida de proteínas antiapoptóticas MCL1 y BCL-XL en las células con *STAG2* mutado. Por el contrario, las líneas con alteraciones en *NRAS* mostraron una infraexpresión de la proteína antiapoptótica BCL2. Además, las mutaciones en *NRAS* se asociaron con resistencia a tratamientos basados en venetoclax. El perfil de expresión génica mediante secuenciación de ARN mostró que las alteraciones en *STAG2* se asociaron con una desregulación de la reorganización del citoesqueleto durante la mitosis, así como

defectos en la segregación de las cromátidas hermanas. Sin embargo, las alteraciones en *NRAS* se correlacionaron con alteraciones en las vías de señalización Pi3K y MYC.

Conclusiones

Estos resultados muestran que las mutaciones en *STAG2* alteran la viabilidad celular, mientras que las mutaciones en *NRAS* se asocian con una desregulación de vías de señalización especializadas y resistencia a tratamientos. El análisis de este modelo de transformación sugiere que los pacientes con SMD en estadios tempranos de la enfermedad (genes en complejo cohesinas), podrían beneficiarse de terapias basadas en venetoclax, mientras que etapas más avanzadas de la enfermedad (con mutaciones en la vía de RAS) podrían presentar resistencia a estos tratamientos. Anticipar el tratamiento en pacientes con SMD en estadios iniciales y con riesgo de transformación leucémica, podría ser beneficioso para prevenir la aparición de resistencias a tratamientos.

Original article

STAG2 AND NRAS MUTATIONS COOPERATE IN THE TRANSFORMATION OF MDS TO AML BY REDUCING CELL VIABILITY AND INDUCING RESISTANCE TO VENETOCLAX-BASED THERAPIES

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ABSTRACT

Myelodysplastic syndromes (MDS) are heterogeneous clonal disorders with variable risk of leukemic transformation. Recently, sequential acquisition of somatic mutations has been identified as a key player to the MDS transformation into acute myeloblastic leukemia (AML), but it is still unclear how all these mutations are necessary for disease progression. To evaluate whether critical biological events caused by the acquisition of somatic mutations might determine AML transformation, an *in vitro* model with mutations in *STAG2*, *NRAS* and co-occurrence of both *STAG2* and *NRAS* alterations in the SKM1 cell line was carried out. *STAG2*-deficient cells exhibited increased apoptosis and reduced cell proliferation, while the *NRAS_G12D* mutation favored resistance to venetoclax. In addition, the co-occurrence of *STAG2* and *NRAS* mutations synergistically reduced cell viability and induced resistance to venetoclax-based therapies. Expression profile analysis by RNA-seq revealed that chromatin segregation and C-MYC and Pi3K pathways were deregulated in the double-mutant clone (*STAG2*^{mut}, *NRAS*^{mut}). In summary, the presence of *STAG2* and *NRAS* mutations leads to a treatment-resistant phenotype. These results suggest that early-stage MDS patients showing *STAG2* mutations could benefit from the approved and new therapies of MDS, while patients in more advanced stages may show resistance to these therapies.

INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal disorders characterized by genetic instability and ineffective hematopoiesis¹⁻³. Clinical outcome of patients is highly heterogeneous. Approximately, 30% of patients progress to secondary acute myeloblastic leukemia (sAML), which may occur in a few months or several years from MDS diagnosis⁴.

The risk of sAML progression is related to several features described in the International Prognostic Scoring System (IPSS) and in the later revisions by improving cytogenetics score (IPSS-R) and including information about somatic mutations (IPSS-M)⁵⁻⁷. Indeed, next-generation sequencing (NGS) have demonstrated to play an important role in the identification of variants involved in the prognosis of MDS patients in the last 10 years^{3,7}.

The transformation of MDS into sAML have been extensively studied. However, a deeper understanding of the mechanisms involved in this process, is still needed. The accumulation of somatic mutations has been described as one of the main reasons of MDS progression, making sAML associated to genomic instability. Hence, clonal evolution of MDS giving rise to sAML has been widely studied⁸⁻¹². In this regard, the acquisition of mutations in genes related to DNA methylation, splicing factors or the cohesin complex, has been described in the early stages of the disease⁸. By contrast, alterations in signaling genes or transcription factors, are related to advance disease stages, suggesting their role in MDS progression^{9,12}. Specifically, the presence of mutations in RAS pathways genes, such as *NRAS*, have been found to be enriched in sAML^{8,12}. In addition, as long as 15-20% of patients with sAML with prior diagnosis of MDS, showed co-operation between genes involved in the cohesin and RAS pathways¹². The presence of a high concurrency among cohesin-complex and RAS-pathway mutations in sAML suggests that correlations between these mutations might have functional interactions which dictate disease progression.

Advances in the identification of sequential mutations involved in AML transformation have been described recently. However, the treatment of progressive disease remains a major challenge and, in fact, few therapeutic options are approved for the therapy of MDS. In low-risk patients, treatment with lenalidomide in patients with del(5q)^{13,14} remains as the only targeted therapy. In high-risk patients, the use of hypomethylating agents (such as azacytidine – AZA) or chemotherapy regimens – similar to those used in AML – still remains as the standard therapy^{13,15}. Currently, drug combination with venetoclax (VEN), an inhibitor of BCL2 used in AML, are being evaluated as a promising therapeutic approach in high risk MDS¹⁶⁻¹⁸. Therefore, the identification of somatic mutations driving treatment resistances, should be further investigated.

The aim of this study was to analyze the molecular mechanisms by which *STAG2* and *NRAS* mutations cooperate in the transformation of MDS cells into a leukemic

phenotype, favoring the progression to sAML, as well as to determine whether these mutations might play a role in treatment resistances.

METHODOLOGY

Cell line, culture conditions and drugs

The human AML-derived cell line SKM-1 was donated by Mills K Lab, at Queen's University Belfast. SKM-1 cells were cultured in RPM1 1640 medium (Life Technologies) supplemented with 20% inactivated Fetal Bovine Serum (FBS), 1% glutaMAX and 1% penicillin/streptomycin antibiotics (all from Life Technologies). SKM-1 cells were incubated at 37°C in a 5% CO₂ atmosphere. The presence of mycoplasma was recurrently tested with the MycoAlert Kit (Lonza) and only mycoplasma-free cells were used in all the experiments.

5-Azacytidine (A2385) and cytarabine (PHR1787) were purchased from Sigma and venetoclax (ABT-199) was obtained from LC Laboratories. Compounds were diluted with Dimethyl Sulfoxide (DMSO) from Sigma and final concentrations were obtained with serial media dilutions.

Generation of cell models

Cell lines with the somatic mutation G12D in *NRAS* were generated by Homology-directed repair (HDR) using CRISPR/Cas9 technology and following manufacturer instructions (IDT). First, a guide RNA for G12D mutation was design following instructions of the webs <http://crispr.mit.edu/> and <https://eu.idtdna.com>, as previously reported¹⁹. Then, CRISPR/Cas9 was carried out by the codelivery of an Ultramer single-stranded oligodeoxynucleotide (ssODN) and a CRISPR/Cas9 ribonucleoprotein (RNP) complex in the SKM-1 cell line using the Neon Transfection System (ThermoFisher). Single cell clones were expanded and screened for the presence of the *NRAS*_G12D somatic mutation by NGS²⁰.

The restoration of STAG2 protein expression was performed by lentiviral overexpression as previously described²¹. Briefly, lentiviral transduction was carried out by co-transfection of the vsvg and pPax plasmids, together with a plasmid containing the full sequence of the wild type *STAG2* gene, a GFP reporter gene and puromycin-resistance (Supplementary Figure S1). After 24h of culture, the supernatant of cells contained lentivirus, which were used to transduce the myeloid cell line SKM-1. After lentiviral infection, single cell population were generated and expanded. For STAG2-wild type cells screening, total protein was extracted to analyze the restoration of STAG2 protein expression by western blot.

Functional analyses

MTT viability and growth assays

Cell viability and proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich). Cells were counted and seeded in 96-well plates at a density of 1.5×10^4 cells/well for up to 96h in proliferation experiments or 72 and 120h when treated by different drugs. At the time of the analysis, MTT was added and cells were incubated for 2-4h at 37°C, 5%CO₂. Subsequently, a 1:2 solution of SDS-HCl was added and plates were maintained in agitation for at least 4 hours, RT. Absorbance was read on an Infinite® F500 Tecan plate reader (Tecan) at 570nm.

Apoptosis and cell cycle analysis

Apoptosis was measured by flow cytometry. Cells were stained with annexin V-Dy-634 (Immunostep) following manufacturer's instructions: 3×10^5 cells were labeled with 100 ul of a solution containing annexin V and propidium iodide (IP).

The distribution of cells within the cycle phases was also analyzed by flow cytometry. DNA content was measured by IP labelling after cell permeabilization with ethanol.

DNA and RNA extraction

DNA and RNA were obtained using AllPrep DNA/RNA Mini Kit (Qiagen, Germany) according to manufacturer's standard protocol. The concentration of the extracted DNA and RNA was assessed by Qubit 2.0 Fluorometer system (Life Technologies, Carlsbad, CA, USA) and the integrity was analyzed by using a TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA) and a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) by measuring absorbance ratio at 260/230 and 260/280 nm. RNA quality was also assessed using Bioanalyzer 2100 (Agilent Technologies) and Integrity Number (RIN) was assessed to ensure an optimal quality for RNA sequencing.

Protein extraction and western blotting

Subcellular fractionation for protein extraction was performed using the Subcellular Protein Fractionation Kit (ThermoFisher Scientific) following manufacturer's instructions. For whole cell-lysates, cells were washed with PBS and lysed in ice-cold lysis buffer (140 mmol/l NaCl, 50 mmol/l EDTA, 10% glycerol, 1% Nonidet P-40, 20 mmol/l TrisHCl pH 7) containing both protease inhibitors (cOmplete™, Roche) and phosphatase inhibitors (PhosSTOP™, Roche).

Thirty µg of protein were separated by 7-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The following primary antibodies were used in the western blot experiments: anti-PI3K (rabbit; ab278545, Abcam), anti-phospho-c-RAF (rabbit; 9241T, CellSignaling), anti-STAG2 (rabbit; 4239, CellSignaling), anti-β-actin (rabbit; 8457, CellSignaling), anti-β-tubulina (rabbit, 2146, CellSignaling), anti-BCL2 (rabbit, 4223, CellSignaling), anti-BCLXL (rabbit, 2764, CellSignaling) and anti-MCL1 (rabbit, 5453, CellSignaling). Horseradish peroxidase-linked anti-rabbit antibody (7074, Cell Signaling) was used as secondary antibody at 1:5000 dilution. Antibody signal was detected using ECLTM Western Blotting Detection Reagents (RPN2209, GE Healthcare). Level expression of analyzed proteins was calculated by testing the ratios of each protein in relation to the loading control (β-actin) using the ImageJ software.

Next-Generation Sequencing

DNA samples underwent targeted-deep sequencing using a previously validated in-house custom capture-enrichment panel (*SureSelect XT HS Target Enrichment System*, Agilent Technologies, Santa Clara, CA, USA), including 92 genes related to the pathogenesis of myeloid malignancies as previously described¹⁹. Sequencing libraries were prepared according to manufacturer's instructions, using specific barcodes for each cell-line sample, multiplexed and sequenced on Illumina MiSeq sequencer. Variant alignment, calling, annotation and analyses were assessed as previously described.

RNA libraries for sequencing were generated using the TruSeq Stranded mRNA library prep kit (Illumina) from a total amount of 200 ng. Sequencing was carried out in Nextseq500 sequencer. Raw fastq files were first quality filtered using fastp (v0.23.2). Then, processed fastq files were aligned to the human genome (hg19) using STAR aligner (v2.7.8a). FeatureCounts (v1.50) was used to summarize the read counts across the genes. Differential expression analysis between experimental groups was performed using DESeq2 (v2.11.40.6) and plotted with ggplot'heatmap.2 function. Significant differential expression was determined at FDR<0.1. Gene set enrichment analysis (GSEA) was performed on a matrix of normalized counts using the GSEA desktop application v4.2.3. Our data was aligned with Hallmark Signatures (h.all.v2022.1), Reactome (reactome.v2022.1) and KEGG (kegg.v2022.1) gene sets using 1000 permutations.

ddPCR

Gene expression of target genes in SKM-1-edited cell lines was assessed by droplet digital PCR (ddPCR) using Bio-Rad equipments and reagents, following manufacturer's instructions.

RESULTS

Cells with coexisting mutations in *STAG2* and *NRAS* exhibit a markedly abnormal morphology and undifferentiated phenotype

To characterize the biological implications of individual and co-occurrent mutations in *STAG2* and *NRAS*, we generated an *in vitro* model of MDS progression by CRISPR-Cas9 and lentiviral overexpression. For that purpose, we used the SKM-1 derived cell line, which harbors a loss-of-function mutation in *STAG2*, while showing a wild-type *NRAS* gene. SKM-1 parental cells underwent homology direct repair by simultaneous delivery of RNP complexes and a guide RNA with the G12D mutation in *NRAS*. Then, both SKM-1 parental cells and *NRAS*-edited cells, were transduced to stably express functional *STAG2* protein, with >60% efficiency (Figure 1; Supplementary Figure S2a,b). Single-cell FACS sorted clones were expanded and screened for the presence of *NRAS* G12D mutation and the expression of *STAG2* protein (Supplementary Figure S2c). In total, we generated three to six different single-cell clones of the following conditions: *STAG2*^{WT}-*NRAS*^{WT} (double-wild type), *STAG2*^{MUT}-*NRAS*^{WT} (*STAG2*-mutated cell line), *STAG2*^{WT}-*NRAS*^{MUT} (*NRAS*-mutated cell line) and *STAG2*^{MUT}-*NRAS*^{MUT} (double-mutant cell line). NGS of all generated clones revealed that no off-target indels were found in any of the edited clones (Supplementary Table S1). Additionally, further validation of the functionality of the *NRAS*_G12D mutation insertion was assessed by Pi3K protein quantification (Supplementary Figure S2d), which resulted overexpressed in both clones with the G12D mutation (*NRAS*-mutated and double mutant cell lines).

Morphological analyses of all edited cell lines revealed that the double-mutant SKM-1 cells (*STAG2*^{MUT}-*NRAS*^{MUT}) were markedly enlarged compared to the other 3 conditions (Figure 1b). Indeed, Giemsa staining showed that these atypical cells were abnormally multinucleated and showed a monoblast phenotype, suggesting a defect in mitosis as well as in cell differentiation. By contrast, the double wild type SKM-1 cell line (*STAG2*^{WT}-*NRAS*^{WT}) showed a phenotype more likely differentiated monocyte, instead of monoblast. To note, the *NRAS*-mutated clone (*STAG2*^{WT}-*NRAS*^{MUT}) showed a differential morphology with respect to the rest of generated cell lines. Specifically, these cells have fine granulation and small DNA deposits (in purple) in the cytoplasm (Figure 1b). Moreover, *STAG2*-mutated cell line showed a high proportion of apoptotic cells, compared to the other SKM-1-edited clones.

Regarding phenotypic characterization by flow cytometry, all cell lines showed expression of undifferentiated biomarkers, regardless of the mutational status of *STAG2* and *NRAS*. To note, both cell lines with *NRAS* mutations (*NRAS*-mutated and the double-mutant) showed a higher expression of differentiated markers by flow cytometry (HLA-DR^{low}, CD117^{low}, CD11b^{high} or CD13^{high}), compared to the *NRAS*-wild type SKM-1 cell lines (*STAG2*-mutated and the double-wild type). Lymphoid markers, such as CD56 and CD7, was

expressed in all edited SKM-1 cells indicating a highly aberrant phenotype (Supplementary Figure S3).

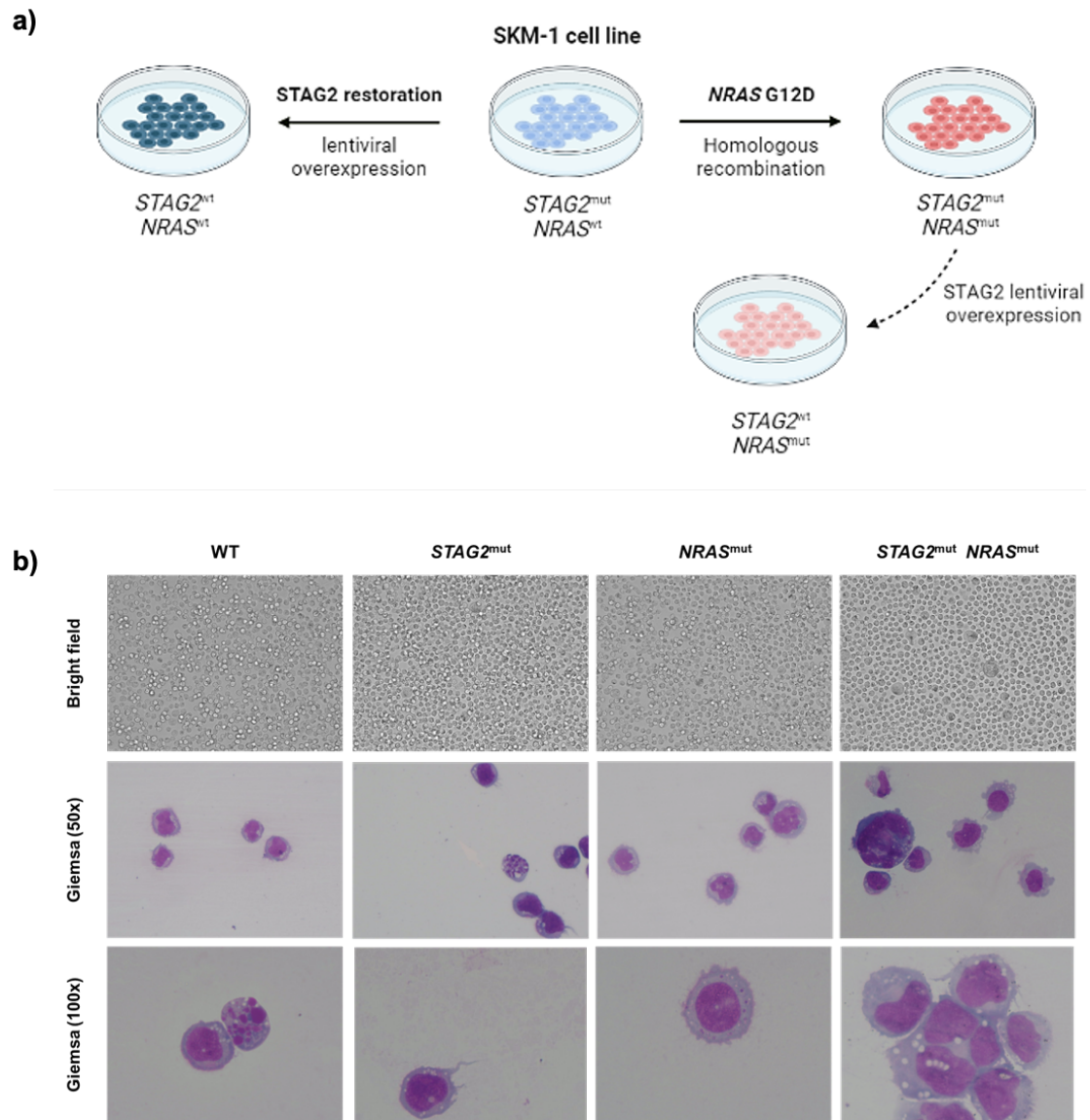


Figure 1: In vitro generation of SKM-1 cell model of AML transformation. a) The SKM1-parental cell line harbors a loss of function-STAG2 alteration. To restore STAG2 protein expression, SKM-1 cell line was transduced with lentiviral vectors containing a plasmid with a STAG2-wild type sequence. NRAS_G12D mutation was introduced in SKM1 cells by CRISPR-Cas9 homology repair; b) Bright field and Giemsa stained representative images of SKM1-edited cell lines.

Cells with *STAG2* mutation showed an increased apoptosis and reduced proliferation

In order to characterize these edited cell lines, we analyzed apoptosis, proliferation and cell cycle in all SKM-1-mutant cells generated (Figure 2).

Apoptosis analysis revealed a higher proportion of apoptotic cells in the double-mutant cell line ($p=0.03$) and *STAG2*-mutated clones ($p=0.08$), compared to the *NRAS*-mutated cells (Figure 2a). To further assess the impaired apoptosis in these cell lines, we next evaluated the expression of pro-apoptotic and anti-apoptotic proteins. No differences were found in the level expression of pro-apoptotic proteins between *STAG2*-mutated and *NRAS*-mutated SKM1 cell lines (data not shown). By contrast, a reduced expression of the anti-apoptotic proteins MCL1 and BCL-XL was observed in the *STAG2*-mutated clones, compared to the *NRAS*-mutated cell lines. Conversely, the expression of BCL-2 was underexpressed in both cell lines with *NRAS* mutations (*NRAS*-mutated and the double-mutant clones) (Figure 3).

MTT and growth assays revealed a similar proliferation ratio between the double-wild type and the *NRAS*-mutated clones (*STAG2*^{WT}-*NRAS*^{MUT}). By contrast, cell lines with altered *STAG2* (*STAG2*-mutated and the double-mutant) showed a reduced proliferation compared to the *NRAS*-mutated cells (Figure 2b).

Regarding cell cycle analysis, no differences were found between *STAG2*-mutated and *NRAS*-mutated clones, showing a similar proportion of cells in all cycle phases. However, a higher proportion of cells in phase G0/G1 were observed in the double-mutant (*STAG2*^{MUT}-*NRAS*^{MUT}) compared to *NRAS*-mutated clones, as well as a markedly reduction in the number of cells in both phase S and G2/M (Figure 2c). Otherwise, a higher proportion of double-wild type cells in phase G0/G1 were found, compared to the *NRAS*-mutated cell line.

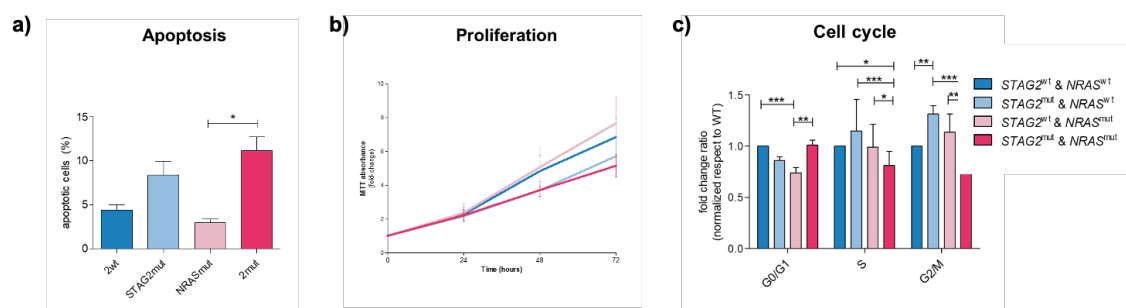


Figure 2: Biological characterization of SKM1-edited cell lines. a) Apoptosis quantification in SKM1-edited cell lines by flow cytometry; b) Cell-growth analysis in SKM1-edited cell lines at four time points every 24 hours; c) Cell cycle analysis in generated cell lines and quantified by flow cytometry.

