Facultad de Ciencias Biológicas

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TESIS DOCTORAL

ESTUDIO MOLECULAR DEL CÁNCER DE PULMÓN NO MICROCÍTICO AVANZADO MEDIANTE TECNOLOGÍAS DE ALTO RENDIMIENTO PARA LA APLICACIÓN DE LA MEDICINA DE PRECISIÓN

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CERTIFICA:

Que la presente Tesis Doctoral, titulada:

"**ESTUDIO MOLECULAR DEL CÁNCER DE PULMÓN NO MICROCÍTICO AVANZADO MEDIANTE TECNOLOGÍAS DE ALTO RENDIMIENTO PARA LA APLICACIÓN DE LA MEDICINA DE PRECISIÓN**"

Ha sido realizada bajo mi dirección por Javier Simarro Farinós, graduado en Biotecnología.

Y para que así conste, a todos los efectos oportunos, expide y firma la presente certificación, en Valencia a 26 de enero de dos mil veintitrés.

Fdo: Dra. Sarai Palanca Suela

La realización de la presente Tesis Doctoral ha sido realizada con las siguientes ayudas: ACIF/2018/258, IIS-LaFe 2016/0129, IIS-LaFe 2015/0713, IIS-LaFe 2017/0070, Acciones Preparatorias IIS-LaFe-UPV 2019-103-1, AEACaP-GIDO 2021-331-1 y GV/2021/144, IIS-LaFe 2017/0070

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LISTADO DE ABREVIATURAS

ADN: Ácido desoxirribonucleico.

ADNtc: ADN tumoral circulante.

ANtc: Ácido nucleico tumoral circulante.

ARN: Ácido ribonucleico.

ATP: Adenosín trifosfato.

CE-IVD: Certificación europea de uso en diagnóstico *in vitro*.

CPCP: Cáncer de pulmón de célula pequeña.

CPNM: Cáncer de pulmón no microcítico.

ddPCR: *Digital droplet PCR*.

ENAC: Entidad Nacional de Acreditación.

FDA: *Food and Drug Administration*.

FFPE: Fijación en formol e inclusión en parafina (*formalin-fixed paraffin-embedded*).

FISH: Hibridación Fluorescente *in situ*.

GTP: Guanosín trifosfato.

HR: *Hazard Ratio*.

IHQ: Inmunohistoquímica.

ITQ: Inhibidor tirosina quinasa.

LOD: Límite de detección *(Limit of detection*).

MAPK: Vía de señalización de las proteínas quinasas activadas por mitógenos.

NGS: Secuenciación masiva (*Next Generation Sequencing*).

PCR: Reacción en cadena de la polimerasa.

PCRd: PCR digital.

PNA: Ácido nucleico peptídico (*Peptide Nucleic Acid*)

RT-PCR: PCR en tiempo real.

SG: Supervivencia global.

SLP: Supervivencia libre de progresión.

TAT: Tiempo de respuesta (*Turnaround time*).

VNC: Variación en el número de copias.

WT: *Wild Type.*

INTRODUCCIÓN

1. MEDICINA DE PRECISIÓN EN CÁNCER DE PULMÓN NO MICROCÍTICO.

El cáncer de pulmón es una de las principales causas de morbimortalidad en nuestro país. Las estimaciones realizadas para el año 2022 lo sitúan como el tercer tipo tumoral más frecuentemente diagnosticado con 30.948 nuevos casos, y los registros del año 2020 lo consolidan como la principal causa de muerte por cáncer con 21.918 fallecimientos registrados [1]. A nivel histológico el cáncer de pulmón no microcítico (CPNM) comprende entre el 80-85% de los casos [2,3] y aproximadamente un 40% de los mismos son diagnosticados en enfermedad avanzada o metastásica [4].

En las últimas décadas el abordaje terapéutico del CPNM avanzado ha experimentado una revolución sin precedentes. Los estudios *ómicos* como la secuenciación masiva o *Next-Generation Sequencing* (NGS), han permitido un mejor conocimiento de las bases genómicas del cáncer, identificado alteraciones moleculares responsables del desarrollo y proliferación de los tumores; siendo factible plantear una clasificación del CPNM en subgrupos o entidades moleculares [5–7] (Figura 1). Estos avances en la biología molecular del CPNM han permitido el desarrollo de fármacos dirigidos capaces de bloquear de forma específica la señalización proteica aberrante que lidera la proliferación tumoral [8,9].