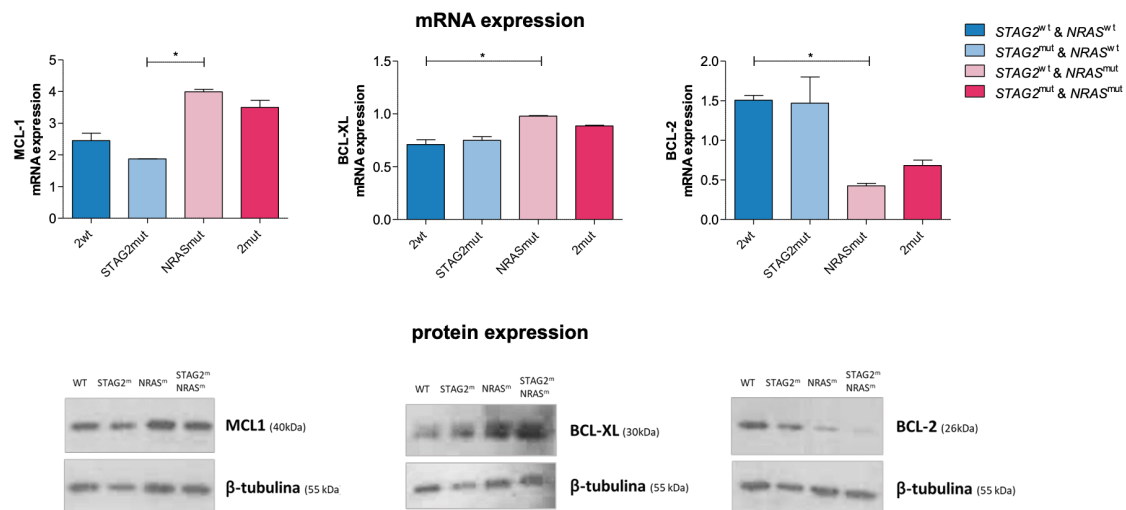


Figure 3: Level expression of anti-apoptotic proteins MCL1, BCL-XL and BCL-2. a) mRNA expression levels of anti-apoptotic proteins analyzed by ddPCR; b) Western blots images of MCL1, BCL-XL and BCL-2 proteins expression.

Dysregulated signaling pathways are enriched in the double-mutant skm1 clones, combining all the altered gene sets caused by individual mutations

To further assess the molecular mechanisms underlying *STAG2* and *NRAS* alterations, transcriptomic analyses of the modified SKM-1 cell lines with and without mutations in both genes by RNA-seq were carried out. The double-wild type SKM-1 cell line for *STAG2* and *NRAS*, was used as a control. Compared to WT clones a total of 1, 50 and 57 transcripts were significantly dysregulated (FDR<0.05) in *STAG2*^{mut}, *NRAS*^{mut} and double-mutant (*STAG2*^{mut}-*NRAS*^{mut}) cell lines, respectively. Gene-set enrichment analyses (GSEA) revealed altered expression of several biological pathways in all clones. Thus, the *STAG2*-mutant cells showed upregulation of gene sets related to spindle/cytoskeleton reorganization in mitosis, chromatid segregation and G2/M cell cycle checkpoint. Otherwise, *NRAS*-mutated clones showed a dysregulation in splicing, immune system activation and signaling pathways (Figure 4). Specifically, macrophage activation and neutrophil degranulation were upregulated in *NRAS*^{mut}-SKM1, as well as the Pi3K-AKT-mTOR and MYC pathways. All of these gene sets, including those related with spindle reorganization and chromatid segregation, were also upregulated in the double-mutant with mutations in both *STAG2* and *NRAS* (Supplementary Figure S4).

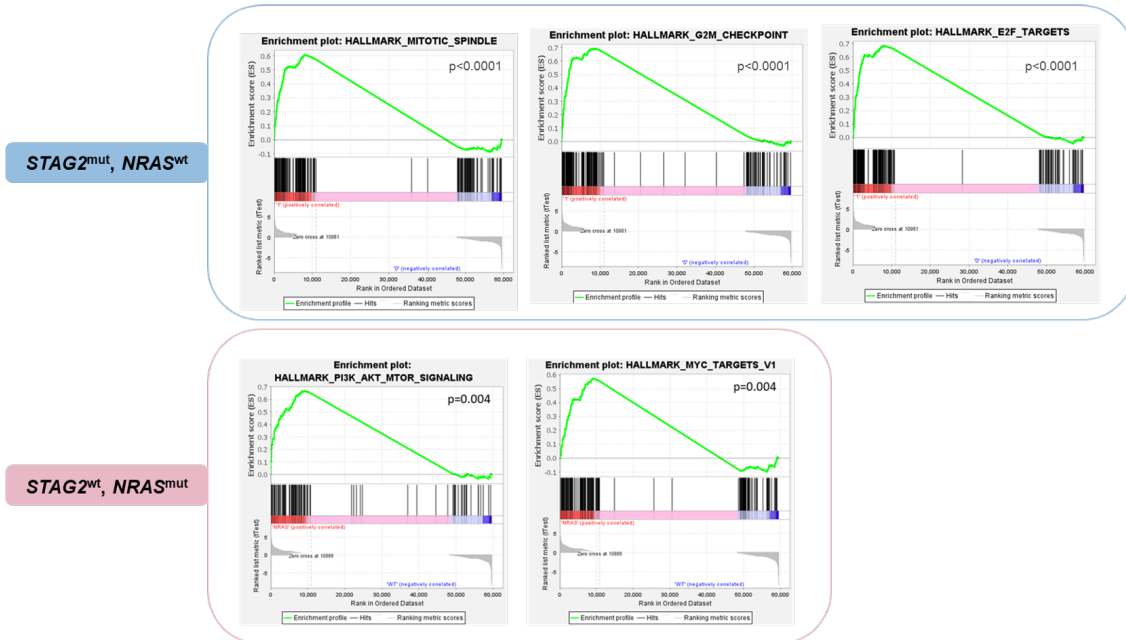


Figure 4: GSEA analysis of deregulated gene sets in *STAG2*-mutated and *NRAS*-mutated SKM1-edited cell lines.

Cells with *STAG2* mutations were sensitive to drug combinations, while *NRAS* mutations conferred resistance to venetoclax-based therapies

We next evaluated whether *STAG2* and/or *NRAS* mutations might have an impact on drug responses in these cell lines harboring high risk and disease progression alterations. For that purpose, we tested the responses of all generated cell lines to AZA and AraC and no differences were found between all cell lines after 72h or 120h of treatment with these drugs (Supplementary Figure S5). Furthermore, we tested the response to these drugs in combination with VEN, as emerging potential therapies for high risk MDS and AML-MR. Remarkably, both clones with *NRAS* mutation (the *NRAS*-mutated SKM-1 and the double mutant cell line) showed resistance to treatment, with consistently higher drug combination index (CI) values compared to *NRAS*-wild type cell lines after 72 hours of treatment (detailed CI can be found in Supplementary Table S2), revealing a synergistic effect of the drug combination notably observed at low doses of the drugs (Figure 5).

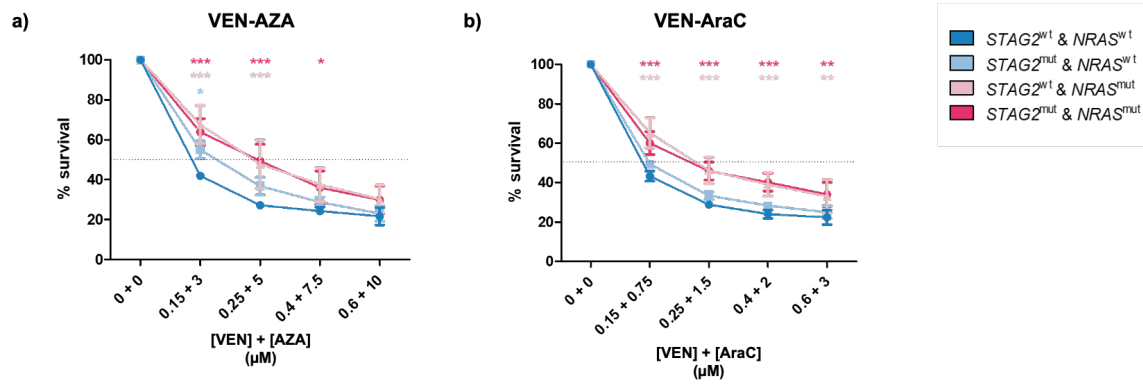


Figure 5: Response of SKM1-edited cell lines to drug combinations. a) Survival in all-edited cell lines after 72h of treatment with venetoclax in combination with azacytidine (VEN-AZA); b) Survival in SKM1-generated cell lines in response to the combination of venetoclax+cytarabine (VEN-AraC) after 72 hours of treatment.

DISCUSSION

MDS are disorders with variable risk of leukemic transformation and the presence of somatic mutations is associated with disease progression. However, the mechanisms are poorly understood. This research confirmed the presence of relevant biological abnormalities in cells displaying mutations in *NRAS* in addition to *STAG2* alterations. These mutations cooperate to increase apoptosis and confer resistance to current therapies.

Somatic mutations have been largely related to biological dysregulation, altering from the most basic cell functions to the most complex cellular processes. In our MDS transformation model, we found that cells with *STAG2* mutations showed alterations in the basis of cell functionality, such as cell death. The apoptosis has been extensively reported to operate as a key physiological mechanism with a dual role in cancer. On the one hand, prevents cell population expansion by removing potentially harmful cells. On the other hand, promotes cell survival and resistance to therapy by limiting tumor cells death²². In this sense, *NRAS* mutations have been associated with increased resistance to apoptosis in several research, both by the hyperactivation of MAPK and PI3K pathways, and the mitigation of reactive oxygen species^{23,24}. Moreover, *NRAS* was also associated with NF-KB constitutive activation, which correlated with resistance to apoptosis in AML cells²⁵, accordingly to our findings (Figure 2a). By contrast, a significant higher proportion of apoptotic *STAG2*-mutated cells were found, as well as infraexpression of anti-apoptotic proteins MCL1 and BCL-XL (Figure 2a; Figure 3). Unfortunately, there are no studies evaluating the role of *STAG2*-loss of function mutations in apoptosis and this is, to our knowledge, the first research describing enhanced apoptosis in cells with truncated *STAG2* alterations. Whether this fact is a normal process to maintain body homeostasis and, therefore, an antitumor immune response, or not, should be further investigated.

In addition to apoptosis, other basics functions, such as cell proliferation and cell cycle were explored. The cohesin subunit *STAG2* plays a role in the cohesion of the sister chromatids following DNA replication during cell division. It has been reported that *STAG2* is involved in the early prophase, once DNA is replicated and the cell enters in G2/M phase²⁶⁻²⁹. Inactivating mutations in *STAG2* are frequently observed in cancer, leading to replication fork stalling and collapse, and therefore interfering in cell proliferation^{26,27,30}. In this regard, we found that SKM-1 cells with *STAG2* mutations were associated to a reduced proliferation, but with a similar cell cycle than the *NRAS*-mutated cells. The collapse of the mitotic spindle that prevent cell division during the early prophase, could explain that both *STAG2*-altered cell lines in our model (*STAG2*-mutated and the double-mutant) showed a reduced viability compared to the *NRAS*-mutated cell line, without affecting the cell's progression through the cell cycle stages. According to our model (Figure 2b), previous studies also reported that *STAG2* loss of function in cells was associated to a reduced proliferation²⁸. Our results were

also consistent with previous studies suggesting a G2/M phase arrest in cells with loss of function mutations in *STAG2*^{28,31,32} (Figure 2c).

NRAS mutations have been largely related to higher proliferation ratios³³⁻³⁶. However, several publications revealed that oncogenic levels of *NRAS* are important determinants of leukemic behavior and the *NRAS_G12D* mutation have a bimodal effect on hematopoietic stem cells^{36,37}. Indeed, intermediate levels of *NRAS* induced maximal proliferation, while higher levels led to attenuated proliferation and maximal self-renewal capacity³⁶. In relation to that, differentiation markers expression showed that clones with the *NRAS*-G12D mutation were slightly more differentiated than edited-cell lines without this alteration (Supplementary Figure S3), which were consistent with prior published data³⁶⁻³⁸.

Expression profile analysis by RNA-seq in our MDS transformation model, revealed a sequential alteration of the biological processes according to the leukemogenesis stages. Indeed, the *STAG2*-mutated cell line showed deregulated pathways related to chromatid segregation and failure in spindle reorganization in mitosis (Figure 4), which have been extensively reported^{27,30}. This data was consistent with previous findings, in which the evaluation of the transcriptional profile in an AML model with chronic *STAG2* protein loss, showed altered chromatin structure and deregulated transcription³⁹. In this regard, the alteration of processes involved in cell division is consistent with the reduction of hematopoietic cells count and cytopenias observed in MDS patients. Otherwise, *NRAS*-mutated clones showed an upregulation in Pi3K and MYC pathways (Figure 4), which was also reported by Shi et al. in a murine model with cooperating mutations in *DNMT3A* and *NRAS*⁴⁰. They also showed that up-regulation of the MYC transcription factor contributed to promote leukemogenesis⁴⁰. In the present study all these deregulated pathways were synergistically upregulated in the double-mutant with *STAG2* and *NRAS* mutations (Supplementary Figure S4), which might potentially represent a leukemic stage of the disease, following MDS progression.

The acquisition of somatic mutations is not only involved in disease progression, but also in resistance to therapies. In this regard, we tested different drugs used in MDS, including novel combinations with venetoclax, to evaluate if the presence of mutations in *STAG2*, *NRAS* or their combination, could show differential responses to therapies. Regarding current treatments in high risk MDS patients, no differences were observed between all edited cell lines when treated with an hypomethylating (AZA) or a chemotherapeutic agent (AraC) (Supplementary Figure S5). However, current clinical trials are evaluating the efficacy of drug combinations with venetoclax, showing significant clinical benefits in AML patients^{16,18,41}. Consequently, the safety and efficacy of VEN+AZA and VEN+AraC are being studied in phase 1/2 trials in high risk MDS patients^{17,18,41,42}. In this study, we evaluated the response of SKM1-edited cell lines to these combinations and,

remarkably, we found that *NRAS*-mutated clones (single and double-mutants) were resistant to these combinations. However, the “*NRAS*-wild type”, were sensitive to VEN-AZA and VEN-AraC (Figure 5 a,b). In addition, transcriptomic studies showed that the anti-apoptotic protein BCL-2 was significantly under-expressed in both cell lines with *NRAS* mutations, which is the main target of venetoclax (Figure 3). For this reason, cells with *NRAS* mutations were resistant to these drug combinations. These results remarked the importance of evaluating the mutational profile of patients to understand whether a therapy would be effective or not. Moreover, this data suggests that treating patients prior to a high risk MDS could be useful in prevent leukemic transformation, even when they only have low-risk mutations (such as *STAG2*) but with an increasing risk of malignant progression.

In summary, in our MDS transformation model, we found that cells with *STAG2* mutations showed alterations in cell viability. Conversely, when acquiring *NRAS* mutations, cells showed dysregulation of specialized pathways, as observed by expression profile analysis. Biological events caused individually by *STAG2* and *NRAS* mutations, coexist in a cell with both alterations, leading to a reduced proliferation, increased apoptosis and treatment-resistant phenotype. This study highlights the importance of studying in depth the role of mutations in cell biology, as well as their involvement in drug resistance. Moreover, these results might suggest that early-stage MDS patients with increasing risk of transformation, might benefit from VEN-AZA and/or VEN-AraC therapies, while more advanced stages of the disease may show resistance to these therapies. Therefore, anticipating treatment in early-stage MDS patients with increasing risk of leukemic transformation could be beneficial for patient’s management and might prevent the acquisition of resistances to therapies. Further research on the role of somatic mutations in treatment resistances is needed.

SUPPLEMENTARY MATERIALS

The following supporting information can be found in the **Appendix 4** of the Doctoral Dissertation; **Supplementary Table S1**: Somatic mutations detected in the four generated cell lines by NGS with an in-house custom capture-enrichment panel; **Supplementary Table S2**: Combination index of VEN-AZA (a) and VEN-AraC (b) drug combinations in all four edited-SKM1 cell lines; **Supplementary Figure S1**: Lentiviral plasmid containing *STAG2*-wild type gene (green) and GFP (blue) and puromycin resistant (red) as gene reporters; **Supplementary Figure S2**: Generation efficiency and validation of edited-SKM1 cell lines with *STAG2* restoration and *NRAS*-G12D mutation; **Supplementary Figure S3**: Differentiation markers expression in all SKM-1-edited cell lines analyzed by flow cytometry; **Supplementary Figure S4**: RNA-seq GSEA analysis of deregulated gene sets in the double-mutant SKM1-edited cell lines (*STAG2*^{mut}-*NRAS*^{mut}); **Supplementary Figure S5**: Drug response to AZA and AraC in SKM1-generated cell lines.

AUTHOR CONTRIBUTIONS

MDC and STC conceptualized the study and design research. STC and CPC performed the research and analyzed the data. STC, CP and MMI undertook the methodology. IT, RA, DS, ARS, SS and SM provided technical support for methodology or data analysis. STC, MDR wrote the paper. MDC, JMHR contributed to the scientific discussion, data interpretation and paper revision. MDC supervised the study and acquired the funding. All authors have read and agreed to the current version of the manuscript.

DECLARATION OF INTERESTS

All other authors declare no competing interests related to this project.

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DISCUSIÓN

El estudio genético de los SMD durante la última década ha permitido profundizar en el conocimiento de las bases moleculares de la patología. Sin embargo, la implementación de la medicina personalizada sigue suponiendo un reto en los SMD, a pesar de que resulta fundamental para el correcto manejo terapéutico de los pacientes, dada la alta heterogeneidad de la enfermedad y las escasas opciones de tratamientos eficaces en estos pacientes. El presente trabajo de tesis doctoral ha profundizado en el papel que juegan las mutaciones somáticas en el impacto clínico de los SMD a través de la NGS, tendiendo a la individualización del manejo de los pacientes, tanto en la clasificación y pronóstico de los mismos, como en la búsqueda del tratamiento óptimo para cada uno de ellos.

En este sentido, el primer trabajo de esta tesis doctoral se centra en el estudio del perfil mutacional de un grupo de pacientes con SMD y características citogenéticas homogéneas. Las anomalías del cariotipo tienen un papel importante en el pronóstico de la enfermedad, algo que se ha reportado en numerosas ocasiones y que se hizo evidente con la clasificación del riesgo citogenético publicada en el año 2012⁹⁰. En este estudio, la +8 fue una de las alteraciones citogenéticas más frecuentes en los SMD que se clasificó con un riesgo intermedio⁹⁰. Los pacientes que presentan +8 aislada muestran una gran diversidad de manifestaciones clínicas y, a pesar de ser un grupo muy específico con una alteración citogenética común, no se ha encontrado aún la causa de esta variabilidad¹⁰⁵⁻¹⁰⁸. En 2018, se describió por primera vez un subgrupo de pacientes con SMD y +8 aislada con características mieloproliferativas y una mayor incidencia de mutaciones somáticas en *STAG2*, *EZH2* o *ASXL1* que presentó menor SG que aquellos pacientes que no presentaban estas características¹⁰⁹. Sin embargo, no se determinó si mutaciones específicas pueden actuar como marcadores pronósticos de la enfermedad. En el presente trabajo, un análisis en profundidad del perfil mutacional de los SMD con +8 aislada ha permitido identificar qué alteraciones actúan como marcadores predictores de peor pronóstico de los pacientes, siendo éstos independientes del número de mutaciones observadas. De esta manera, el primer capítulo de esta tesis doctoral ha permitido conocer la implicación de las mutaciones somáticas en la mejora de la clasificación pronóstica de pacientes con riesgo intermedio, en concreto, con +8 aislada.

Los pacientes con SMD y +8 aislada mostraron una incidencia de mutaciones en genes como *STAG2* o *RUNX1* mayor que en el resto de subgrupos de SMD. Si bien, antes de este estudio no se había publicado una descripción exhaustiva del perfil mutacional de estos pacientes, sí se había asociado una mayor incidencia de mutaciones en *STAG2* con la presencia de +8, acorde a lo observado en el capítulo 1 de esta tesis¹⁰⁹. Además, la presencia de mutaciones en estos genes (*STAG2* y *RUNX1*), así como en *SRSF2*, se asoció con un menor

tiempo hasta la progresión a LMA y una SG más corta, distinguiéndose de esta manera dos grupos de riesgo dentro de los pacientes con SMD y +8 aislada. Los pacientes con mutaciones en alguno de estos 3 genes presentaron, además, un mayor número de mutaciones por paciente. Previamente, se han publicado datos que demuestran que un mayor número de mutaciones (3 o más) se asocia significativamente con una menor SLP y SG^{7,123,173}. Sin embargo, los análisis multivariantes demostraron que no era la presencia de un mayor número de mutaciones, sino las alteraciones específicamente localizadas en los genes *STAG2*, *SRSF2* o *RUNX1* las que se asociaron con un peor pronóstico de estos pacientes. Estos resultados demuestran que, si bien los intentos de refinar las clasificaciones pronósticas con la incorporación de datos moleculares son un paso imprescindible para el avance del manejo de los SMD, es preciso evaluar la presencia de mutaciones en contextos específicos. En este sentido, *STAG2* se incluye en el IPSS-M como un gen residual en la escala pronóstica y, sin embargo, este trabajo demuestra que es un predictor independiente de transformación a LMA cuando se encuentra concomitante con la +8 como única alteración citogenética en el SMD²⁷. En la misma dirección, recientemente se ha descrito que las mutaciones en *SF3B1*, tradicionalmente asociadas a buen pronóstico, reducen la SG en pacientes con SMD y del(5q), en comparación con aquellos enfermos que no presentan mutaciones en este gen^{103,104}. Estos estudios, junto con los resultados de nuestro primer capítulo de esta tesis, demuestran que es fundamental conocer qué tipo de alteraciones presentan los pacientes en el contexto específico de su SMD, ya que un gen mutado podría tener distinta implicación pronóstica en los diferentes subtipos de SMD^{103,104}.

El estudio en conjunto de pacientes con +8 aislada permitió reclasificar como SMD de alto riesgo a un 23,5% y 52,4% de los pacientes clasificados de riesgo bajo e intermedio, respectivamente, según el IPSS-R. De igual forma, se refinó el pronóstico en un total de 37,5% de pacientes clasificados por el IPSS-M con un riesgo intermedio-bajo. La aplicación de estos resultados a la clínica permitiría redirigir a los pacientes con SMD y +8 aislada a una terapia más adaptada al riesgo de la enfermedad. El único tratamiento curativo de los SMD es el TPH, siendo candidatos a esta terapia solamente los pacientes de alto riesgo y buen estado físico. Al clasificar a los pacientes con +8 aislada como riesgo intermedio, en muchos casos los profesionales médicos retrasarán la indicación del TPH en los pacientes candidatos hasta que tuvieran evidencias clínicas de progresión o empeoramiento del SMD. Por tanto, la identificación temprana mediante la identificación de un perfil mutacional de riesgo de los pacientes candidatos a TPH o, en su defecto, candidatos a HMA, sería fundamental para tratarlos de manera precoz y poder mejorar su pronóstico adverso. De hecho, se ha demostrado que una intervención temprana de los SMD de alto riesgo mediante TPH o HMA,

se correlaciona con mejores resultados de supervivencia global y de respuesta a tratamientos^{59,60,174}. Por ello, el tratamiento precoz de estos pacientes de mal pronóstico con +8 aislada podría asociarse con una mayor supervivencia global en este grupo de pacientes, anticipándonos al deterioro de los enfermos e incrementando las posibilidades de obtener una respuesta al mismo.

Los tratamientos disponibles para tratar los síntomas derivados de la anemia en los pacientes con SMD de menor riesgo suelen ser más efectivos en conseguir el objetivo de mejora del bienestar del paciente (calidad de vida) cuando se utilizan antes de la aparición de sintomatología severa, como la dependencia transfusional (DT)^{43,93,175,176}. Esto se debe a que las tasas de respuesta al tratamiento son inferiores cuando los pacientes se encuentran en régimen transfusional periódico, especialmente cuando presentan altos requerimientos transfusionales^{38,96}. El tratamiento precoz en los SMD de bajo riesgo cuando el paciente presenta anemia, pero no DT, consigue mejorar la sintomatología que motiva el tratamiento (astenia, disnea o fatiga derivadas de la anemia). Además, ha demostrado alargar el tiempo hasta la DT, un evento que acontece en la mayoría de los pacientes y siempre se asocia a una supervivencia más corta de los enfermos, aparición y/o deterioro de comorbilidades preexistentes y disminución de la calidad de vida^{43,177-180}. En este sentido, el ensayo clínico SintraRev ha demostrado recientemente que el tratamiento precoz con dosis bajas de lenalidomida (Lena) es eficaz para retrasar la aparición de la DT en pacientes con SMD de bajo riesgo con del(5q), anemia sintomática y sin DT⁴³. Además, incrementa los niveles de hemoglobina, mejora las respuestas citogenéticas obtenidas en comparación a cuando se utiliza en gases más avanzadas y, todo ello, podría no sólo mejorar la calidad de vida, sino incluso alargar la supervivencia de estos pacientes^{43,93}. En resumen, todos estos motivos han llevado a una adopción cada vez mayor de este enfoque terapéutico precoz.

Aunque las terapias farmacológicas, como la Lena en pacientes con SMD y delección del cromosoma 5q, son modificadores de la enfermedad, algunos médicos pueden ser reticentes a su utilización en ausencia de sintomatología grave. Esto puede deberse a la incertidumbre sobre el impacto que estos tratamientos puedan tener en la expansión de clones malignos, lo que podría acelerar la progresión de la enfermedad. Para abordar esta inquietud, es fundamental evaluar la evolución clonal de las mutaciones durante el tratamiento en ensayos clínicos. Esto permitiría aplicar estas terapias de manera precoz y preservar la seguridad del paciente. Al monitorizar de manera precisa la respuesta clonal, se pueden tomar decisiones informadas sobre el uso de estas terapias y minimizar el riesgo de progresión de la enfermedad.

En este sentido, el capítulo 2 de la tesis doctoral estudia la dinámica de las mutaciones durante el tratamiento de un grupo homogéneo de pacientes con una terapia estándar: los SMD con del(5q) y la Lena. Los pacientes analizados pertenecían al ensayo clínico SintraRev que demostró el beneficio asociado al tratamiento precoz con Lena en pacientes sin DT, viviendo más tiempo sin necesidad de transfusiones, con una mediana de tiempo hasta la DT no alcanzada en el grupo de tratamiento (a una mediana de seguimiento de 5 años), frente a 11,6 meses en el grupo comparador sin tratamiento, lo cual tiene una repercusión directa en la calidad de vida de los mismos, acorde a lo que se ha reportado previamente^{43,176}. El estudio del perfil mutacional en los pacientes del ensayo clínico SintraRev llevado a cabo en esta tesis demuestra que la administración precoz con Lena es, además de efectiva, segura a nivel molecular, pues no sólo no favorece la expansión de los clones aberrantes, sino que además reduce la carga mutacional de las alteraciones observadas en los pacientes al inicio del estudio. Aunque ya se ha reportado previamente la disminución de la VAF en las mutaciones somáticas de pacientes con y sin del(5q) tratados con Lena^{181,182}, resultó especialmente interesante la reducción de la VAF en las mutaciones localizadas en *SF3B1* y *TP53* bajo tratamiento con Lena (que fueron, además, los genes más frecuentemente mutados en estos enfermos) ya que ambos genes se han asociado con un peor pronóstico. En concreto, las mutaciones en *SF3B1* se han correlacionado tradicionalmente con un buen pronóstico en los SMD de bajo riesgo en ausencia de clones con alta complejidad genética, pero en el contexto específico de SMD con del(5q), recientemente se han asociado con un pronóstico desfavorable, en comparación con los pacientes con del(5q) que no presentan alteraciones en este gen^{103,104}. En el presente estudio, todos los pacientes que mostraron mutaciones en *SF3B1* alcanzaron respuestas eritroides y citogenéticas en una proporción similar al grupo global. Sin embargo, la duración de la respuesta citogenética fue inferior en pacientes con mutaciones en *SF3B1*, en comparación con los que no presentaban estas alteraciones (12 vs 24,6 meses, respectivamente; $p < 0,05$), aunque esto no afectó al tiempo hasta la DT o progresión a LMA, que fue similar en ambos grupos. Por otro lado, los pacientes con mutaciones en *SF3B1* tratados con Lena mostraron una menor incidencia de necesidades transfusionales (37,5%) y un mayor tiempo hasta la DT (mediana de 21,8 meses) en comparación con el grupo placebo – sin tratamiento y con *SF3B1* mutado (100% de incidencia de DT, mediana de 6,3 meses). Además, la administración precoz de Lena redujo a la mitad la incidencia y el tiempo hasta la progresión de LMA en los pacientes con *SF3B1* mutado. Estos resultados indican que, a pesar de que los pacientes con *SF3B1* alcanzan respuestas más cortas, el tratamiento precoz con Lena prolonga de manera eficaz el tiempo hasta la DT y la progresión a LMA. Son necesarios

estudios adicionales para conocer la causa de la pérdida de respuesta temprana en estos pacientes, así como la implicación clínica que pueda tener.

Por otro lado, las mutaciones en *TP53* se han asociado en numerosas ocasiones con mal pronóstico, no sólo en los SMD, sino en el conjunto de las neoplasias hematológicas^{27,46,100,183}. Existe una gran controversia acerca de la administración de Lena en pacientes con del(5q) y mutaciones en *TP53*, ya que varias investigaciones han reportado una baja tasa de respuestas en pacientes con *TP53* mutado, así como la expansión de clones con alteraciones en este gen tras la administración de Lena^{45,46,184}. Jädersten y colaboradores describieron por primera vez este efecto de la Lena sobre *TP53*, basándose en muestras secuenciales analizadas en únicamente 2 pacientes (de un total de 55) en los que se observó un aumento gradual de la expresión proteica de P53⁴⁶. También, en el ensayo clínico de fase 2 MDS-004, donde se estudió la eficacia del tratamiento con Lena en pacientes con SMD, del(5q) y DT, se describió una asociación entre la sobreexpresión proteica de P53 y un mayor riesgo de transformación leucémica¹⁸⁴. En el segundo capítulo de esta tesis doctoral, se evaluó el estado mutacional de *TP53* en diferentes tiempos y de manera secuencial durante el tratamiento con Lena y se observó una disminución de la frecuencia alélica. Únicamente un 20% de pacientes con mutaciones en *TP53* y tratamiento con Lena progresó a LMA tras varios años de tratamiento, un porcentaje similar al observado en el grupo placebo y al de pacientes tratados en el ensayo clínico MDS-004⁹⁸. Además, en dos pacientes se observaron nuevas mutaciones en *TP53* con un VAF del 5% tras la pérdida de respuesta a Lena, pero ninguno de ellos progresó a LMA en más de 40 meses de seguimiento después de la discontinuación del tratamiento. Al igual que en este estudio, da Silva-Coelho y colaboradores reportaron un caso de SMD con del(5q) tratado con Lena en el que se observó una nueva mutación de *TP53* tras la pérdida de respuesta al tratamiento. Este paciente no progresó a LMA en más de 10 años de seguimiento¹⁸¹. En concordancia con estos resultados, se conoce que clones transitorios sin repercusión pronóstica pueden aparecer en los SMD con del(5q)⁴⁶. Por otro lado, el mal pronóstico tradicionalmente asociado a mutaciones en *TP53* en los SMD, recientemente se ha modificado, siendo la presencia de varias mutaciones o la pérdida de heterocigosidad por mutaciones en *TP53* y del(17p), (estado *multihit* o inactivación bialélica), así como VAF superiores al 22%, las que confieren un mal pronóstico a los pacientes^{5,24,100}. Ninguno de los casos incluidos en el ensayo clínico presentó mutaciones en *TP53 multihit* ni inactivación bialélica. Además, en todos ellos la carga mutacional de *TP53* al inicio del estudio fue inferior al 20%, lo que podría explicar la eficacia del tratamiento con Len en estos pacientes. Todos estos datos demuestran que la administración de Lena en pacientes con del(5q) y mutaciones en *TP53* no compromete la seguridad del paciente, ya que no hay estudios sólidos que

demuestren la expansión clonal de estas alteraciones ni su asociación con un mayor riesgo de transformación a LMA debido al tratamiento. Además, las respuestas a tratamiento observadas en estos pacientes, no sólo en el ensayo clínico SintraRev, sino también en los estudios anteriormente expuestos, así como la reducción de la frecuencia alélica observada, podrían indicar que los pacientes con mutaciones en *TP53* también podrían beneficiarse del tratamiento con Lena. Los resultados obtenidos en esta tesis doctoral abren, por tanto, una nueva opción terapéutica en pacientes con del(5q). Sin embargo, sería preciso ampliar y estudiar en profundidad el efecto de Lena en pacientes con inactivación bialélica y/o VAF aumentadas en *TP53* antes de administrar el tratamiento.

Resultó destacable que un bajo porcentaje de mutaciones en los pacientes de SintraRev, mostraron un incremento de la VAF tras la administración de Lena (17,6%). En total, tres mutaciones localizadas en el gen *DNMT3A*, se expandieron durante la administración de Lena. En ninguno de los casos, la respuesta al tratamiento se vio alterada por la persistencia e incremento de estas mutaciones y, además, el 100% de pacientes con alteraciones en *DNMT3A* mostraron tanto respuestas eritroides, como citogenéticas. Este hallazgo es consistente con un estudio previo en pacientes con SMD sin del(5q) que mostraron respuesta al tratamiento con Lena, donde se describió la presencia de mutaciones somáticas que se mantienen estables e, incluso, aumentan la VAF. Todas las mutaciones que aumentaron el VAF bajo tratamiento con Lena, estaban localizadas en *DNMT3A* y *TET2*¹⁸². Estos genes, son, de hecho, los más frecuentemente mutados en individuos con CHIP, en los que se han descrito como factores que aumentan el riesgo de padecer neoplasias mieloides, pero que requieren de eventos adicionales para el desarrollo de estas neoplasias¹⁴¹⁻¹⁴³. Las mutaciones observadas en *DNMT3A* que no se vieron afectadas por el tratamiento podrían, por tanto, tratarse de mutaciones asociadas a CHIP en lugar de clones con alto potencial patogénico, de manera que su persistencia no alteraría la respuesta a Lena ni tampoco favorecería la progresión del paciente. Además, la presencia de respuesta citogenética en los pacientes analizados en SintraRev, junto con la expansión simultánea de las mutaciones en *DNMT3A*, podría indicar que estas alteraciones no estarían presentes en el clon con la del(5q), clon que se redujo a causa del tratamiento con Lena.

A pesar de que una intervención terapéutica temprana puede retrasar la aparición de sintomatología adversa como la DT o prolongar notablemente la SLP, con frecuencia el SMD continúa el curso clínico hasta la progresión de la enfermedad. Las mutaciones somáticas, de nuevo, han demostrado tener una implicación muy relevante en el transcurso de la enfermedad, especialmente en la transformación leucémica. De hecho, numerosos estudios

han reportado que la adquisición secuencial de mutaciones en las células aberrantes de los SMD, son la causa de la progresión de la enfermedad^{12,137,139,151}. Si bien se reconoce la importancia de las mutaciones somáticas en la patogenia de los SMD, aún existe una gran falta de conocimiento acerca del papel que juegan a nivel biológico en la transformación de la enfermedad. Por ello, la generación *in vitro* de modelos de evolución es de gran utilidad para estudiar los procesos de jerarquía clonal y los mecanismos moleculares a través de los cuales un SMD se transforma en una LMA. La traslación de estos resultados a la clínica posibilitaría anticiparse al avance del SMD mediante estrategias terapéuticas dirigidas.

Debido a ello, los pacientes con mayor riesgo de transformación leucémica son difíciles de manejar, pues las únicas opciones terapéuticas disponibles son el TPH – no apto para la mayoría de ellos por edad avanzada y/o comorbilidades asociadas – y los HMA. Alrededor de un 50% de los pacientes acaban mostrando resistencias a estos tratamientos^{185–188} y la escasez de otras opciones de tratamiento hace que, en muchas ocasiones, los pacientes de alto riesgo no tengan una terapia de rescate. El desarrollo de nuevas terapias, así como la identificación de dianas terapéuticas, es fundamental para mejorar el tratamiento de los SMD. La aparición de clones aberrantes durante el transcurso del SMD se ha relacionado en numerosas ocasiones con terapias ineficaces o resistencias a tratamientos. En este sentido, la generación de modelos de evolución *in vitro* resulta una herramienta muy útil para el estudio de las neoplasias hematológicas, ya que permite investigar la implicación del perfil mutacional en los mecanismos de resistencia a tratamientos, así como identificar vulnerabilidades en las células aberrantes de estos pacientes para establecer nuevas dianas terapéuticas^{189–195}.

En el capítulo 3 de este trabajo de tesis doctoral, se ha generado un modelo *in vitro*, utilizando el sistema CRISPR-Cas9 y sobreexpresión génica mediante lentivirus, para recrear la evolución de un SMD a una LMA mediante la adquisición de mutaciones en *STAG2* y *NRAS*. Estudios previos han descrito en numerosas ocasiones que las alteraciones en genes del complejo cohesinas se observan en etapas tempranas del SMD, mientras que las mutaciones en genes implicados en la transducción de señales, como la vía de RAS, se encuentran en un estadio más avanzado de la enfermedad y presentan un alto riesgo de progresión a LMA, por lo que se han asociado con el proceso de transformación leucémica^{33,137,138,140,151,196}. En el modelo *in vitro* presentado en este trabajo, las células con mutaciones de pérdida de función en *STAG2* mostraron una reducción significativa de la proliferación, lo que resulta consistente con publicaciones previas donde asocian las alteraciones en la formación del huso mitótico y segregación de las cromátidas hermanas con mutaciones en *STAG2*^{134,197,198}. Este hecho podría ser responsable de que las células con mutación en *STAG2* se acumulen en la fase G2 del ciclo

celular, siendo la acumulación de células disfuncionales la causa del aumento en la apoptosis observado en estas células. A nivel clínico, el incremento de muerte celular de estas células en MO podría explicar la presencia de citopenias en la SP de estos pacientes^{199,200}. Las mutaciones en *NRAS*, sin embargo, provocan la sobreexpresión proteica de la vía Pi3K, así como la desregulación de la expresión génica de Pi3K y MYC. Estos resultados demuestran el potencial leucémico de estas mutaciones, ya que las alteraciones en esta vía de señalización se han relacionado con progresiones de la enfermedad, así como con procesos leucémicos más agresivos^{196,201}. Además, no sólo en los SMD en transformación, sino también en LMA secundarias y *de novo*, la presencia de mutaciones en *NRAS* se ha relacionado con un peor pronóstico de los pacientes^{137,202,203}. Por tanto, la coexistencia de mutaciones en *STAG2* y *NRAS* llevan a una desregulación de la proliferación, apoptosis y ciclo celular que podría estar relacionado con la hematopoyesis aberrante que presentan los enfermos con SMD, así como al desarrollo de un fenotipo más agresivo por la desregulación de vías de señalización de Pi3K y MYC.

La respuesta a tratamientos estudiada en este modelo de transformación pone de manifiesto que, si bien no existen diferencias en el tratamiento con AZA o Ara-C entre los distintos perfiles moleculares, sí se observó, sin embargo, diferente sensibilidad a la combinación de estos tratamientos con VEN. En la práctica clínica, actualmente los pacientes con SMD de alto riesgo son tratados – en caso de no ser elegibles a TPH – con HMAs o bajas dosis de quimioterapia³⁷. La resistencia a estas terapias ha propiciado el desarrollo de combinaciones con VEN, las cuales han mostrado respuestas prometedoras en pacientes con LMA y, ahora, con SMD de alto riesgo que están siendo estudiadas en ensayos clínicos^{35,71,73–76}. Sin embargo, en el presente trabajo, son precisamente los perfiles moleculares con *NRAS* mutado y, por tanto, de alto riesgo, los que han mostrado una mayor resistencia a las combinaciones con VEN. Estos resultados parecen indicar que las mutaciones en *NRAS*, mediante la infraexpresión de la diana de este fármaco (BCL2), inhiben la acción de VEN y confieren resistencia a las células tratadas. Estos datos fueron consistentes con estudios previos donde se observó una menor sensibilidad a VEN en pacientes con LMA y mutaciones en la vía de RAS^{204–210}. En concreto, varios estudios retrospectivos que analizaron la respuesta a VEN en combinación con HMAs o bajas dosis de citarabina en pacientes con LMA refractaria, describieron las mutaciones en *NRAS* como predictoras de menor SG, sugiriendo que el beneficio de estos tratamientos se limitaría a pacientes sin mutaciones en este gen^{205–208}. Recientemente, las alteraciones en *NRAS* se han asociado también con baja SG en pacientes con LMA secundarias a una neoplasia mieloide²⁰⁹. Asimismo, una investigación reciente mostró una asociación entre la resistencia a VEN+AZA en pacientes con LMA de nuevo

diagnóstico y la presencia de mutaciones en *SRSF2*. Cabe destacar que se reportó una correlación directa entre las mutaciones en genes de *splicing* con una alta incidencia de alteraciones en *NRAS*, sugiriendo que la elección de este tratamiento debe guiarse por el análisis de las mutaciones que presentan los pacientes²¹⁰. Por el contrario, el modelo de transformación presentado en esta tesis mostró que el perfil asociado con un menor riesgo de la enfermedad (sin alteraciones en *NRAS*), muestra sensibilidad a las terapias en combinación con VEN, tratamientos a los que estos pacientes no serían candidatos por el menor riesgo del SMD. Así pues, conocer la sensibilidad a los tratamientos en función del perfil mutacional de los pacientes, permitiría anticipar las terapias más efectivas de manera personalizada y evitar la transformación leucémica de los enfermos. La administración temprana de tratamientos modificadores de la enfermedad en los SMD de bajo riesgo sería beneficiosa en pacientes cuyo perfil mutacional estuviera apuntando hacia una posible transformación leucémica; de otra manera, estos pacientes podrían mostrar resistencias a las terapias en el momento de la transformación y no se dispondría de opciones de rescate para ellos.

Los resultados presentados en esta tesis doctoral demuestran que la alta heterogeneidad de los SMD requiere de análisis individualizados para cada paciente pues, como se ha demostrado en el capítulo 1, el papel de las mutaciones somáticas varía según el contexto en que se encuentren. Además, la presencia de ciertas mutaciones puede influir en la elección del tratamiento, por lo que el estudio de la dinámica clonal, como se muestra en el capítulo 2, es imprescindible para no descartar pacientes que podrían beneficiarse de una terapia. Por último, las alteraciones genéticas generan ventajas selectivas en las células malignas y modifican la sensibilidad a los fármacos actuales, lo que se ha demostrado en el capítulo 3, de manera que retrasar la administración de un tratamiento puede hacer que las células adquieran resistencias causadas por la evolución clonal de la enfermedad.

En resumen, la gran variabilidad genética de los SMD dificulta enormemente establecer estándares de tratamiento en los pacientes. Por eso, el futuro de la enfermedad debe mirar hacia el desarrollo de la medicina de precisión y personalizada. Los resultados presentados sugieren que la aplicación de técnicas de secuenciación en contextos específicos de un SMD permite optimizar el manejo clínico de los pacientes, clasificándolos de manera precisa y anticipando el tratamiento a la evolución de la enfermedad, traducándose estos avances en una posible mejora en la calidad de vida y un aumento en la supervivencia global de los pacientes.

CONCLUSIONES

1. La presencia de mutaciones en *STAG2*, *SRSF2* y/o *RUNX1* permite identificar a un subgrupo de pacientes con SMD y +8 aislada que tienen un riesgo de transformación leucémica similar a los pacientes de alto riesgo sin esta alteración citogenética. Por el contrario, la ausencia de estas mutaciones somáticas en pacientes con SMD y +8 como única alteración de cariotipo, muestran una SG similar a los pacientes con SMD de bajo riesgo y sin +8.
2. Las mutaciones en *STAG2*, *SRSF2* y/o *RUNX1* modifican la estratificación del riesgo en los SMD con +8 como única alteración citogenética, y su presencia permite reclasificar al 37,5% de los pacientes de riesgo moderado-bajo en el IPSS-M como alto riesgo.
3. La administración precoz de Lena en los pacientes con SMD de bajo riesgo y del(5q) sin DT incluidos en el ensayo clínico SintraRev, es segura a nivel molecular, ya que no promueve la expansión clonal de las mutaciones somáticas observadas al inicio del tratamiento y, además, reduce su frecuencia de variación alélica.
4. Los pacientes con SMD de bajo riesgo y del(5q) con mutación en *TP53* y sin DT incluidos en el ensayo clínico SintraRev que fueron tratados con Lena, muestran respuestas eritroides y citogenéticas duraderas al tratamiento y no presentan mayor riesgo de progresión a LMA tras la administración de Lena que los pacientes que no presentan mutaciones en este gen.
5. Los pacientes con SMD, del(5q), mutaciones en *SF3B1*, anemia, sin DT y tratados de manera precoz con Lena, alcanzan respuestas de menor duración que los pacientes que no presentan alteraciones en *SF3B1*. A pesar de ello, prolonga eficazmente el tiempo hasta la DT y la progresión de la enfermedad y reduce la incidencia de LMA.
6. Las alteraciones de pérdida de función en *STAG2* juegan un papel fundamental en el desarrollo del fenotipo tumoral, desregulando procesos básicos para la supervivencia celular, como la apoptosis y la proliferación celular.
7. Las mutaciones en *NRAS* confieren resistencia a VEN en combinación con AZA o AraC debido a la infraexpresión proteica de BCL2 en las células mutadas. Además, estas mutaciones son responsables de alteraciones en las vías de señalización Pi3K y MYC, y contribuyen a la transformación leucémica de la célula.