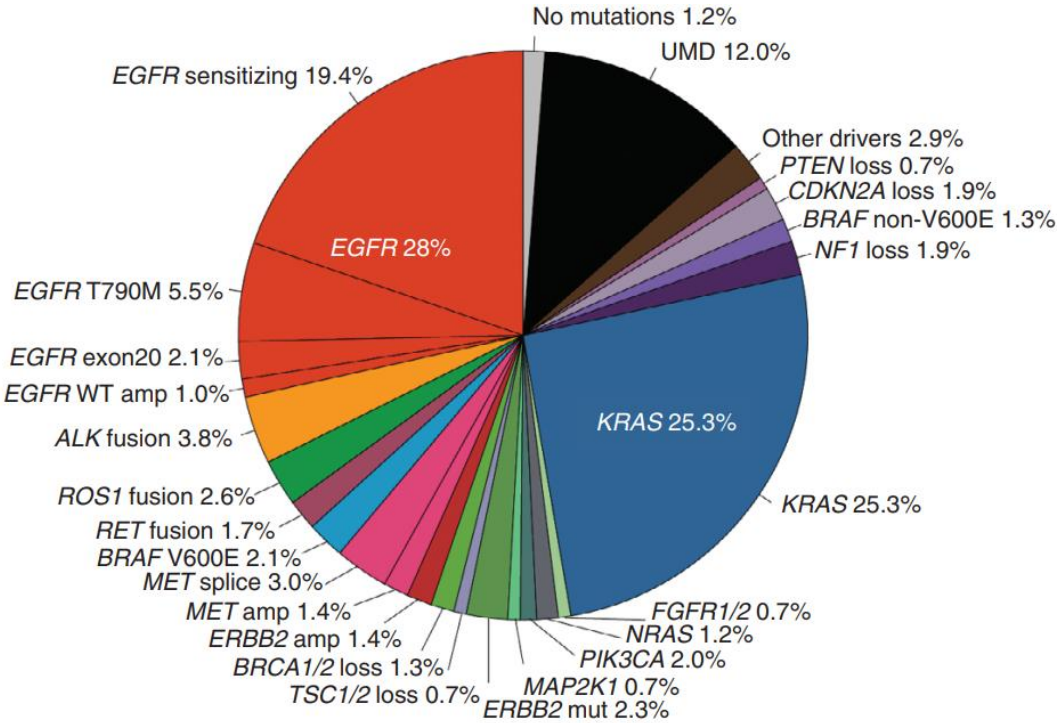

Figura 1. Clasificación del CPNM en entidades moleculares (Adaptada de Jordan EJ *et al*., 2017).

Con ello, se ha producido un cambio paradigmático en el enfoque clínico del CPNM conocido como medicina de precisión. La transición del empleo de esquemas de tratamiento inespecíficos a la selección del tratamiento basado en marcadores moleculares predictivos ha permitido mejorar la eficacia terapéutica en términos de supervivencia global (SG) así como disminuir la toxicidad asociada a los tratamientos [10–12]. Este nuevo enfoque, ha consolidado a los laboratorios de diagnóstico molecular como un elemento esencial para el manejo clínico del paciente debido a la creciente necesidad de una mayor caracterización molecular de la enfermedad.

Sin embargo, los laboratorios deben enfrentarse a la necesidad de ofrecer un perfil molecular de la enfermedad superando diversos retos tecnológicos. Las limitaciones intrínsecas a los tipos de muestras biológicas, junto con la inclusión de nuevos biomarcadores relevantes y la necesidad de evaluar la enfermedad a la progresión, plantean un escenario cambiante que requiere de la investigación traslacional para incorporar y garantizar la calidad de nuevos estudios moleculares a la realidad asistencial [13].