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ANEXOS Y MATERIAL SUPLEMENTARIO

ANEXO I

Bases de datos

Para la realización de esta tesis doctoral, se han utilizado tres bases de datos de pacientes: dos de ellas generadas en el Hospital Universitario de Salamanca con la colaboración de centros nacionales e internacionales adicionales; y una tercera obtenida de publicaciones en revistas de alto impacto.

Capítulo 1:

Cohorte principal del estudio: Se utilizó una base de datos en la que se incluyeron 2602 pacientes con SMD primario, diagnosticados entre 1997 y 2022. Esta base de datos pertenece al Hospital Universitario de Salamanca y su creación ha sido posible gracias a la colaboración de distintos centros nacionales. Para el estudio concreto que se ha llevado a cabo en esta tesis doctoral, el Hospital Clínic de Barcelona, el Hospital La Fe de Valencia, el Instituto Catalán de Oncología (ICO)-Hospital Duran i Reynals y el Instituto de Investigación Contra la Leucemia Josep Carreras, han colaborado estrechamente con el envío de muestras y cesión de datos clínicos de los pacientes.

Se analizó la incidencia de + 8 en los pacientes con SMD incluidos en la base de datos. En total, 124 pacientes presentaron +8 (4,7%). Se seleccionaron los pacientes con presencia de +8 aislada y se llevó a cabo la secuenciación masiva de las muestras disponibles.

Cohorte de validación: se hizo uso de una cohorte externa de 2494 pacientes con SMD, procedente del Grupo de Trabajo Internacional de Pronóstico de los SMD. Un total de 94 pacientes mostraron +8 aislada. Los datos de la cohorte se extrajeron de: Bernard, E.; Tuechler, H.; Greenberg, P.L.; Hasserjian, R.P.; Arango Ossa, J.E.; Nannya, Y.; Devlin, S.M.; Creignou, M.; Pinel, P.; Monnier, L.; et al. Molecular International Prognostic Scoring System for Myelodysplastic Syndromes. NEJM Evid. 2022, 1.

Capítulo 2:

Se utilizó la base de datos del ensayo clínico de fase III SintraRev. Un total de 62 pacientes fueron incluidos en el estudio entre febrero de 2010 y febrero de 2018, procedentes de 22 centros hospitalarios de España, Francia y Alemania. De ellos, 61 pacientes se aleatorizaron en una proporción 2:1 en una rama de tratamiento con Len y un grupo placebo control. Para el presente trabajo, se utilizaron los datos clínicos y moleculares de 51 pacientes en los que se disponían muestras analizadas mediante NGS.

ANEXO II

Material Suplementario del Capítulo 1

Article

Mutational Profile Enables the Identification of a High-Risk Subgroup in Myelodysplastic Syndromes with Isolated Trisomy 8

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- **Figure S1:** (a) Time to AML progression in newly described isolated +8 MDS subgroups compared to non-trisomy 8 MDS patients with normal and complex karyotypes; (b) overall survival in newly defined risk subgroups in isolated +8 MDS patients compared to non-trisomy 8 MDS patients with normal and complex karyotypes.
- **Figure S2:** (a) Multivariate analysis of patients with isolated +8 depending on number of mutations per patient (≥ 4) in AML progression; (b) multivariate

analysis of OS in isolated +8 MDS patients depending on the number of mutations per patient; (c) multivariate analysis of OS in isolated +8 MDS considering 10% of BM blasts in patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1*; (d) multivariate analysis of patients with isolated +8 MDS regarding percentage of BM blasts (>10%) and the number of mutations per patient (≥ 4).

- **Figure S3:** (a) Percentage of patients with isolated +8 MDS with and without mutations in *STAG2*, *SRSF2* and/or *RUNX1* stratified by the IPSS-R; (b) percentage of patients with MDS and isolated +8 with and without mutations in *STAG2*, *SRSF2* and/or *RUNX1* stratified by the IPSS-M.
- **Figure S4:** Mutational pattern of mutated genes in more than 5% of isolated +8 MDS patients of the International Working Group for the Prognosis of MDS.
- **Figure S5:** (a) Time to AML progression in newly defined prognostic subgroups in isolated +8 compared to the control MDS without +8 stratified by IPSS-R risk subgroups; (b) OS of isolated +8 MDS patients reclassified in prognostic groups according to mutational profile within IPSS-R MDS control groups; (c) multivariate analysis for OS in isolated +8 patients of the validation cohort.
- **Figure S6:** (a) Percentage of patients from the validation cohort stratified within IPSS-R and IPSS-M categories reconsidered as low risk and high risk based on mutational profile; (b) percentage of patients classified by the IPSS-R and IPSS-M in patients with isolated +8 MDS depending on mutational status of *STAG2*, *SRSF2* and *RUNX1*.

Table S1: Genes included in the in-house custom capture-enrichment panel used in the study.

Gene	Exons	NM
ABL1	4,5,6,7,8,9,10	NM_005157
ACD	12	NM_022914.2
ANKRD26	5'UTR,1	NM_014915
	1 (c.-113-c.-134)	NM_014915
ARID2	Full	NM_152641.3
ASXL1	1,2,3,4,5,6,7,8,9,10,11,12,13,14	NM_015338.5
	5	NM_001164603.1
ATM	5,9,13,28,37,41,42,52,56	NM_000051.3
ATRX	8,9,10,11,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32	NM_000489.3
BCOR	2,3,4,5,6,7,9,10,11,12,13,14,15	NM_017745
	8	NM_001123385.1
BCORL1	1,2,3,4,5,6,7,8,9,10,11,12	NM_021946.4
BRAF	3,10,11,12,13,15	NM_004333
BRCC3	1,4,5, 8	NM_001018055.2
	6,7,10,11	NM_024332.3
CALR	8,9	NM_004343
CBL	2,3,4,5,7,8,9,16	NM_005188.3
CEBPA	Full	ENST00000498907.3
CSF3R	17	NM_156039
	10,18	NM_172313
	14,15,16	NM_000760
CSNK1A1	3,4	NM_001025105; NM_001271741; NM_001892
CTCF	Full	NM_006565
CUX1	15,16,17,18,19,20,21,22,23, 24	NM_001202543
DCK	Full	NM_000788
DDX41	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17	NM_016222
DNAJC21	12	NM_001012339.2
DNMT3A	2,3,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23	NM_022552
	1,2	NM_153759
	4	NM_175630

EED	Full	NM_003797
ELA2/ELANE	Full	NM_001972
EP300	Full	NM_001429
ESCO2	3	NM_001017420.2
ETNK1	3	NM_018638
ETV6	1,2,3,4,5,6,7,8	NM_001987
EZH2	2,3,4,5,6,7,8,9,10,11,12,13, 14,15,16,17,18,19,20	ENST00000483967
FLT3	13,14,15,20	NM_004119.2
GATA1	2	NM_002049
GATA2	Full	NM_032638
GNAS	8,9,10,11,12,13	NM_000516.5
GNB1	6	ENST00000378609.4
HRAS	2,3,4	NM_005343
IDH1	3,4	NM_005896
IDH2	4,6,8	NM_002168
IRF1	2,3,4,5,6,7,8,9,10	NM_002198.2
JAK1	9,10,14,15,17,18	NM_002227
JAK2	12,13,14,15,16,17,19,20,21, 22,23,24,25	NM_004972
JAK3	3,11,13,15,18,19	NM_000215
JARID2	2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18	NM_004973.4
KDM6A	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18, 19,20,21,22,23,24,25,26,27,28,29	NM_021140
	14	NM_001291415
KIT	1,2,5,8,9,10,11,12,13,14,15, 17,18	NM_000222
KMT2A/MLL	1,2,3,4,5,6,7,8,9,10,11,12,13,15,16,17,18,19,20,21,22, 23, 24,25,26,27,28,29,30,31,32, 33,34,35,36	NM_005933
	14	NM_001197104
KRAS	1,2,3,4	NM_004985
LUC7L2	2,7,8	NM_016019
MAP2K1	2,3	NM_002755
MPL	10,12	NM_005373
NCOR2	21,23,35,	NM_001077261

NF1	1,2,3,4,5,6,7,8,9,10,11,12,13,14,16,17,18,19,20,21,22, 23, 24,25,26,27,28	NM_000267
	15	NM_001128147
	31	NM_001042492
NIPBL	7,11,12,21,33	NM_015384
NPM1	2,3,4,5,6,7,8,9,10,11	NM_002520.6
NRAS	1,2,3,4,5	NM_002524
PAX5	1,2,3,4,5,6,7,8,9,10	
PDGFRA	12,13,14,15,16,17,18	NM_006206
PDGFRB	Full	NM_002609
PDS5b	10,20,31	NM_015032.3
PHF6	1,2,3,4,5,6,7,8,9	NM_032335
	10	NM_001015877
	9	NM_032458
PIGA	2,3,4,5,6	NM_002641
PPM1D	6	NM_003620
PRPF8	6,26,27,28,29,30,31	NM_006445
PTPN11	3,4,7,8,12,13	NM_002834
	11	NM_080601
RAD21	2,3,4,5,6,7,8,9,10,11,12,13, 14	NM_006265
RBBP6	Full	NM_006910
RUNX1	1,2,3,4,5,6,7,8,9	NM_001754
	1,5	NM_001122607
SAMD9	2	NM_001193307
SAMD9L	4,5	NM_152703: c.2640C>A
SBDS	Full	NM_016038.2
SETBP1	4	NM_015559
SF1	Full	NM_201995
SF3A1	13,5,4,3	NM_005877
SF3B1	10,11,12,13,14,15,16,17,18, 19,20,21	NM_012433
SH2B3(LNK)	2,3,4,5,6,7,8	NM_005475
SMC1A	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21, 22, 23,24,25	NM_006306
	2	NM_001281463
SMC3	10,13,19,23,25,28	NM_005445
SRP72	6,1	NM_006947.3
SRSF2	1,2	NM_003016

STAG1	Full	NM_005862
STAG2	Full	NM_006603
	32	NM_001042749
SUZ12	14,15,intron15,16	NM_015355
TERC	1	NR_001566
TERT	15,14,13,12,11,10,9,8,7,6,5,4,3,2,1	NM_001193376
TET2	2,3,4,5,6,7,8,9,10,11	NM_001127208
	3	NM_017628
TP53	1,2,3,4,5,6,7,8,9,10,11	NM_000546
	10	NM_001276696
	10	NM_001276695
TYK2	Full	NM_003331
U2AF1	2,6,7	NM_006758
	6	NM_001025204
U2AF2	Full	NM_007279
WAS	Full	NM_000377
WT1	Full	NM_000378
	8	NM_001198552
XPO1	15,16,18	NM_003400
ZRSR2	Full	NM_005089

Table S2: Somatic mutations found in patients with MDS and isolated +8

Patient ID	Gene	Chr	Start	End	Ref	Alt	Total depth	Reference depth	Alt depth	FREQ	Function	Exonic consequence	AA change & Transcript
1	BRAF	chr7	140783116	140783116	G	T	423	372	51	12.06	exonic	nonsynonymous SNV	NM_004333:c.C1219A:p.P407T
1	RUNX1	chr21	34880580	34880580	C	T	126	100	26	20.63	exonic	nonsynonymous SNV	NM_001001890:c.G404A:p.R135K
4	IDH2	chr15	90088606	90088606	C	T	211	199	12	5.69	exonic	nonsynonymous SNV	NM_001290114:c.G125A:p.R42K
4	U2AF1	chr21	43104346	43104346	G	T	467	322	137	29.34	exonic	nonsynonymous SNV	NM_001025203:c.C101A:p.S34Y
5	SF3B1	chr2	197402110	197402110	T	C	576	385	190	32.99	exonic	nonsynonymous SNV	NM_012433:c.A2098G:p.K700E
5	TET2	chr4	105275668	105275668	A	T	469	333	136	29	exonic	stopgain	NM_001127208:c.A518T:p.K1720X
5	SMC3	chr10	110582633	110582643	TAAAATG GAGG	-	367	316	51	13.9	exonic	frameshift deletion	NM_005445:c.795_804del:p.D265fs
5	ASXL1	chr20	32435574	32435574	C	A	371	199	172	46.36	exonic	nonsynonymous SNV	NM_015338:c.C2862A:p.D954E
5	CTUX1	chr7	102201990	102201990	C	-	271	252	19	7.01	exonic	frameshift deletion	NM_001202543:c.2726delC:p.T909fs
6	NRAS	chr1	114716123	114716123	C	T	466	446	20	4.29	exonic	nonsynonymous SNV	NM_002524:c.G38A:p.G13D
6	TET2	chr4	105234774	105234774	C	T	420	241	178	42.38	exonic	stopgain	NM_001127208:c.C832T:p.Q278X
6	EZH2	chr7	148810343	148810343	G	T	578	333	245	42.39	exonic	nonsynonymous SNV	NM_001203249:c.C1851A:p.N617K
6	EZH2	chr7	148810385	148810385	T	G	454	274	180	39.65	exonic	nonsynonymous SNV	NM_001203249:c.A1809C:p.R603S
6	PTPN11	chr12	112450395	112450395	C	T	340	316	24	7.06	exonic	nonsynonymous SNV	NM_002834:c.C215T:p.A72V
6	PHLPP1	chr18	62716129	62716129	C	T	180	93	85	47.22	exonic	nonsynonymous SNV	NM_194449:c.C446T:p.S149L
6	ZRSR2	chrX	15804174	15804174	C	T	224	43	181	80.8	exonic	stopgain	NM_005089:c.C376T:p.R126X
6	STAG2	chrX	124086590	124086590	C	T	334	169	165	49.4	exonic	stopgain	NM_006603:c.C3097T:p.R1033X
8	DNMT3A	chr2	25246660	25246660	-	C	338	272	65	19.23	exonic	frameshift insertion	NM_153759:c.671dupG:p.G224fs
8	SMC3	chr10	110601791	110601793	GAA	-	637	549	90	14.08	exonic	nonframeshift deletion	NM_005445:c.2799_2801del:p.933_934del
9	STAG2	chrX	124086717	124086717	C	G	98	79	19	19.39	exonic	stopgain	NM_006603:c.C3224G:p.S1075X
10	STAG2	chrX	124083521	124083521	A	C	94	82	12	12.77	exonic	nonsynonymous SNV	NM_006603:c.A3025C:p.K1009Q
10	ARID2	chr12	45731297	45731298	AA	-	217	207	10	4.61	exonic	frameshift deletion	NM_152641:c.267_268del:p.L89fs
10	ETV6	chr12	11839157	11839157	T	A	193	170	23	11.92	exonic	nonsynonymous SNV	NM_001987:c.T181A:p.W61R
10	ARID2	chr12	45730054	45730054	A	-	254	225	29	11.42	exonic	frameshift deletion	NM_152641:c.103delA:p.K35fs
10	CTCF	chr16	67621585	67621585	G	T	136	109	27	19.85	exonic	nonsynonymous SNV	NM_001191022:c.G367T:p.D123Y
10	U2AF1	chr21	43104346	43104346	G	T	216	172	43	19.91	exonic	nonsynonymous SNV	NM_001025203:c.C101A:p.S34Y
12	SETBP1	chr18	44951948	44951948	G	A	298	284	14	4.7	exonic	nonsynonymous SNV	NM_015559:c.G2608A:p.G870S

12	KRAS	chr12	25245277	25245277	T	C	316	305	11	3.48	exonic	nonsynonymous SNV	NM_004985:c.A108G;p.I36M
12	EGFR	chr7	55173092	55173092	C	T	167	142	25	14.97	exonic	nonsynonymous SNV	NM_005228:c.C2029T;p.R677C
12	C7orf55- LUC7L2, LUC7L2	chr7	139402152	139402152	C	T	347	288	58	16.71	exonic	stopgain	NM_016019:c.C271T;p.Q91X
12	SRSF2	chr17	76736877	76736877	G	C	308	255	53	17.21	exonic	nonsynonymous SNV	NM_001195427:c.C284G;p.P95R
12	STAG2	chrX	124045347	124045347	C	T	175	145	30	17.14	exonic	stopgain	NM_006603:c.C646T;p.R216X
14	TET2	chr4	105275330	105275330	C	G	246	7	239	97.15	exonic	stopgain	NM_001127208:c.C4820G;p.S1607X
14	NF1	chr17	31230850	31230850	T	A	163	82	81	49.69	exonic	nonsynonymous SNV	NM_000267:c.T3122A;p.M1041K
14	SRSF2	chr17	76736877	76736877	G	T	143	77	66	46.15	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
14	ASXL1	chr20	32434812	32434812	T	G	277	131	146	52.71	exonic	stopgain	NM_015338:c.T2100G;p.Y700X
14	STAG2	chrX	124083530	124083530	C	T	89	44	45	50.56	exonic	stopgain	NM_006603:c.C3034T;p.R1012X
14	STAG2	chrX	124077999	124077999	A	-	71	59	12	16.9	exonic	frameshift deletion	NM_006603:c.T716del;Ap.K906fs
15	SRSF2	chr17	76736877	76736877	G	T	445	274	171	38.43	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
15	SETBP1	chr18	44951948	44951948	G	A	661	457	204	30.86	exonic	nonsynonymous SNV	NM_015559:c.G2608A;p.G870S
15	ASXL1	chr20	32435018	32435021	CTTC	-	721	694	25	3.47	exonic	frameshift deletion	NM_015338:c.2306_2309del;p.T769fs
17	IDH2	chr15	90088702	90088702	C	T	455	435	20	4.4	exonic	nonsynonymous SNV	NM_001290114:c.G29Ap.R10Q
17	NRAS	chr1	114716126	114716126	C	T	540	369	170	31.48	exonic	nonsynonymous SNV	NM_002524:c.G35Ap.G12D
17	SMC3	chr10	110593107	110593107	A	G	205	108	97	47.32	exonic	nonsynonymous SNV	NM_005445:c.A1847G;p.N616S
17	ETV6	chr12	11839280	11839280	T	-	447	267	178	39.73	exonic	frameshift deletion	NM_001987:c.304delT;p.F102fs
17	SRSF2	chr17	76736877	76736877	G	T	215	126	89	41.4	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
17	ASXL1	chr20	32434600	32434622	CACCACT GCCATAG AGAGCG GC	-	66	52	14	21.21	exonic	frameshift deletion	NM_015338:c.1888_1910del;p.H630fs
18	ETV6	chr12	11839280	11839280	T	-	447	267	178	39.73	exonic	frameshift deletion	NM_001987:c.304delT;p.F102fs
18	IDH2	chr15	90088702	90088702	C	T	455	435	20	4.4	exonic	nonsynonymous SNV	NM_001290114:c.G29Ap.R10Q
18	NRAS	chr1	114716126	114716126	C	T	540	369	170	31.48	exonic	nonsynonymous SNV	NM_002524:c.G35Ap.G12D
18	SRSF2	chr17	76736877	76736877	G	T	215	126	89	41.4	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
21	SRSF2	chr17	76736877	76736877	G	A	490	442	48	9.8	exonic	nonsynonymous SNV	NM_001195427:c.C284T;p.P95L
21	STAG2	chrX	124061291	124061291	G	A	425	356	69	16.24	exonic	stopgain	NM_006603:c.G1484A;p.W495X
21	STAG2	chrX	124081529	124081529	G	A	379	358	21	5.54	exonic splicing	NA	NA
22	SETBP1	chr18	44951942	44951942	G	A	816	800	15	1.84	exonic	nonsynonymous SNV	NM_015559:c.G2602A;p.D868N
22	RUNX1	chr21	34880641	34880641	-	GG	748	709	37	4.95	exonic	frameshift insertion	NM_001001890:c.342_343insCC;p.A115fs
22	EZH2	chr7	148810350	148810350	A	T	682	228	454	66.57	exonic	nonsynonymous SNV	NM_001203249:c.T1844A;p.L615Q

22	CBL	chr11	119278529	119278529	G	A	852	577	275	32.28	exonic	nonsynonymous SNV	NM_005188:c.G1247A:p.C416Y
22	PRPF8	chr17	1659995	1659995	C	T	655	451	204	31.15	exonic	nonsynonymous SNV	NM_006445:c.G4792A:p.D1598N
22	STAG2	chrX	124063194	124063194	C	T	222	116	106	47.75	exonic	stopgain	NM_006603:c.C1810T:p.R604X
23	TET2	chr4	105275172	105275173	AG	-	622	405	217	34.89	exonic	frameshift deletion	NM_001127208:c.4662_4663del:p.T1554fs
23	TET2	chr4	105275487	105275487	T	G	650	268	382	58.77	exonic	stopgain	NM_001127208:c.T4977G:p.Y1659X
23	EP300	chr22	41155114	41155114	G	T	469	277	192	40.94	splicing	NA	NA
24	STAG2	chrX	124065947	124065947	G	A	141	124	16	11.35	splicing	NA	NA
24	RUNX1	chr21	34880621	34880621	G	-	402	386	15	3.73	exonic	frameshift deletion	NM_001001890:c.363delC:p.T121fs
24	RUNX1	chr21	34880662	34880662	-	AGCA	385	370	15	3.9	exonic	frameshift insertion	NM_001001890:c.321_322insTGGCT:p.G108fs
24	GATA2	chr3	128481269	128481269	C	T	314	174	140	44.59	exonic	nonsynonymous SNV	NM_001145662:c.G1151A:p.R384Q
24	GATA2	chr3	128485852	128485852	-	AG	310	210	95	30.55	exonic	frameshift insertion	NM_001145662:c.745_746insCT:p.Y249fs
24	EZH2	chr7	148809351	148809351	C	T	595	310	285	47.9	exonic	nonsynonymous SNV	NM_001203249:c.G1901A:p.R634H
24	EZH2	chr7	148810363	148810368	TGTAAT	-	363	224	142	38.8	exonic	nonframeshift deletion	NM_001203249:c.1826_1831del:p.609_611del
24	RUNX1	chr21	34792387	34792387	-	TGGA	502	377	123	24.45	exonic	frameshift insertion	NM_001001890:c.1109_1110insTCCA:p.Q370fs
24	ZRSR2	chrX	15815835	15815836	TC	-	201	13	189	93.56	exonic	frameshift deletion	NM_005089:c.716_717del:p.F239fs
27	PDGFRB	chr5	150132940	150132940	T	C	334	184	149	44.61	exonic	nonsynonymous SNV	NM_002609:c.A937G:p.S313G
27	EZH2	chr7	148829779	148829779	A	C	609	374	235	38.59	exonic	nonsynonymous SNV	NM_001203247:c.T433G:p.F145V
27	PRPF8	chr17	1659995	1659995	C	T	588	369	218	37.07	exonic	nonsynonymous SNV	NM_006445:c.G4792A:p.D1598N
27	STAG2	chrX	124081464	124081464	C	T	776	706	69	8.89	exonic	nonsynonymous SNV	NM_006603:c.C2860T:p.R954C
28	EZH2	chr7	148814964	148814964	C	A	949	694	255	26.87	exonic	nonsynonymous SNV	NM_001203247:c.G1607T:p.C536F
28	EZH2	chr7	148817262	148817262	C	T	739	568	171	23.14	exonic	nonsynonymous SNV	NM_001203249:c.G1328A:p.C443Y
28	ARID2	chr12	45849666	45849666	G	A	713	374	338	47.41	exonic	nonsynonymous SNV	NM_152641:c.G1802A:p.R601Q
28	IDH2	chr15	90088702	90088702	C	T	671	496	175	26.08	exonic	nonsynonymous SNV	NM_001290114:c.G29A:p.R10Q
29	TET2	chr4	105235831	105235831	A	G	468	246	222	47.44	exonic	nonsynonymous SNV	NM_001127208:c.A1889G:p.K630R
29	ASXL1	chr20	32435899	32435899	C	T	381	293	87	22.83	exonic	stopgain	NM_015338:c.C3187T:p.Q1063X
29	EP300	chr22	41129910	41129910	C	T	309	222	87	28.16	exonic	stopgain	NM_001429:c.C1189T:p.R397X
29	ZRSR2	chrX	15820247	15820247	C	T	200	79	121	60.5	exonic	stopgain	NM_005089:c.C868T:p.R290X
29	ASXL1	chr20	32435739	32435739	-	A	386	368	18	4.66	exonic	frameshift insertion	NM_015338:c.3028dupA:p.L1009fs
30	TET2	chr4	105269619	105269619	G	A	657	627	29	4.41	exonic	nonsynonymous SNV	NM_001127208:c.G4054A:p.E1352K
30	DNM3A	chr2	25245257	25245257	C	T	488	294	194	39.75	exonic	nonsynonymous SNV	NM_153759:c.G983A:p.C328Y

30	SF3B1	chr2	197402110	197402110	T	C	772	455	317	41.06	exonic	nonsynonymous SNV	NM_012433:c.A2098G;p.K700E
30	TET2	chr4	105243710	105243710	C	G	643	574	69	10.73	exonic	stopgain	NM_001127208:c.C3735G;p.Y1245X
30	CUX1	chr7	102204518	102204518	A	G	682	362	319	46.77	exonic	nonsynonymous SNV	NM_001202543:c.A3068G;p.Q1023R
32	TET2	chr4	105235977	105235977	A	-	851	475	378	44.26	exonic	frameshift deletion	NM_001127208:c.2035delA;p.T679fs
32	EZH2	chr7	148846512	148846513	CA	-	456	54	402	88.16	exonic	frameshift deletion	NM_001203249:c.203_204del;p.V68fs
32	STAG2	chrX	124061781	124061781	-	A	167	125	43	25.44	exonic	frameshift insertion	NM_006603:c.1546dupA;p.D515fs
32	STAG2	chrX	124086685	124086685	-	AGCCGGG	632	606	26	4.11	exonic	frameshift insertion	NM_006603:c.3192_3193insAGCCGGG;p.S1064fs
33	SF3B1	chr2	197402636	197402636	T	G	989	632	356	36	exonic	nonsynonymous SNV	NM_012433:c.A1997C;p.K666T
35	EZH2	chr7	148817276	148817276	G	C	869	532	336	38.67	exonic	stopgain	NM_001203249:c.C1314G;p.Y438X
35	RUNX1	chr21	34859483	34859483	C	A	685	544	141	20.58	exonic	stopgain	NM_001001890:c.G523T;p.E175X
36	IDH2	chr15	90088702	90088702	C	T	581	333	248	42.69	exonic	nonsynonymous SNV	NM_001290114:c.G29A;p.R10Q
36	CTCF	chr16	67621528	67621528	G	T	813	486	327	40.22	exonic	stopgain	NM_001191022:c.G310T;p.E104X
36	ZRSR2	chrX	15823034	15823037	GTCG	-	354	208	146	41.24	exonic	frameshift deletion	NM_005089:c.1241_1244del;p.S414fs
36	SMC1A	chrX	53409482	53409482	G	A	269	228	41	15.24	exonic	nonsynonymous SNV	NM_006306:c.C1276T;p.R426W
40	TET2	chr4	106157861	106157861	T	TA	579	433	146	25.22	exonic	frameshift insertion	NM_001127208.2:c.2763_2764insAp.His922fs
40	RUNX1	chr21	36206824	36206824	G	A	620	532	88	14.19	exonic	nonsynonymous SNV	NM_001754.4:c.688C>T;p.Gln230Ter
40	UZAF1	chr21	36206824	36206824	G	A	647	395	252	38.95	exonic	nonsynonymous SNV	NM_006758.2:c.101C>T;p.Ser34Phe
40	BCOR	chrX	39922189	39922190	TG	T	227	107	120	52.86	exonic	frameshift deletion	NM_001123385.1:c.3982delC;p.Gln1328fs
40	STAG2	chrX	123220614	123220614	T	TCA	140	59	81	57.86	exonic	frameshift insertion	NM_001042749.2:c.3274_3275insAC;p.Leu1092fs
43	NRAS	chr1	115256529	115256529	T	C	1997	1923	74	3.71	exonic	nonsynonymous SNV	exonic:NM_002524.5
43	IDH1	chr2	209113113	209113113	G	A	1723	1631	92	5.34	exonic	nonsynonymous SNV	exonic:NM_005896.4
43	TET2	chr4	106180837	106180837	T	G	1998	1107	891	44.59	exonic	nonsynonymous SNV	exonic:NM_001127208.3
43	EZH2	chr7	148511041	148511054	TGTGCTC ACCTTT	T	1982	143	1839	92.79	splicing	frameshift deletion	spliceite_5:NM_004456.5
43	RUNX1	chr21	36252849	36252852	CGTA	CTT	1992	1584	408	20.48	splicing	frameshift deletion	spliceite_5:NM_001754.5
43	STAG2	chrX	123179197	123179197	C	T	828	774	54	6.52	exonic	nonsynonymous SNV	exonic:NM_001042749.2
44	EZH2	chr7	148828746	148828746	G	A	1106	647	459	41.5	exonic	stopgain	NM_001203249:c.C592T;p.R198X
44	EZH2	chr7	148846529	148846529	G	A	911	480	431	47.31	exonic	stopgain	NM_001203249:c.C187T;p.R63X
44	IDH2	chr15	90088702	90088702	C	T	666	369	297	44.59	exonic	nonsynonymous SNV	NM_001290114:c.G29A;p.R10Q
44	ASXL1	chr20	32434649	32434649	-	C	1218	668	521	42.78	exonic	frameshift insertion	NM_015338:c.1938dupC;p.G646fs
44	STAG2	chrX	124057954	124057954	-	T	477	267	206	43.19	exonic	frameshift insertion	NM_006603:c.1394dupT;p.V465fs

45	BCOR	chrX	40074432	40074432	G	-	710	505	206	28.93	exonic	frameshift deletion	NM_001123385:c.914delC:p.P305fs
46	TET2	chr4	105234764	105234764	C	-	1456	1405	50	3.43	exonic	frameshift deletion	NM_001127208:c.822delC:p.I274fs
46	DNMT3A	chr2	25234401	25234401	G	A	696	438	258	37.07	exonic	nonsynonymous SNV	NM_153759:c.C2050T:p.H684Y
46	SF3B1	chr2	197402110	197402110	T	C	786	512	274	34.86	exonic	nonsynonymous SNV	NM_012433:c.A2098G:p.K700E
48	STAG2	chrX	124049076	124049076	C	A	129	119	10	7.75	exonic	stopgain	NM_006603:c.C891A:p.Y297X
48	EZH2	chr7	148818030	148818030	-	CCGT	938	608	329	35.07	exonic	frameshift insertion	NM_001203249:c.1044_1045insACGG:p.L349fs
48	ETV6	chr12	11869588	11869588	C	T	1390	696	692	49.78	exonic	nonsynonymous SNV	NM_001987:c.C628T:p.R210C
48	ASXL1	chr20	32434836	32434836	-	GC	1922	1260	659	34.27	exonic	frameshift insertion	NM_015338:c.2124_2125insGC:p.Q708fs
48	RUNX1	chr21	34859525	34859525	T	C	1502	914	588	39.15	exonic	nonsynonymous SNV	NM_001001890:c.A481G:p.T161A
48	ZRSR2	chrX	15820317	15820317	G	A	708	88	619	87.43	splicing	NA	NA
48	STAG2	chrX	124086590	124086590	C	T	870	713	157	18.05	exonic	stopgain	NM_006603:c.C3097T:p.R1033X
49	TET2	chr4	105237250	105237254	ATTTT	-	1140	739	405	35.4	exonic	frameshift deletion	NM_001127208:c.3308_3312del:p.N1103fs
49	EZH2	chr7	148815014	148815014	G	T	1380	810	570	41.3	exonic	nonsynonymous SNV	NM_001203247:c.C1557A:p.N519K
49	SRSF2	chr17	76736877	76736877	G	T	691	406	285	41.24	exonic	nonsynonymous SNV	NM_001195427:c.C284A:p.P95H
49	RUNX1	chr21	34834601	34834601	C	A	312	244	68	21.79	exonic	nonsynonymous SNV	NM_001001890:c.G533T:p.R178I
49	STAG2	chrX	124042619	124042619	C	T	182	129	53	29.12	exonic	stopgain	NM_006603:c.C436T:p.R146X
49	STAG2	chrX	124061342	124061342	G	T	284	150	134	47.18	splicing	NA	NA
51	DNMT3A	chr2	25245318	25245321	AGGA	-	520	341	178	34.23	exonic	frameshift deletion	NM_153759:c.919_922del:p.S307fs
51	SF3B1	chr2	197402110	197402110	T	C	782	476	305	39	exonic	nonsynonymous SNV	NM_012433:c.A2098G:p.K700E
51	RUNX1	chr21	34887015	34887015	G	A	797	421	374	46.93	exonic	nonsynonymous SNV	NM_001001890:c.C98T:p.A33V
52	SRSF2	chr17	76736854	76736877	GGCGGCT GTGGTGT GAGTCCG GGG	-	346	248	96	27.83	exonic	nonframeshift deletion	NM_001195427:c.284_307del:p.95_103del
53	EZH2	chr7	148826613	148826613	G	A	859	749	109	12.69	exonic	stopgain	NM_001203249:c.C721T:p.Q241X
53	EZH2	chr7	148832751	148832751	C	T	513	440	73	14.23	splicing	NA	NA
53	ASXL1	chr20	32433783	32433783	C	T	1052	797	253	24.05	exonic	stopgain	NM_015338:c.C1585T:p.Q529X
53	ZRSR2	chrX	15804121	15804122	AG	-	316	229	88	27.76	exonic	frameshift deletion	NM_005089:c.323_324del:p.K108fs
54	CBL	chr11	119278191	119278191	T	A	456	393	63	13.82	exonic	nonsynonymous SNV	NM_005188:c.T1121A:p.M374K
54	CBL	chr11	119278200	119278200	C	T	503	370	133	26.44	exonic	nonsynonymous SNV	NM_005188:c.C1130T:p.T377I
54	IDH2	chr15	90088702	90088702	C	T	601	330	271	45.09	exonic	nonsynonymous SNV	NM_001290114:c.G29A:p.R10Q
54	CEBPA	chr19	33302051	33302051	C	G	187	93	94	50.27	exonic	nonsynonymous SNV	NM_001285829:c.G7C:p.G3R
54	U2AF1	chr21	43094667	43094667	T	G	201	120	81	40.3	exonic	nonsynonymous SNV	NM_001025203:c.A470C:p.Q157P

54	PHF6	chrX	134393919	134393919	C	T	260	199	61	23.46	exonic	stopgain	NM_001015877:c.385T;p.R129X
54	PHF6	chrX	134417275	134417275	T	C	397	235	162	40.81	exonic	nonsynonymous SNV	NM_001015877:c.1941C;p.I314T
55	SRSF2	chr17	76736877	76736877	G	T	598	351	246	41.14	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
56	NRAS	chr1	114713911	114713911	C	T	561	491	70	12.48	exonic	nonsynonymous SNV	NM_002524:c.G179A;p.G60E
56	TET2	chr4	105243621	105243621	C	T	405	228	177	43.7	exonic	stopgain	NM_001127208:c.C3646T;p.R1216X
56	TET2	chr4	105276211	105276212	TT	-	949	580	374	39.2	exonic	frameshift deletion	NM_001127208:c.5701_5702del;p.F1901fs
56	EZH2	chr7	148809351	148809351	C	T	831	115	716	86.16	exonic	nonsynonymous SNV	NM_001203249:c.G1901A;p.R634H
56	STAG2	chrX	124065875	124065875	G	A	307	176	131	42.67	splicing	NA	NA
57	SETBP1	chr18	44951942	44951942	G	A	946	900	34	3.59	exonic	nonsynonymous SNV	NM_015559:c.G2602A;p.D868N
57	ETV6	chr12	11853525	11853525	C	T	830	603	224	26.99	exonic	stopgain	NM_001987:c.C427T;p.Q143X
57	ETV6	chr12	11884481	11884481	T	-	816	765	51	6.25	exonic	frameshift deletion	NM_001987:c.1046delT;p.L349fs
57	ETV6	chr12	11885938	11885938	A	G	493	448	45	9.13	exonic	nonsynonymous SNV	NM_001987:c.A1165G;p.M389V
57	NF1	chr17	31226459	31226459	-	C	530	486	45	8.38	exonic	frameshift insertion	NM_000267:c.2027dupC;p.T676fs
57	NF1	chr17	31233155	31233155	A	G	851	299	550	64.63	exonic	nonsynonymous SNV	NM_000267:c.A3650G;p.D1217G
57	NF1	chr17	31338092	31338092	C	T	919	837	82	8.92	exonic	stopgain	NM_000267:c.C6709T;p.R2237X
57	SRSF2	chr17	76736877	76736877	G	T	642	561	80	12.46	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
57	SETBP1	chr18	44951948	44951948	G	A	935	861	72	7.7	exonic	nonsynonymous SNV	NM_015559:c.G2608A;p.G870S
57	ZRSR2	chrX	15804198	15804198	G	T	407	37	368	90.42	splicing	NA	NA
58	TET2	chr4	105237281	105237281	-	A	1142	666	498	42.75	exonic	frameshift insertion	NM_001127208:c.3340dupA;p.D1113fs
58	TET2	chr4	105259667	105259669	CTT	-	967	569	398	41.16	exonic	nonframeshift deletion	NM_001127208:c.3852_3854del;p.1284_1285del
58	SRSF2	chr17	76736877	76736877	G	T	694	417	277	39.91	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
58	RUNX1	chr21	34792535	34792535	-	G	1111	924	185	16.65	exonic	frameshift insertion	NM_001001890:c.961dupC;p.H321fs
58	STAG2	chrX	124061848	124061848	C	-	513	423	89	17.38	exonic	frameshift deletion	NM_006603:c.1612delC;p.P538fs
59	SRSF2	chr17	76736877	76736877	G	T	484	247	237	48.97	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
59	RUNX1	chr21	34792608	34792608	-	CGCTGCAg	796	528	269	33.62	exonic	frameshift insertion	NM_001001890:c.888_889insCTGACG;p.A297fs
59	STAG2	chrX	124037566	124037566	C	T	419	399	20	4.77	exonic	stopgain	NM_006603:c.C328T;p.R110X
60	SF3B1	chr2	197402636	197402636	T	G	993	612	381	38.37	exonic	nonsynonymous SNV	NM_012433:c.A1997C;p.K666T
61	TET2	chr4	105234764	105234764	C	-	1016	628	390	38.31	exonic	frameshift deletion	NM_001127208:c.872delC;p.I274fs
61	RUNX1	chr21	34880569	34880569	G	C	945	874	71	7.51	exonic	nonsynonymous SNV	NM_001001890:c.C415G;p.R139G
61	STAG2	chrX	124049042	124049042	-	T	281	223	59	20.92	exonic	frameshift insertion	NM_006603:c.858dupT;p.N286fs

62	NFI	chr17	31259031	31259031	G	A	309	287	21	6.8	splicing	NA	NA
62	SRF2	chr17	76736877	76736877	G	T	521	278	243	46.64	exonic	nonsynonymous SNV	NM_001195427:c. C284A:p. P95H
62	STAG2	chrX	124049076	124049076	C	G	36	10	25	69.44	exonic	stopgain	NM_006603:c. C891G:p. Y297X
63	EP300	chr22	41176347	41176347	G	A	960	880	80	8.33	exonic	nonsynonymous SNV	NM_001429:c. G4880A:p. R1627Q
64	TET2	chr4	105269672	105269672	A	-	1006	579	425	42.29	exonic	frameshift deletion	NM_001127208:c. 4107delA:p. S1369fs
64	EZH2	chr7	148809351	148809351	C	T	1013	575	438	43.24	exonic	nonsynonymous SNV	NM_001203249:c. G1901A:p. R634H
64	EZH2	chr7	148810341	148810341	A	C	839	476	362	43.15	exonic	nonsynonymous SNV	NM_001203249:c. T1853G:p. L618W
64	PTPN11	chr12	112450398	112450398	C	T	1136	1053	82	7.22	exonic	nonsynonymous SNV	NM_002834:c. C218T:p. T73I
65	EZH2	chr7	148810392	148810392	T	C	792	31	760	95.96	exonic	nonsynonymous SNV	NM_001203249:c. A1802G:p. D601G
65	CEBPA	chr19	33301979	33301979	G	-	147	108	39	26.53	exonic	frameshift deletion	NM_001285829:c. 79delC:p. L27fs
65	RUNX1	chr21	34887038	34887038	-	A	1070	605	463	43.27	exonic	frameshift insertion	NM_001001890:c. 74dupT:p. M25fs
65	ZRSR2	chrX	15809266	15809266	C	T	476	17	459	96.43	exonic	stopgain	NM_005089:c. C505T:p. R169X
65	NRAS	chr1	114716126	114716126	C	A	1106	1070	29	2.62	exonic	nonsynonymous SNV	NM_002524:c. G35T:p. G12V
65	PTPN11	chr12	112450361	112450361	G	A	1421	1405	16	1.13	exonic	nonsynonymous SNV	NM_002834:c. G181A:p. D61N
65	TET2	chr4	105243571	105243571	-	G	535	19	527	96.34	exonic	frameshift insertion	NM_001127208:c. 3597dupG:p. V1199fs
66	TET2	chr4	105235696	105235696	C	A	1594	1298	296	18.57	exonic	stopgain	NM_001127208:c. C1754A:p. S585X
66	EZH2	chr7	148810355	148810355	G	C	988	678	309	31.28	exonic	nonsynonymous SNV	NM_001203249:c. C1839G:p. S613R
66	RUNX1	chr21	34792418	34792418	C	-	1393	1309	84	6.03	exonic	frameshift deletion	NM_001001890:c. 1079delG:p. G360fs
66	RUNX1	chr21	34886907	34886907	T	C	948	752	196	20.68	exonic	nonsynonymous SNV	NM_001001890:c. A206G:p. N69S
66	RUNX1	chr21	34886928	34886928	-	G	933	753	188	19.98	exonic	frameshift insertion	NM_001001890:c. 184dupC:p. L62fs
66	ZRSR2	chrX	15790932	15790932	A	C	274	160	113	41.39	splicing	NA	NA
67	TET2	chr4	105269640	105269640	C	T	872	807	65	7.45	exonic	nonsynonymous SNV	NM_001127208:c. C4075T:p. R1359C
67	TET2	chr4	105272774	105272774	C	T	1129	744	384	34.01	exonic	stopgain	NM_001127208:c. C4393T:p. R1465X
67	SRF2	chr17	76736877	76736877	G	C	715	440	275	38.46	exonic	nonsynonymous SNV	NM_001195427:c. C284G:p. P95R
68	DNMT3A	chr2	25240418	25240418	G	T	774	484	290	37.47	exonic	nonsynonymous SNV	NM_153759:c. C1639A:p. R547S
68	SF3B1	chr2	197401794	197401794	A	C	752	468	284	37.77	exonic	nonsynonymous SNV	NM_012433:c. T2318G:p. L773R
68	TET2	chr4	105236679	105236679	C	T	1735	1684	51	2.94	exonic	stopgain	NM_001127208:c. C2737T:p. Q913X
68	JAK2	chr9	5073770	5073770	G	T	1165	1127	38	3.26	exonic	nonsynonymous SNV	NM_004972:c. G1849T:p. V617F
69	TET2	chr4	105235892	105235893	TC	-	866	96	806	89.26	exonic	frameshift deletion	NM_001127208:c. 1950_1951del:p. H650fs
69	EZH2	chr7	148810332	148810332	C	A	474	238	236	49.79	splicing	NA	NA

69	EH2	chr7	148826633	148826633	C	A	510	311	199	39.02	splicing	NA	NA
69	STAG2	chrX	124068630	124068630	-	ACTC	143	27	116	80	exonic	frameshift insertion	NM_006603:c.2332_2333insACTC;p.N778fs
70	SRSF2	chr17	76736877	76736877	G	T	808	461	346	42.82	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
70	STAG2	chrX	124051330	124051330	A	G	199	38	161	80.9	exonic	nonsynonymous SNV	NM_006603:c.A1132G;p.M378V
70	STAG2	chrX	124063180	124063180	-	A	281	35	247	87.59	exonic	frameshift insertion	NM_006603:c.1797dupA;p.I599fs
71	TET2	chr4	105243616	105243616	G	A	513	308	205	39.96	exonic	nonsynonymous SNV	NM_001127208:c.G3641A;p.R1214Q
71	TET2	chr4	105259678	105259678	G	T	945	583	362	38.31	exonic	nonsynonymous SNV	NM_001127208:c.G3863T;p.G1288V
72	SRSF2	chr17	76736877	76736877	G	T	646	408	238	36.84	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
72	ASXL1	chr20	32435308	32435308	T	-	1546	1019	537	34.51	exonic	stopgain	NM_015338:c.2596delT;p.L866X
72	STAG2	chrX	124050205	124050205	C	T	715	485	230	32.17	exonic	stopgain	NM_006603:c.C913T;p.R305X
74	DNMT3A	chr2	25234374	25234374	C	T	na	na	na	45	exonic	nonsynonymous SNV	NM_022552:c.C2644T;p.R882C
74	U2AF1	chr21	43104346	43104346	C	T	na	na	na	44	exonic	nonsynonymous SNV	NM_006758:c.C101T;p.S34F
74	TET2	chr4	105269743	105269743	C	T	na	na	na	45	exonic	nonsynonymous SNV	NM_001127208:c.C4178T;p.T1393I
75	ASXL1	chr20	32434789	32434789	C	T	na	na	na	31	exonic	nonsynonymous SNV	NM_015338:c.C2077T;p.R693*
75	SRSF2	chr17	76736877	76736877	C	A	na	na	na	45	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
75	IDH2	chr15	90088702	90088702	G	A	na	na	na	11	exonic	nonsynonymous SNV	NM_002168:c.G419A;p.R140Q
75	RUNX1	chr21	34799441	34799441	G	-	na	na	na	34	exonic	frameshift deletion	NM_001754:c.830del;p.P277Hfs*34
75	JAK2	chr9	5022126	5022126	C	G	na	na	na	11	exonic	nonsynonymous SNV	NM_004972:c.C139G;p.L47V
76	SRSF2	chr17	76736878	76736878	C	G	na	na	na	43	exonic	nonsynonymous SNV	NM_001195427:c.C283G;p.P95A
76	TET2	chr4	105269662	105269662	G	A	na	na	na	44	exonic	nonsynonymous SNV	NM_001127208:c.G4097A;p.R1366H
76	RUNX1	chr21	34859494	34859494	A	G	na	na	na	37	exonic	nonsynonymous SNV	NM_001754:c.A593G;p.D198G
77	ZRSR2	chrX	na	na	na	na	na	na	na	40	exonic	frameshift deletion	NA
78	ZRSR2	chrX	na	na	na	na	na	na	na	61	exonic	NA	NM_005098:p.Q652*
78	TET2	chr4	na	na	na	na	na	na	na	25	exonic	NA	NM_001127208:p.T80fs*48
79	DNMT3A	chr2	25246637	25246637	G	C	na	na	na	11	exonic	nonsynonymous SNV	NM_022552:c.G1261C;p.Gly421Ala
79	TP53	chr17	7674221	7674221	C	T	na	na	na	8	exonic	nonsynonymous SNV	NM_001126114:c.C742T;p.R248W
80	ASXL1	chr20	32434647	32434648	-	T	na	na	na	27	exonic	frameshift insertion	NM_015338:c.1935dup;p.G646Wfs*12
80	TET2	chr4	105275770	105275770	G	C	na	na	na	50	exonic	nonsynonymous SNV	NM_001127208:c.G5260C;p.G1754R
85	TET2	chr4	105269703	105269703	C	T	715	76	639	89.37	exonic	nonsynonymous SNV	NM_001127208:c.C4138T;p.H1380Y
85	KRAS	chr12	25225628	25225628	C	T	718	570	148	20.61	exonic	nonsynonymous SNV	NM_004985:c.G436A;p.A146T

85	ZRSR2	chrX	15799926	15799930	AAGAA	-	268	59	209	77.99	exonic	frameshift deletion	NM_005089:c.176_180del;p.E59fs
85	CBL	chr11	119278541	119278541	G	T	780	746	34	4.36	exonic	nonsynonymous SNV	NM_005188:c.G1259T;p.R420L
85	NFI	chr17	31235729	31235729	G	A	895	869	26	2.91	exonic	nonsynonymous SNV	NM_000267:c.G3827A;p.R1276Q
85	NRAS	chr1	114716126	114716126	C	T	636	619	17	2.67	exonic	nonsynonymous SNV	NM_002524:c.G35A;p.G12D
86	TET2	chr4	105261769	105261769	T	C	415	248	167	40.24	exonic	nonsynonymous SNV	NM_001127208:c.T3965C;p.L1322P
86	EZH2/EZH2	chr7	148826468	148826468	C	T	443	134	308	69.53	exonic-splicing	nonsynonymous SNV	NM_004456:c.G893A;p.R298H
86	SRSF2	chr17	76736878	76736878	G	C	587	364	223	37.99	exonic	nonsynonymous SNV	NM_001195427:c.C283G;p.P95A
86	SETBP1	chr18	44951942	44951942	G	A	903	570	333	36.88	exonic	nonsynonymous SNV	NM_015559:c.G3602A;p.D868N
86	ASXL1	chr20	32433844	32433844	-	T	883	559	322	36.43	exonic	frameshift insertion	NM_015338:c.1647dupT;p.R549fs
87	NRAS	chr1	114716127	114716127	C	G	672	388	284	42.26	exonic	nonsynonymous SNV	NM_002524:c.G34C;p.G12R
87	IDH2	chr15	90088702	90088702	C	T	673	354	318	47.25	exonic	nonsynonymous SNV	NM_001290114:c.G29A;p.R10Q
87	SRSF2	chr17	76736877	76736877	G	T	599	327	272	45.41	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
87	RUNX1	chr21	34799310	34799310	G	A	747	678	69	9.24	exonic	stopgain	NM_001001890:c.C877T;p.R293X
87	BCOR	chrX	40062138	40062138	C	T	262	142	120	45.8	splicing	NA	NA
87	STAG2	chrX	124077998	124077998	-	A	240	33	207	86.25	exonic	frameshift insertion	NM_006603:c.2716dupA;p.S905fs
88	TET2	chr4	105236308	105236308	-	T	1183	1064	121	10.19	exonic	frameshift insertion	NM_001127208:c.2367dupT;p.N789fs
88	ZRSR2	chrX	15815676	15815676	G	A	325	25	299	92	splicing	NA	NA
90	TET2	chr4	105236426	105236426	C	A	1038	588	449	43.26	exonic	stopgain	NM_001127208:c.C2484A;p.C828X
90	TET2	chr4	105261800	105261800	-	A	503	292	211	41.95	exonic	frameshift insertion	NM_001127208:c.3997dupA;p.L1332fs
90	SFI	chr11	64770293	64770293	C	T	688	390	297	43.23	exonic	nonsynonymous SNV	NM_001178030:c.G727A;p.A243T
90	KRAS	chr12	25225713	25225713	T	A	700	650	50	7.14	exonic	nonsynonymous SNV	NM_004985:c.A351T;p.K117N
90	STAG2	chrX	124042619	124042619	C	T	139	17	122	87.77	exonic	stopgain	NM_006603:c.C436T;p.R146X
91	IDH1	chr2	208248388	208248388	C	T	452	266	186	41.15	exonic	nonsynonymous SNV	NM_005896:c.G395A;p.R132H
91	RUNX1	chr21	34880608	34880608	-	C	673	381	296	43.72	exonic	frameshift insertion	NM_001001890:c.375dupG;p.N126fs
91	STAG2	chrX	124063866	124063866	C	T	244	41	203	83.2	exonic	stopgain	NM_006603:c.C1840T;p.R614X
91	PHF6	chrX	134417193	134417193	G	A	324	247	76	23.46	exonic	nonsynonymous SNV	NM_001015877:c.G859A;p.G287S
92	SRSF2	chr17	76736877	76736877	G	T	496	284	212	42.74	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
92	ASXL1	chr20	32435179	32435179	T	-	978	557	427	43.39	exonic	stopgain	NM_015338:c.2467delT;p.L823X
92	RUNX1	chr21	34880569	34880569	G	A	653	374	277	42.42	exonic	stopgain	NM_001001890:c.C415T;p.R139X
92	STAG2	chrX	124049079	124049079	G	T	64	48	16	25	splicing	NA	NA

92	BCOR	chrX	40055454	40055454	-	CC	430	307	121	28.14	exonic	frameshift insertion	NM_001123385:c.4654_4655insGG;p.Y1552fs
93	IDH1	chr2	208248389	208248389	G	A	621	589	31	4.99	exonic	nonsynonymous SNV	NM_005896:c.C394T;p.R132C
93	TET2	chr4	105236167	105236167	-	G	1746	1062	690	39.29	exonic	frameshift insertion	NM_001127208:c.2226dupG;p.Q742fs
93	SRSF2	chr17	76736360	76736360	C	A	983	481	502	51.07	exonic	nonsynonymous SNV	NM_001195427:c.G467T;p.R156L
93	SRSF2	chr17	76736877	76736877	-	GGC	610	393	219	35.67	exonic	nonframeshift insertion	NM_001195427:c.283_284insGCC;p.P95delinsRP
93	RUNX1	chr21	34886902	34886902	G	-	724	442	288	39.45	exonic	frameshift deletion	NM_001001890:c.211delC;p.L71fs
93	BCOR	chrX	40073128	40073128	C	A	512	230	281	54.88	exonic	stopgain	NM_001123385:c.G2218T;p.E740X
93	BCOR	chrX	40074873	40074873	-	T	590	469	118	19.97	exonic	frameshift insertion	NM_001123385:c.472dupA;p.S158fs
94	RAD21	chr8	116857335	116857338	TTAA	-	840	780	59	7.02	exonic	frameshift deletion	NM_006265:c.617_620del;p.L206fs
95	TP53	chr17	7673802	7673802	C	T	548	530	18	3.28	exonic	nonsynonymous SNV	NM_001126115:c.G422A;p.R141H
95	STAG1	chr3	136359160	136359160	G	C	752	416	336	44.68	exonic	nonsynonymous SNV	NM_005862:c.C2924G;p.A975G
95	CBL	chr11	119278191	119278191	T	A	465	14	449	96.98	exonic	nonsynonymous SNV	NM_005188:c.T1121A;p.M374K
95	SUZ12	chr17	31995572	31995572	G	A	766	427	338	44.13	exonic	nonsynonymous SNV	NM_015355:c.G1604A;p.R535Q
95	PPM1D	chr17	60663262	60663262	-	A	1412	1065	342	24.22	exonic	frameshift insertion	NM_003620:c.1529dupA;p.Q510fs
95	SRSF2	chr17	76736877	76736877	G	T	581	329	252	43.37	exonic	nonsynonymous SNV	NM_001195427:c.C284A;p.P95H
95	ASXL1	chr20	32433384	32433384	C	T	965	505	459	47.56	exonic	stopgain	NM_015338:c.C1186T;p.Q396X
96	KRAS	chr12	25245347	25245347	C	T	975	961	13	1.33	exonic	nonsynonymous SNV	NM_004985:c.G38A;p.G13D
96	EZH2	chr7	148810374	148810374	T	C	789	446	343	43.47	exonic	nonsynonymous SNV	NM_001203249:c.A1820G;p.Y607C
96	EZH2	chr7	148814136	148814136	A	T	709	410	299	42.17	exonic	stopgain	NM_001203247:c.T1659A;p.C553X
96	PTPN11	chr12	112489084	112489084	G	T	768	719	49	6.38	exonic	nonsynonymous SNV	NM_002834:c.G1508T;p.G503V
96	ASXL1	chr20	32433447	32433447	C	T	1260	727	533	42.3	exonic	stopgain	NM_015338:c.C1249T;p.R417X
96	RUNX1	chr21	34792541	34792541	-	G	1103	700	401	36.36	exonic	frameshift insertion	NM_001001890:c.955dupC;p.R319fs

Table S3: Univariate analyses of mutation status for AML progression of patients with isolated +8.

Mutational variable	Mutation status	N	Median time to AML (months)	P value
TET2	MUT	29	NR	0.903
	WT	46	117.4	
STAG2	MUT	26	15.7	0.002*
	WT	49	NR	
SRSF2	MUT	21	14.0	<0.0001***
	WT	54	117.4	
RUNX1	MUT	19	15.7	0.036*
	WT	56	NR	
EZH2	MUT	18	22.0	0.536
	WT	57	NR	
ASXL1	MUT	12	NR	0.156
	WT	62	117.4	
ZRSR2	MUT	13	NR	0.667
	WT	62	117.4	
IDH2	MUT	8	5.2	0.06
	WT	67	117.4	
NRAS	MUT	8	5.9	0.013*
	WT	67	66.2	
SF3B1	MUT	7	NR	0.142
	WT	68	NA	
DNMT3A	MUT	7	26.1	0.729
	WT	68	117.4	
U2AF1	MUT	5	26.1	0.388
	WT	70	117.4	
ETV6	MUT	5	5.2	0.005**
	WT	70	117.4	

NR: not reached; NA: not applicable; * $p < 0.05$; ** $p < 0.01$; *** $p > 0.001$.

Table S4: Clinical characteristic of patients with low risk-like and high risk-like MDS and isolated trisomy 8.

	Isolated +8 MDS without <i>STAG2</i> , <i>SRSF2</i> and <i>RUNX1</i> mutations (n=34)	Isolated +8 MDS with <i>STAG2</i> , <i>SRSF2</i> and/or <i>RUNX1</i> mutations (n=45)	p value	test
% of patients	43%	57%		
Demographics				
Age at diagnosis, years (range)	75.5 (49-94)	72 (33-91)	0.434	a
Gender, n (%)			0.613	b
Female	8 (23.5%)	14 (31.1%)		
Male	26 (76.5%)	31 (68.9%)		
Risk stratification				
IPSS-R, n (%)			0.03	b
Low	13 (43.3%)	4 (12.9%)		
Intermediate 1-2	10 (33.4%)	11 (35.5%)		
High	6 (20%)	11 (35.5%)		
Very high	1 (3.3%)	5 (16.1%)		
IPSS-M, n (%)			<0.0001	b
Low	17 (53.1%)	1 (2.8%)		
Moderate low	5 (15.6%)	3 (8.3%)		
Moderate high	2 (6.3%)	3 (8.3%)		
High	7 (21.9%)	14 (38.9%)		
Very high	1 (3.1%)	15 (41.7%)		
Blood and bone marrow counts at diagnosis				
Hemoglobin (g/dL), median (range)	9.95 (7.7-14.6)	9.45 (5.4-14.6)	0.177	a
Platelets (x10 ⁹ /L), median (range)	150.5 (18-491)	72.5 (2-520)	0.194	a
Neutrophils (x10 ⁹ /L), median (range)	1.95 (0.3-11.8)	1.3 (0.2-9.7)	0.458	a
Bone marrow blasts (%), median (range)	3 (0-16)	6.5 (0-15)	0.020	c
Circulating blasts (%), median (range)	0 (0-2)	0 (0-13)	0.025	c
Ring sideroblasts (%), median (range)	0 (0-72)	0 (0-9)	0.067	a
Outcome and follow-up				
Overall survival, months (range)	46.3 (16.2-76.3)	23.7 (4.6-42.8)	0.005	d
AML progression, n (%)	2 (5.9%)	13 (28.9%)	0.008	b
Time to AML, months (range)	NR	14 (7.7-23.6)	<0.0001	d
NGS data, n (%)				
Mutations per patient, median (range)	2 (0-7)	5 (1-10)	<0.0001	c

NR: not reached; a: Parametric test (T-student); b: Fisher exact test; c: Non-parametric test (U-Mann Whitney); d: Log rank.

Table S5: Clinical features of isolated +8 MDS patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1* compared to high risk IPSS-R and high risk.

	High/very high risk MDS without +8 (n=108)	Isolated +8 MDS with <i>STAG2</i> , <i>SRSF2</i> and/or <i>RUNX1</i> mutations (n=45)	p value
Demographics			
Age at diagnosis, years (range)	73.2 (35.9-93.9)	72 (33-91)	0.990
Gender, n (%)			0.073
Female	52 (48.1%)	14 (31.1%)	
Male	56 (51.9%)	31 (68.9%)	
Blood and bone marrow counts at diagnosis			
Hemoglobin (g/dL), median (range)	8.7 (4.8-13.1)	9.45 (5.4-14.6)	0.003
Platelets (x10 ⁹ /L), median (range)	70 (6-1000)	72.5 (2-520)	0.4
Neutrophils (x10 ⁹ /L), median (range)	0.9 (0.1-15.4)	1.3 (0.2-9.7)	0.236
Bone marrow blasts (%), median (range)	10.3 (0-20)	6.5 (0-15)	0.001
Circulating blasts (%), median (range)	0 (0-7)	0 (0-13)	0.234
Ring sideroblasts (%), median (range)	4 (0-30)	0 (0-9)	0.006
Outcome and follow-up			
Overall survival, months (range)	10.1 (5.3-14.9)	23.7 (4.6-42.8)	0.323
AML progression, n (%)	34 (31.5%)	13 (28.9%)	0.559
Time to AML, months (range)	11.4 (9.5-13.6)	14 (7.7-23.6)	0.296
NGS data, n (%)			
Mutations per patient, median (range)	3 (0-8)	5 (1-10)	0.003

Table S6: Clinical features of isolated +8 MDS patients with mutations in *STAG2*, *SRSF2* and/or *RUNX1* compared to high risk IPSS-R and high risk.

	Low IPSS-R risk MDS without +8 (n=93)	Isolated +8 MDS without <i>STAG2</i> , <i>SRSF2</i> and <i>RUNX1</i> mutations (n=34)	p value
Demographics			
Age at diagnosis, years (range)	79.3 (49.2-92.2)	75.5 (49-94)	0.167
Gender, n (%)			0.025
Female	43 (46.2%)	8 (23.5%)	
Male	50 (53.8%)	26 (76.5%)	
Blood and bone marrow counts at diagnosis			
Hemoglobin (g/dL), median (range)	9.3 (4-15.1)	9.95 (7.7-14.6)	0.09
Platelets (x10 ⁹ /L), median (range)	203 (10-637)	150.5 (18-491)	0.039
Neutrophils (x10 ⁹ /L), median (range)	2.3 (0.2-8.8)	1.95 (0.3-11.8)	0.905
Bone marrow blasts (%), median (range)	1 (0-10)	3 (0-16)	<0.001
Circulating blasts (%), median (range)	0 (0-6)	0 (0-2)	0.927
Ring sideroblasts (%), median (range)	4 (0-95)	0 (0-72)	0.035
Outcome and follow-up			
Overall survival, months (range)	64.8 (34.2-95.4)	46.3 (16.2-76.3)	0.066
AML progression, n (%)	11 (11.8%)	2 (5.9%)	0.213
Time to AML, months (range)	109.3	NR	0.163
NGS data, n (%)			
Mutations per patient, median (range)	3 (0-7)	2 (0-7)	0.178

NR: not reached.

Table S7: Clinical and biological features of isolated +8 MDS cohorts.

	Discovery cohort (n=79)	Validation cohort (n=110)	p	test
Demographics				
Age at diagnosis, years (range)	75 (33-94)	72 (27-87)	0.106	a
Gender, n (%)			0.631	b
Female	22 (27.8%)	35 (31.8%)		
Male	57 (72.2%)	75 (68.2%)		
MDS risk stratification				
IPSS-R category, n (%)			0.471	b
Very low	0 (0%)	1 (0.9%)		
Low	17 (21.5%)	32 (29.1%)		
Intermediate	21 (26.6%)	23 (20.9%)		
High	17 (21.5%)	37 (33.6%)		
Very high	6 (7.6%)	11 (10%)		
IPSS-M category, n (%)			0.488	b
Very low	0 (0%)	2 (2%)		
Low	18 (26.5%)	24 (23.8%)		
Moderate low	8 (11.8%)	14 (13.9%)		
Moderate high	5 (7.4%)	8 (7.9%)		
High	21 (30.8%)	20 (19.8%)		
Very high	16 (23.5%)	33 (32.6%)		
Blood and bone marrow counts at diagnosis				
Hemoglobin (g/dL), median (range)	9.8 (5-15)	9.12 (6-14)	0.099	a
Platelets (x10 ⁹ /L), median (range)	95 (2-520)	128 (13-842)	0.032	a
Neutrophils (x10 ⁹ /L), median (range)	1.5 (0-12)	1.6 (0-10)	0.165	a
Bone marrow blasts (%), median (range)	5 (0-16)	4 (0-19)	0.989	a
Circulating blasts (%), median (range)	0 (0-13)	0 (0-16)	0.864	a
Ring sideroblasts (%), median (range)	0 (0-72)	0 (0-90)	0.098	c
Outcome and follow-up*				
Overall survival, months (range)	7.6 (0-133)	13.3 (0-152)	0.21	d
AML progression, n (%)	16 (20.3%)	24 (21.8%)	0.719	b
Time to AML, months (range)	7.3 (0-133)	13.5 (0-152)	0.089	d
NGS data, n (%)			0.242	b
Low-risk like isolated +8 defined group	34 (43%)	57 (51.8%)		
High-risk like isolated +8 defined group	45 (57%)	53 (48.2%)		

*Data censored in patients underwent TPH; a: Parametric test (T-student); b: Fisher exact test; c: Non-parametric test (U-Mann Whitney); d: Log rank.

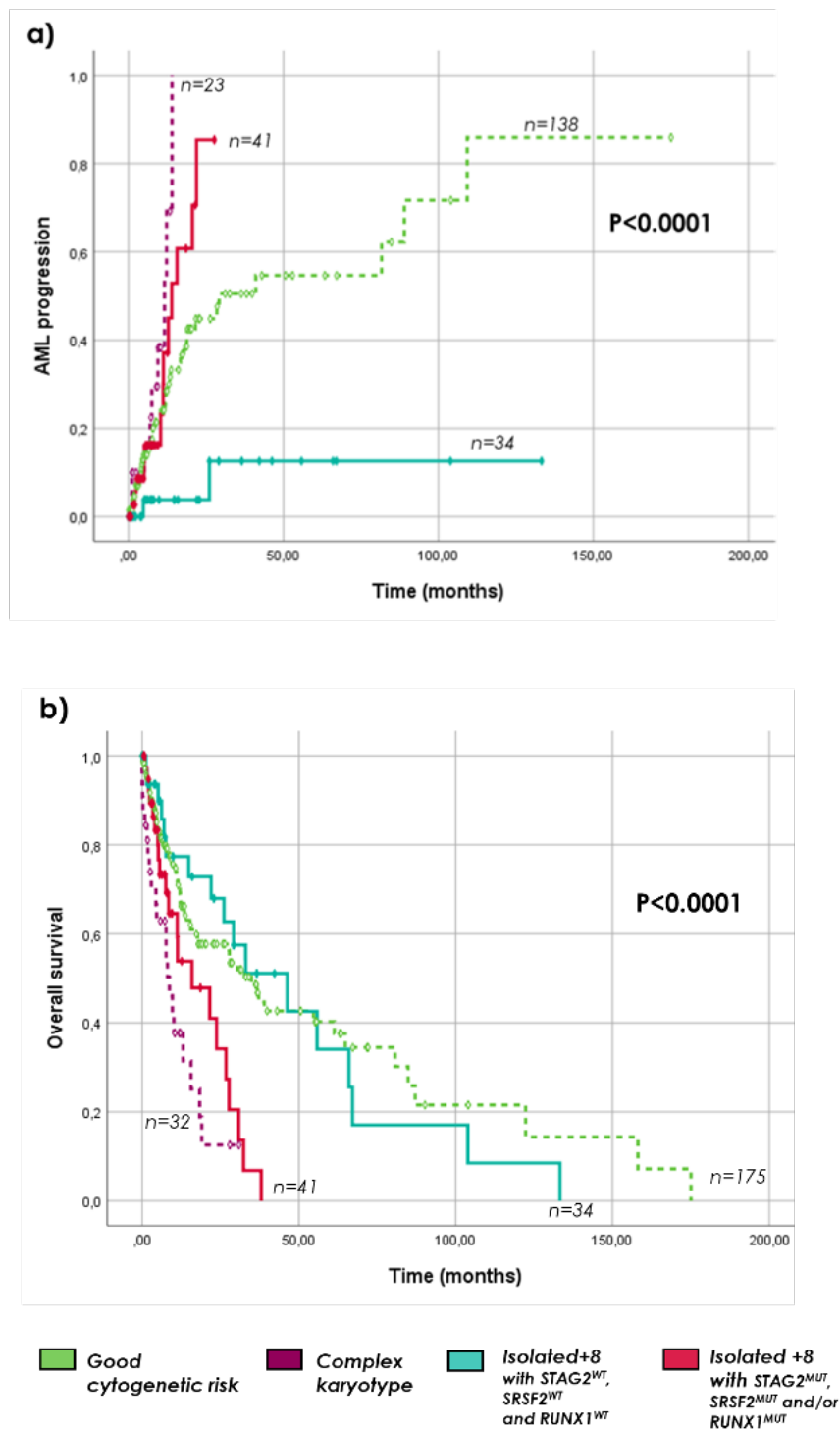


Figure S1: a) Time to AML progression in newly described isolated +8 MDS subgroups compared to non-trisomy 8 MDS patients with normal and complex karyotypes; b) overall survival in newly defined risk subgroups in isolated +8 MDS patients compared to non-trisomy 8 MDS patients with normal and complex karyotypes.

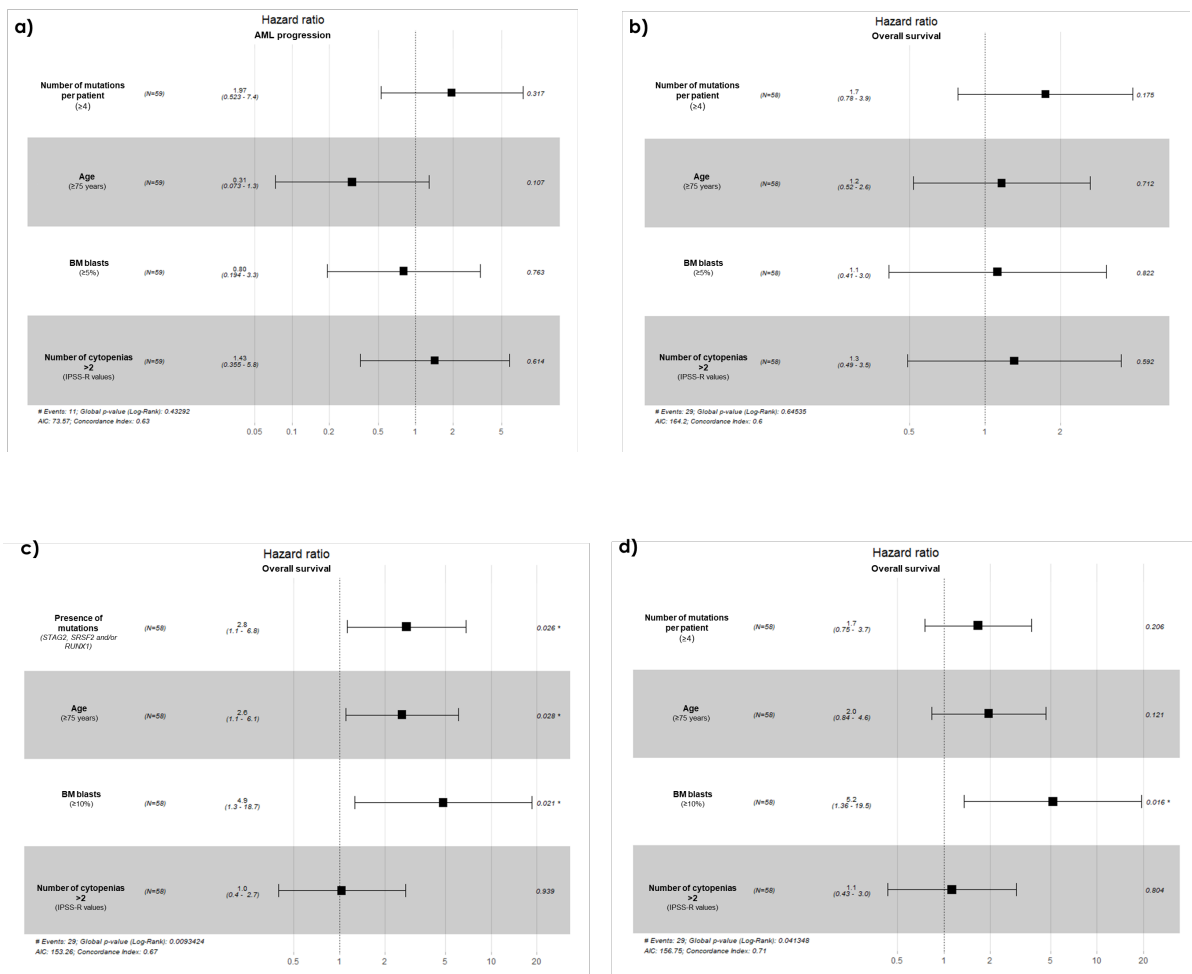


Figure S2: a) Multivariate analysis of patients with isolated +8 depending on number of mutations per patient (≥ 4) in AML progression; b) Multivariate analysis of OS in isolated +8 MDS patients depending on the number of mutations per patient; c) Multivariate analysis of OS in isolated +8 MDS considering 10% of BM blasts in patients with mutations in STAG2, SRSF2 and/or RUNX1; d) Multivariate analysis of patients with isolated +8 MDS regarding percentage of BM blasts ($>10\%$) and the number of mutations per patient (≥ 4).

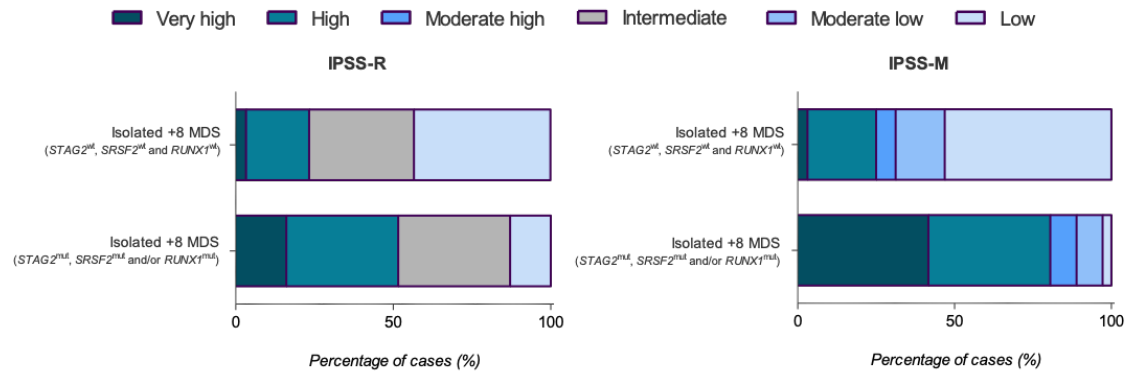


Figure S3: a) Percentage of patients with isolated +8 MDS with and without mutations in STAG2, SRSF2 and/or RUNX1 stratified by the IPSS-R; b) Percentage of patients with MDS and isolated +8 with and without mutations in STAG2, SRSF2 and/or RUNX1 stratified by the IPSS-M.

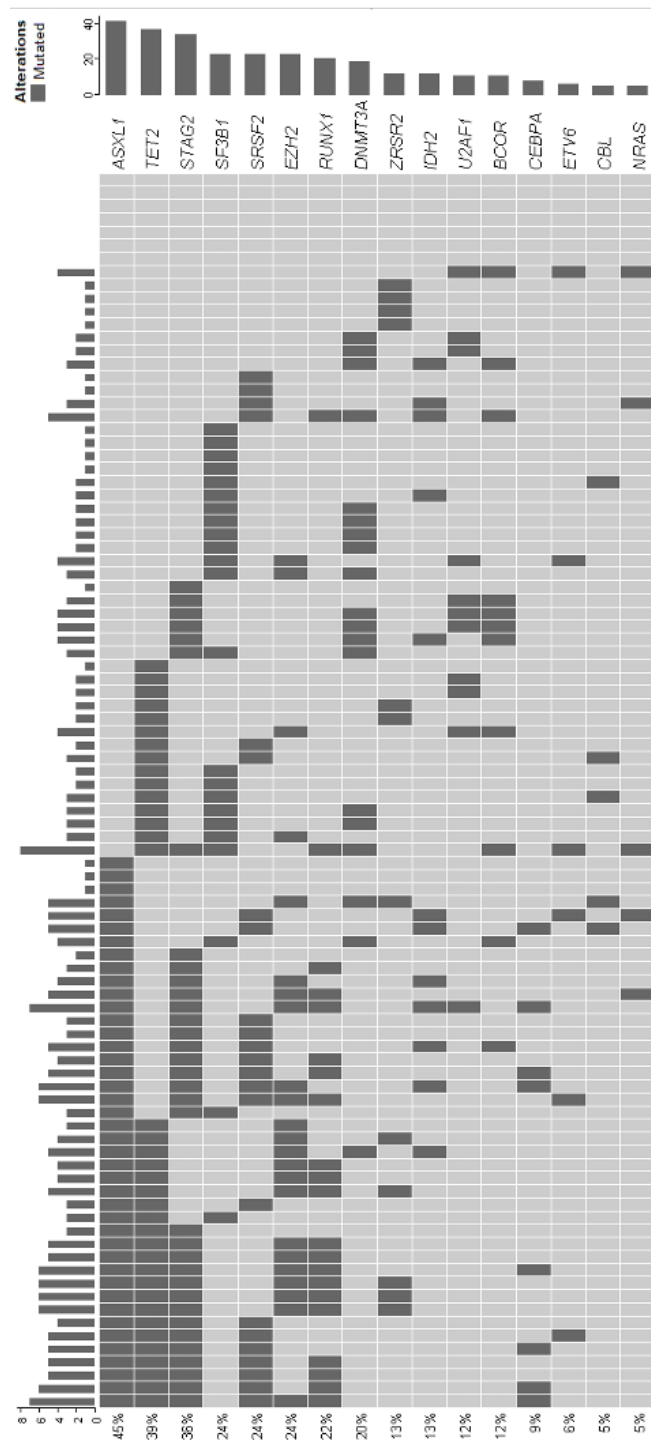


Figure S4: Mutational pattern of mutated genes in more than 5% of isolated +8 MDS patients of the International Working Group for the Prognosis of MDS.

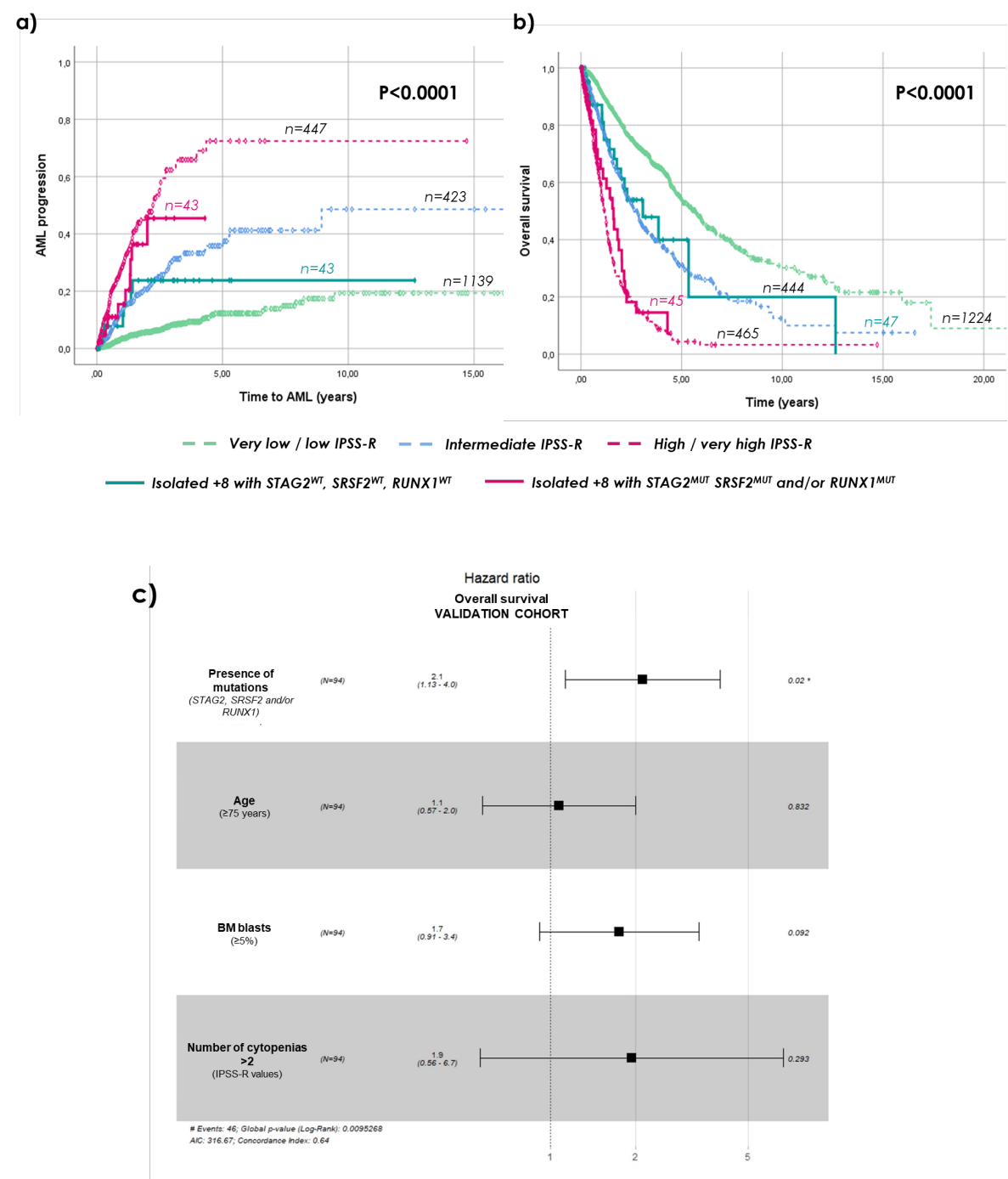


Figure S5: a) Time to AML progression in newly defined prognostic subgroups in isolated +8 compared to the control MDS without +8 stratified by IPSS-R risk subgroups; b) OS of isolated +8 MDS patients reclassified in prognostic groups according to mutational profile within IPSS-R MDS control groups; c) Multivariate analysis for OS in isolated +8 patients of the validation cohort.

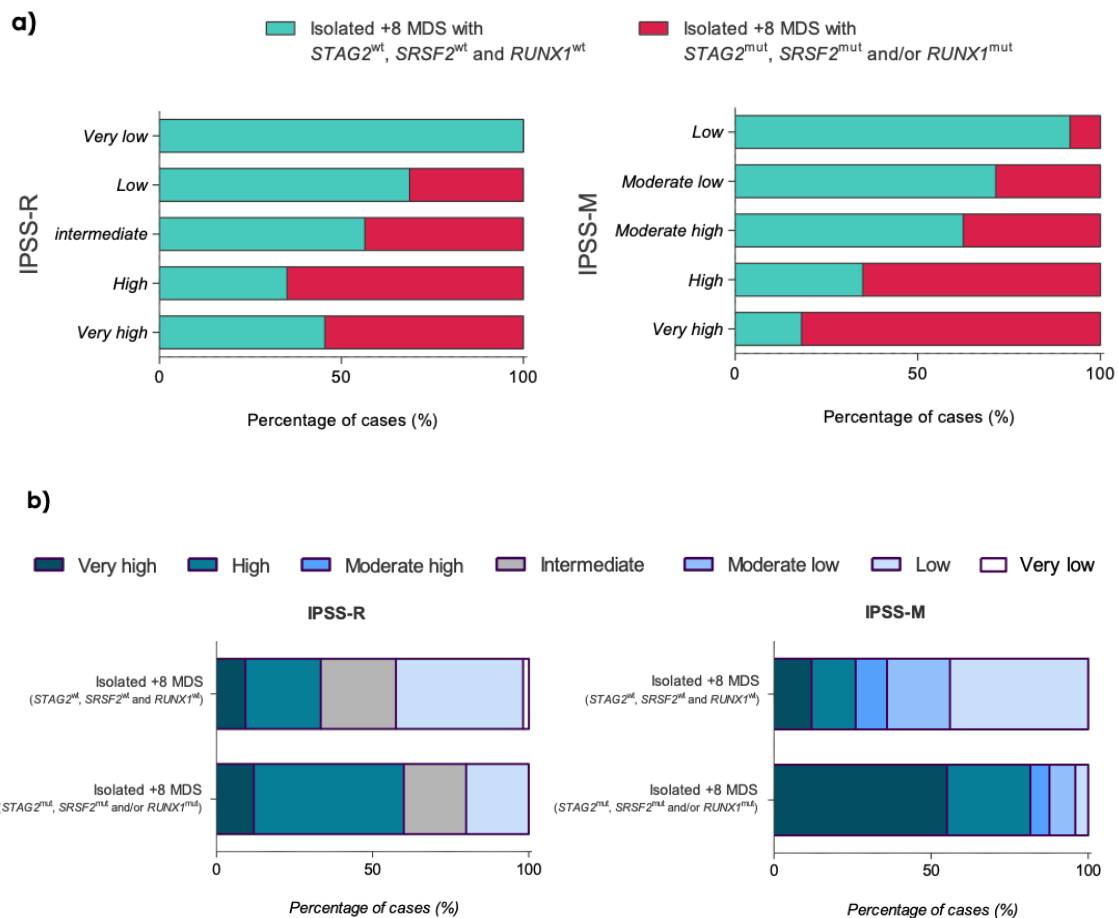


Figure S6: a) Percentage of patients from the validation cohort stratified within IPSS-R and IPSS-M categories reconsidered as low risk and high risk based on mutational profile; b) Percentage of patients classified by the IPSS-R and IPSS-M in patients with isolated +8 MDS depending on mutational status of *STAG2*, *SRSF2* and *RUNX1*.

ANEXO III

Material Suplementario del Capítulo 2

Original article – Supplementary Data

MUTATIONAL DYNAMICS IN PATIENTS WITH DEL(5Q) MDS TREATED WITH LENALIDOMIDE PRIOR TO TRANSFUSION DEPENDENCY – MOLECULAR RESULTS FROM THE SINTRAREV CLINICAL TRIAL

Sofía Toribio Castelló¹, Felix Lopez-Cadenas², Claude Preudhomme³, Teresa González^{1,2}, Angela Villaverde Ramiro¹, Laurene Fenwarth, Mónica Del Rey¹, Blanca Xicoy⁴, Aline Renneville^{3,5}, Joaquín Sánchez-García⁶, Rosa Coll⁷, Bohrane Slama⁸, José Ángel Hernández-Rivas⁹, Sylvain Thepot¹⁰, Teresa Bernal¹¹, Agnès Guerci-Bresler¹², Katharina Götze¹³, Pierre Fenaux¹⁴, Jesús María Hernández-Rivas^{1,2,15*}, María Díez-Campelo^{1,2*^c}.

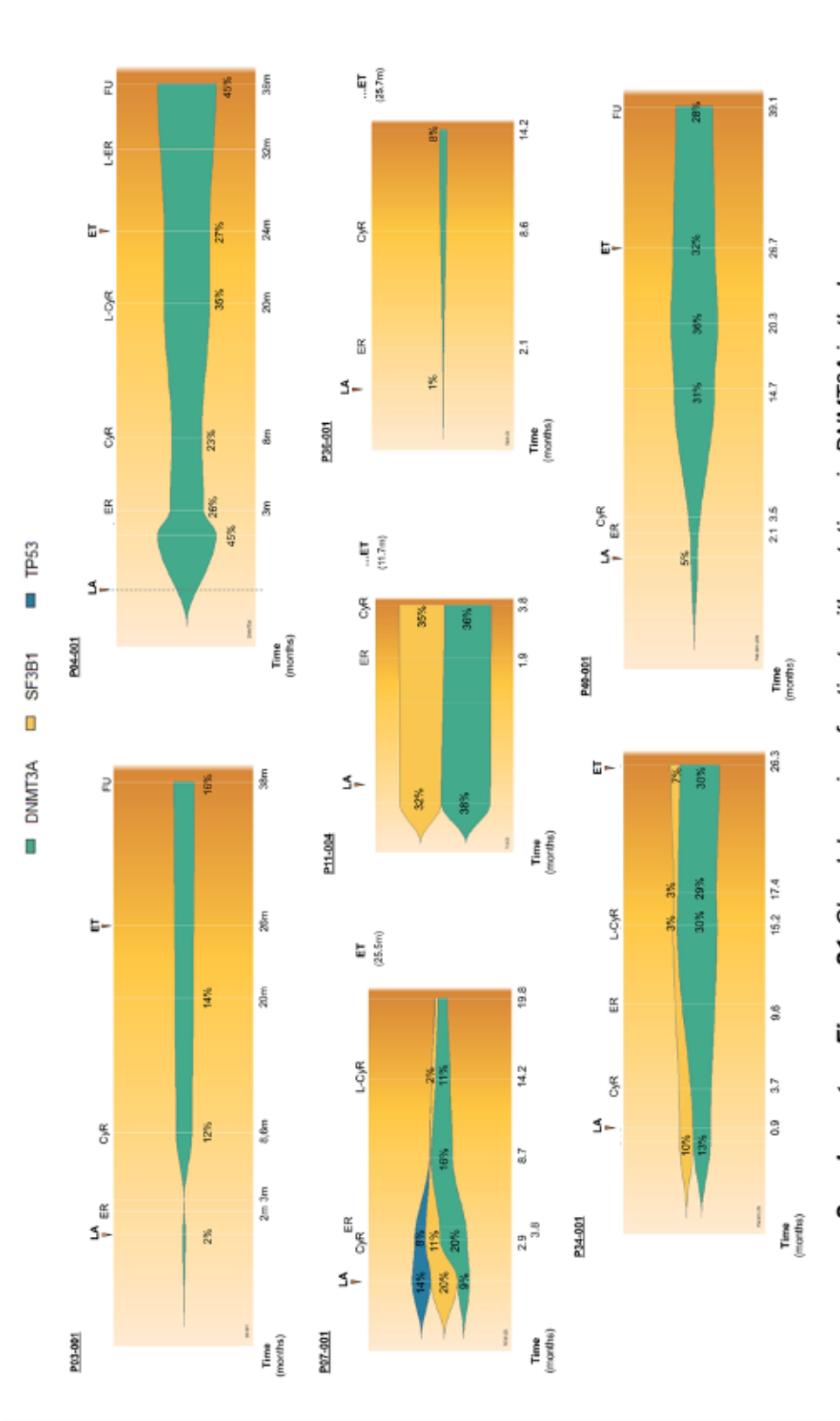
List of supporting information:

- **Supplementary Table S1:** Response ratios and AML progression comparison in patients from the MDS-004 and SintraRev clinical trials.
- **Supplementary Figure S1:** Clonal dynamics of patients with mutations in *DNMT3A* in the Len arm.
- **Supplementary Figure S2:** Acquisition of *TP53* mutations >10 months after the start of Len administration in 2 patients from the treatment (Len) arm.

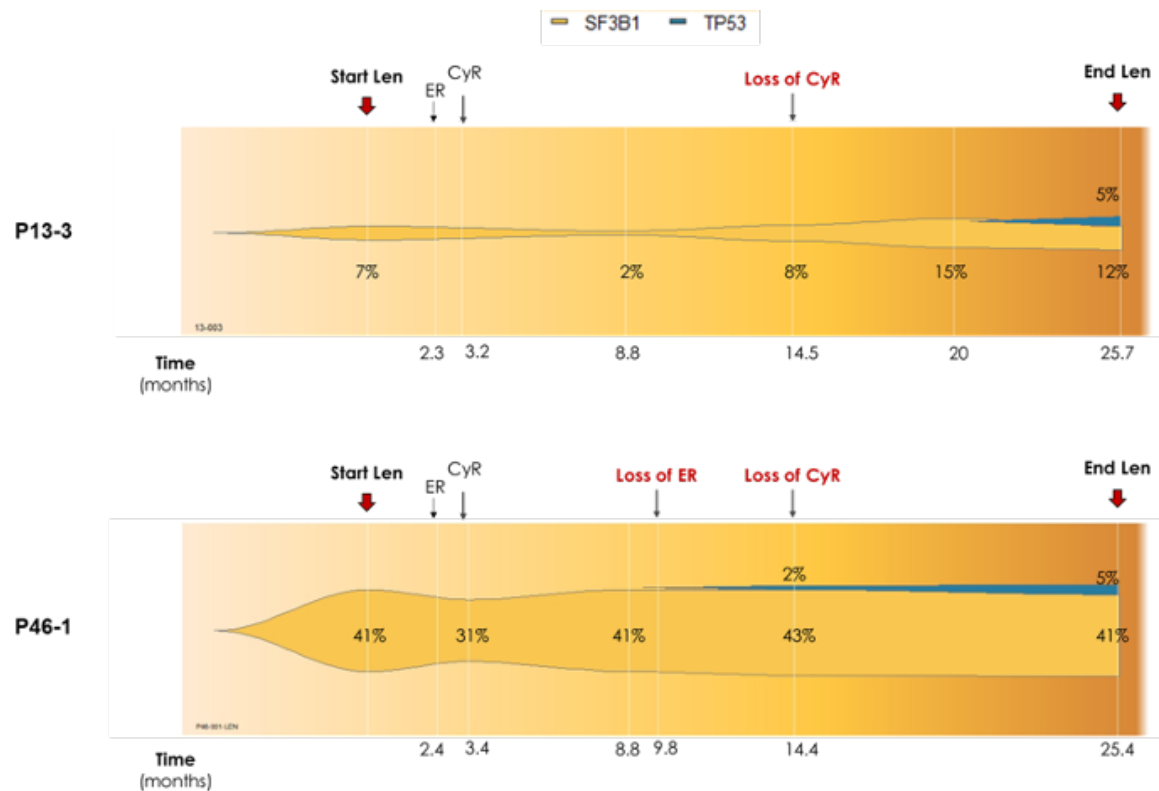
Supplementary Table S1: Response ratios and AML progression comparison in patients from the MDS-004 and SintraRev clinical trials.

	MDS-004 clinical trial		SintraRev clinical trial			
	<i>Len</i> 5mg/day	<i>Len</i> 10mg/day	<i>Len</i> 5mg/day (all patients)	<i>Len</i> 5mg/day <i>DNMT3A</i> ^{mut*}	<i>Len</i> 5mg/day <i>SF3B1</i> ^{mut*}	<i>Len</i> 5mg/day <i>TP53</i> ^{mut*}
ER (%)	42.6	56.1	77.8	100	100	40
Median time ER (months)	10.3 ⁺	20.7 ⁺	23.6	24.9	13.8	41.6
CyR (%)	25	50	94.1	100	100	60
Median time CyR (months)	n.a	n.a	17	21.9	12	11.8
AML progression (%)	23.3	21.7	15	25	25	20
LFS (months)	31.8	36.1	64.77	66.5	62.9	57.9

*Patients with mutations at baseline; +Original data in weeks, recalculated in months dividing by 4.



Supplementary Figure S1: Clonal dynamics of patients with mutations in DNMT3A in the Len arm.
LA: Len administration; ER: erythroid response; CyR: cytogenetic response; L-CyR: loss of ER; L-CyR: loss of CyR; ET: end of treatment; FU: follow-up



Supplementary Figure S2: Acquisition of TP53 mutations >10 months after the start of Len administration in 2 patients from the treatment (Len) arm. ER: erythroid response; CyR: cytogenetic response.

ANEXO IV

Material Suplementario del Capítulo 3

Original article – Supplementary Data

STAG2 AND NRAS MUTATIONS COOPERATE IN THE TRANSFORMATION OF MDS TO AML BY REDUCING CELL VIABILITY AND INDUCING RESISTANCE TO VENETOCLAX-BASED THERAPIES

Sofía Toribio-Castelló¹, Claudia Pérez-Carretero¹, Ignacio Tuñón¹, Marta Martín-Izquierdo¹, Raúl Azibeiro¹, David Sanz¹, Alberto Rodríguez-Sánchez¹, Sandra Santos¹, Sergio Matarraz², Teresa González¹, María Díez Campelo^{3,4}, Mónica Del Rey¹, Jesús María Hernández Rivas^{1,3,4}

List of supporting information:

- **Supplementary Table S1:** Somatic mutations detected in the four generated cell lines by NGS with an in-house custom capture-enrichment panel.
- **Supplementary Table S2:** Combination index of VEN-AZA (a) and VEN-AraC (b) drug combinations in all four edited-SKM1 cell lines.
- **Supplementary Figure S1:** Lentiviral plasmid containing *STAG2*-wild type gene (green) and GFP (blue) and puromicine resistant (red) as gene reporters.
- **Supplementary Figure S2:** Generation efficiency and validation of edited-SKM1 cell lines with *STAG2* restoration and *NRAS*-G12D mutation.
- **Supplementary Figure S3:** Differentiation markers expression in all SKM-1-edited cell lines analyzed by flow cytometry.
- **Supplementary Figure S4:** RNA-seq GSEA analysis of deregulated gene sets in the double-mutant SKM1-edited cell lines (*STAG2*^{mut}-*NRAS*^{mut}).

Supplementary Table S1: Somatic mutations detected in the four generated cell lines by NGS with an in-house custom capture-enrichment panel.

Gene	Chr	Start	End	Ref	Alt	Function	Exonic consequence	AA change & Transcript	NAF STAG2wt, NRASwt	NAF STAG2mut, NRASwt	NAF STAG2wt, NRASmut	NAF STAG2mut, NRASmut
EZH2	chr4	148811635	148811635	T	C	exonic	nonsynonymous SNV	EZH2:NM_001203249:exon15:c. A1769G;p.Y590C	99,22	100	100	99,47
KRAS	chr11	25225713	25225713	T	G	exonic	nonsynonymous SNV	KRAS:NM_004985:exon4:c.A351 C;p.K117N	100	99,64	100	98,72
TP53	chr12	7674220	7674220	C	T	exonic	nonsynonymous SNV	TP53:NM_001126115:exon3:c.G 347A;p.R116Q	100	100	100	99,19
ASXL1	chr17	32434485	32434485	C	A	exonic	stopgain	ASXL1:NM_015338:exon12:c.C1 773A;p.Y591X	99,58	100	100	99,5
BCORL1	chrX	130050716	130050716	G	A	splicing	NA	NA	99,53	100	100	100
BCOR	chr20	40052401	40052401	C	T	splicing	NA	NA	100	99,58	100	100
TET2	chr1	105272633	105272633	-	TT	exonic	frameshift insertion	TET2:NM_001127208:exon10:c.4 252_4253insTT;p.L1418fs	47,26	47,71	48,54	42,57
WT1	chr9	32435055	32435061	GCTCACA	-	exonic	frameshift deletion	WT1:NM_000378:exon1:c.285_2 91del;p.P95fs	47,31	39,69	43,28	52,51
SF1	chr11	64766985	64766985	T	C	exonic	nonsynonymous SNV	SF1:NM_201997:exon12:c.A150 1G;p.M501V	53,01	53,19	47,28	49,55
SF1	chr11	64767012	64767012	T	G	exonic	nonsynonymous SNV	SF1:NM_201997:exon12:c.A147 4C;p.T492P	6,59	7,47	6,67	8,31
NRAS	Chr	114716126	114716126	C	T	exonic	nonsynonymous SNV	NRAS:NM_002524:exon2:c.G35A :p.G12D	NO	NO	48,76	47,11
STAG2	chrX	124047461	124047461	C	T	exonic	stopgain	STAG2:NM_006603:exon8:c.C77 5T;p.R259X	45,86	99,53	42,77	99,49

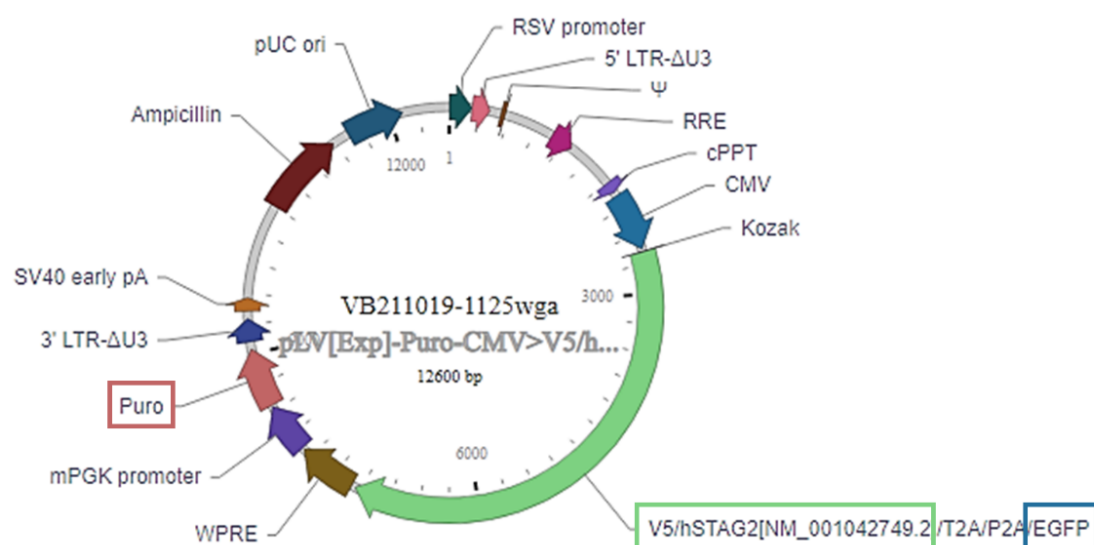
Supplementary Table S2: Combination index of VEN-AZA (a) and VEN-AraC (b) drug combinations in all four edited-SKM1 cell lines.

a) Combination Index VEN-AZA

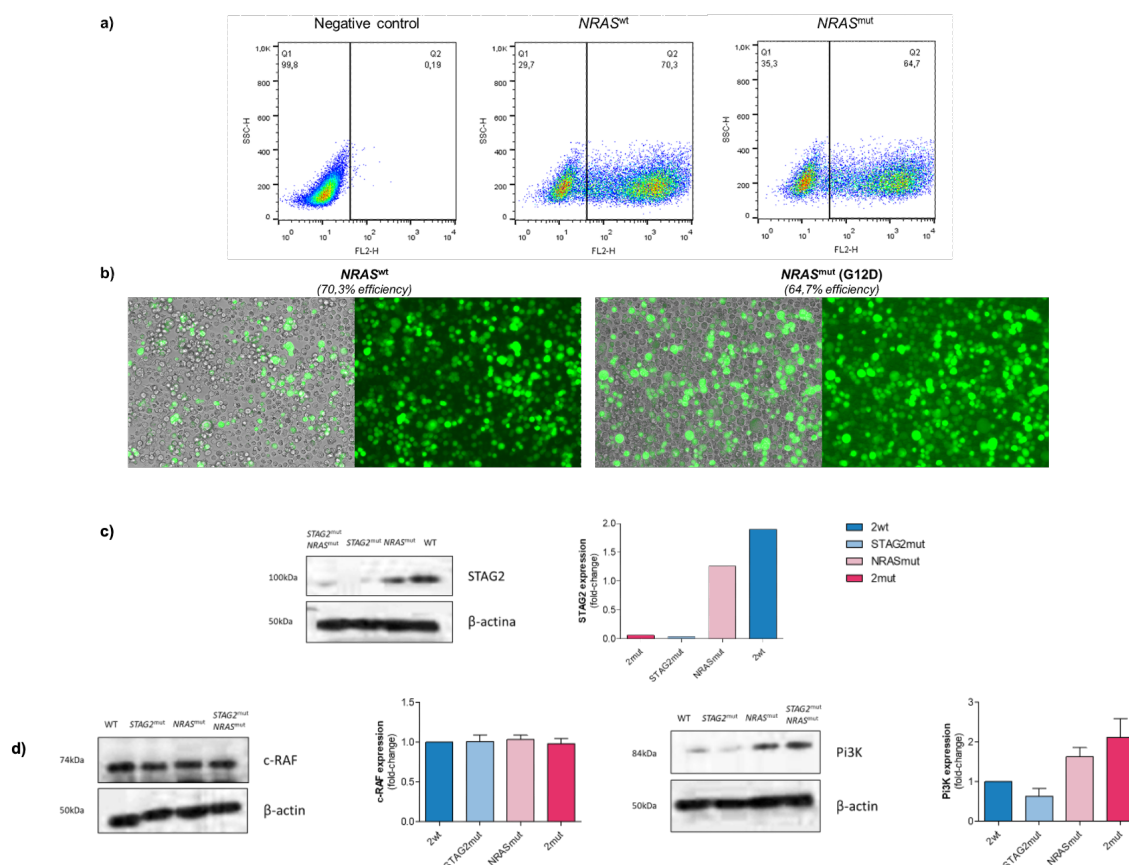
Venetoclax (μ M)	5-azacitidine (μ M)	<i>STAG2</i> ^{wt} <i>NRAS</i> ^{wt}	<i>STAG2</i> ^{mut} <i>NRAS</i> ^{wt}	<i>STAG2</i> ^{wt} <i>NRAS</i> ^{mut}	<i>STAG2</i> ^{mut} <i>NRAS</i> ^{mut}
0.15	3	0.77625	0.91875	1.05525	0.95525
0.25	5	0.79	0.85125	1.03625	1.0925
0.4	7.5	1.12525	0.9815	0.984	1.23725
0.6	10	1.53	1.1575	1.22525	1.61475

b) Combination Index VEN-AraC

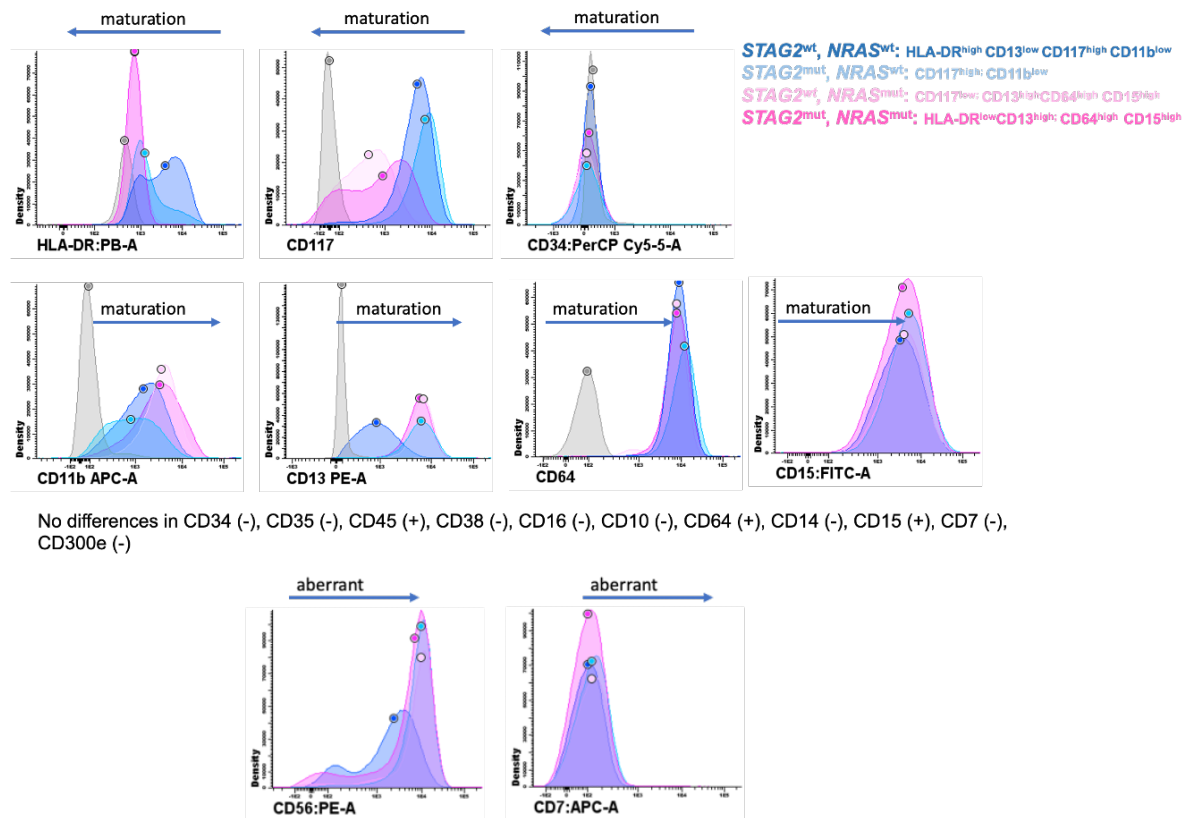
Venetoclax (μ M)	Citarabine (μ M)	<i>STAG2</i> ^{wt} <i>NRAS</i> ^{wt}	<i>STAG2</i> ^{mut} <i>NRAS</i> ^{wt}	<i>STAG2</i> ^{wt} <i>NRAS</i> ^{mut}	<i>STAG2</i> ^{mut} <i>NRAS</i> ^{mut}
0.15	0.75	0.5885	0.67075	0.8465	0.73875
0.25	1.25	0.593	0.63175	0.72375	0.78325
0.4	2	0.78475	0.829	0.91775	1.0475
0.6	3	1.102	1.083	1.11775	1.28125



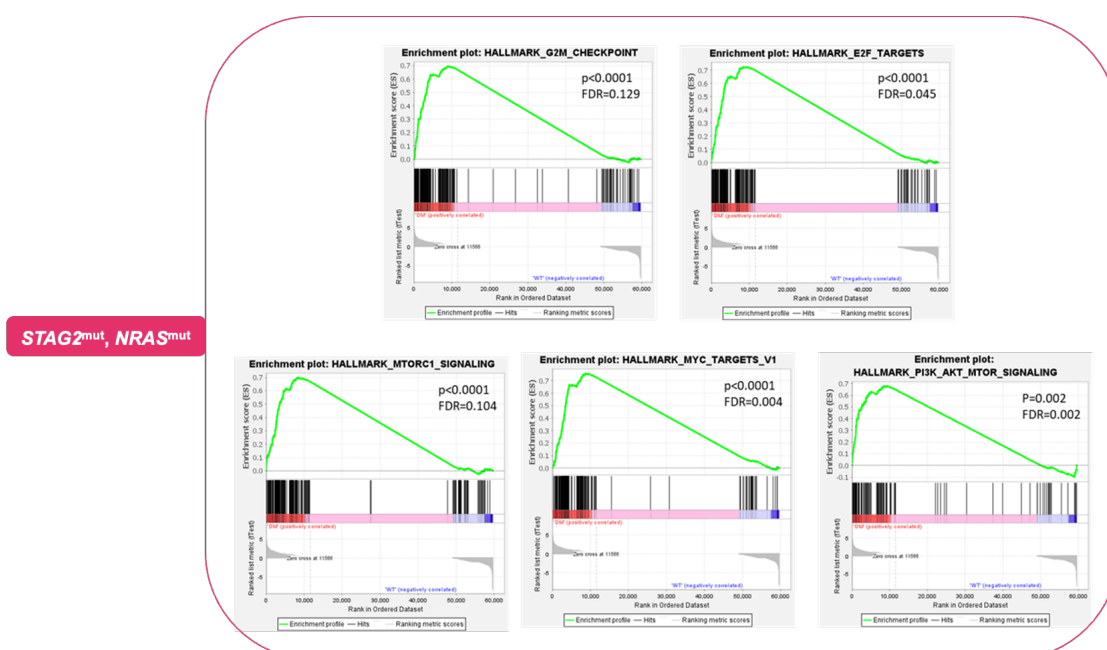
Supplementary Figure S1: Lentiviral plasmid containing STAG2-wild type gene (green) and GFP (blue) and puromycin resistant (red) as gene reporters.



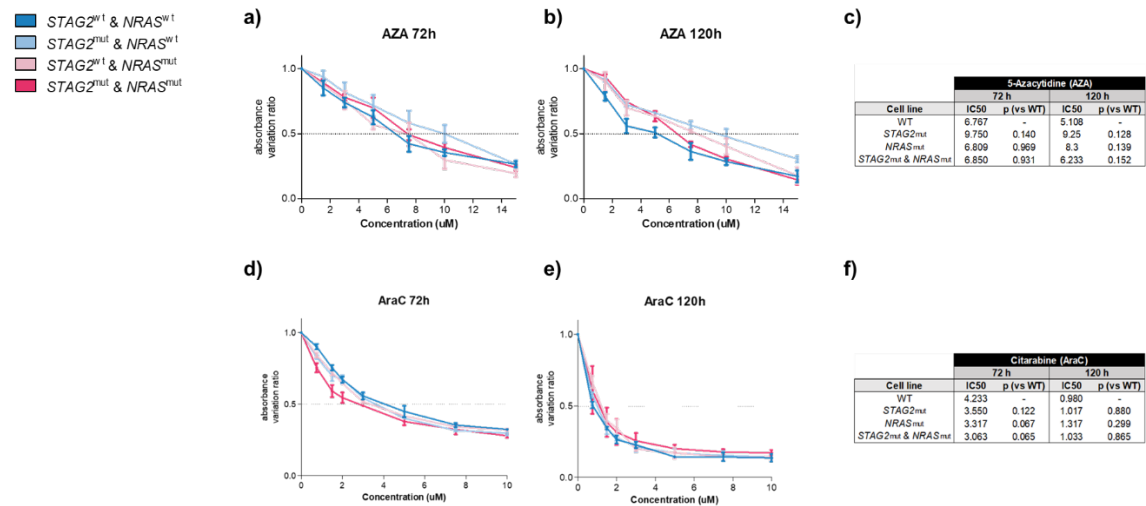
Supplementary Figure S2: Generation efficiency and validation of edited-SKM1 cell lines with STAG2 restoration and NRAS-G12D mutation. *a)* Lentiviral transduction efficiency of SKM-1 cell line by percentage of GFP+ events evaluated by flow cytometry in a non-transduced and NRAS^{wt} and NRAS^{mut} transduced-SKM-1 cells; *b)* Image of NRAS^{wt} and NRAS^{mut} transduced-SKM-1 cell lines with a fluorescent microscopy; *c)* STAG2 protein restoration in clones transduced with lentiviral vectors containing STAG2-wild type gene; *b, d)* Expression of c-Raf (*b*) and Pi3K (*c*) signalling protein to validate the functional insertion of NRAS_G12D mutation.



Supplementary Figure S3: Differentiation markers expression in all SKM-1-edited cell lines analyzed by flow cytometry.



Supplementary Figure S4: RNA-seq GSEA analysis of deregulated gene sets in the double-mutant SKM1-edited cell lines (STAG2^{mut}-NRAS^{mut}).



Supplementary Figure S5: Drug response to AZA and AraC in SKM1-generated cell lines. a) Percentage of survival of SKM1-edited cell lines in response to AZA at 72h, measured as absorbance variation ratio; b) percentage of survival of all generated cell lines in response to AZA after 120h of drug exposition; c) IC50 of AZA in all edited-cell lines after 72h and 120h of treatment; d) Absorbance variation ratio of all edited-cell lines representing the percentage of survival after 72h of AraC treatment; e) Percentage of survival of SKM1-edited cell lines in response to AraC at 120h; f) IC50 of SKM1 cell lines under AraC treatment at 72 and 120 hours.