1.1. Implicaciones terapéuticas de la clasificación molecular del CPNM.

Diversas alteraciones moleculares oncogénicas definen grupos de pacientes que pueden beneficiarse del tratamiento con terapias dirigidas. La medicina de precisión se ha expandido desde 2015, cuando las recomendaciones de diagnóstico molecular únicamente incluían el estudio de *EGFR* y *ALK* [14], hasta la actualidad, con la inclusión de otros grupos moleculares: reordenamientos de *ROS1*, mutaciones en el codón 600 del gen *BRAF,* reordenamientos de *NTRK,* reordenamientos de *RET*, duplicaciones/inserciones del exón 20 de *EGFR*, mutaciones de salto del exón 14 del gen *MET* (*MET*-Ex14) y la mutación G12C en el gen *KRAS* [15] (Figura 2). Actualmente, todos los pacientes con CPNM de histología no escamosa son candidatos a estudio molecular, así como los pacientes con histología escamosa que sean jóvenes (<50 años), no fumadores o con baja carga tabáquica (<10 años/paquete) [16].

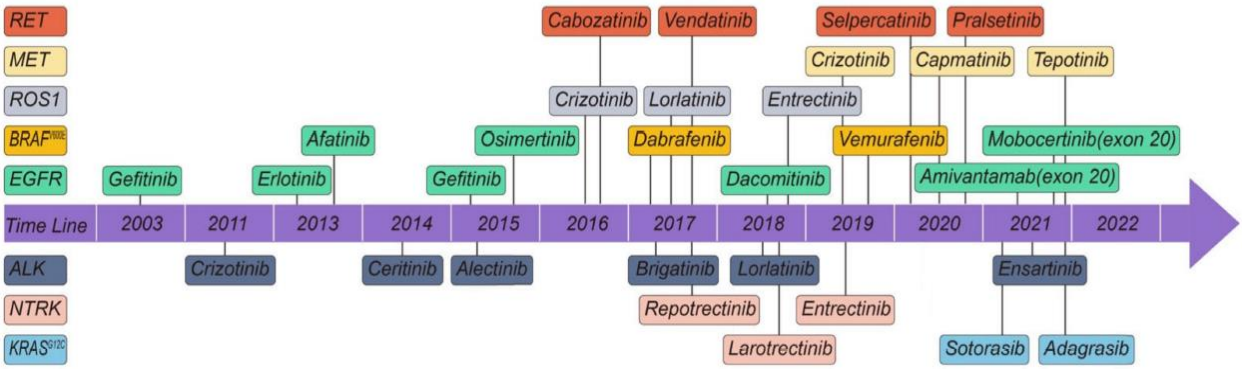

Figura 2. Cronología de la aprobación de terapias dirigidas en CPNM por la *Food and Drug Administration*, FDA (Adaptada de Guo H *et al*., 2022).

1.1.1. Mutaciones en *EGFR*.

Las mutaciones activantes del dominio tirosina quinasa (exones 18, 19, 20 y 21) del receptor del factor de crecimiento epidérmico (*EGFR*), se detectan en aproximadamente un 15% de los pacientes y provocan una activación constitutiva de la vía de señalización de las proteínas quinasas activadas por mitógenos (MAPK). Las células tumorales portadoras de estas mutaciones son sensibles al tratamiento con inhibidores tirosina quinasa de EGFR (EGFR-ITQs) como erlotinib, gefitinib, afatinib, dacomitinib y osimertinib, que son capaces de unirse de forma reversible o irreversible al dominio tirosina quinasa del EGFR mutado bloqueando la unión del ATP y la activación de la cascada de señalización [17,18]. Por otra parte, las duplicaciones/inserciones detectadas en el exón 20 que se asocian a resistencia al tratamiento con EGFR-ITQs, recientemente se han consolidado como biomarcador de respuesta a amivantamab, un anticuerpo bi-específico que mediante la unión al dominio extracelular del receptor bloquea la señalización aberrante de EGFR con estas alteraciones [19].

1.1.2. Fusiones de *ALK*.

Un grupo de pacientes con CPNM que se beneficia de terapias dirigidas muy eficaces son los portadores de reordenamientos del gen *ALK (4-5%)*, siendo el más frecuente aquel que yuxtapone el extremo 5’ del gen *EML4* con el extremo 3’ del gen *ALK*. Las proteínas de fusión que incluyen el extremo 3’ de ALK presentan potencial oncogénico al carecer de la regulación de la expresión del gen *ALK* normal o *wild-type* (WT) y estar constitutivamente activas [20]. Esta alteración molecular predice la sensibilidad al tratamiento con ALK-ITQs como crizotinib, ceritinib, alectinib, brigatinib y lorlatinib.

1.1.3. Salto del exón 14 del gen *MET.*

Otro biomarcador relevante en CPNM es el gen *MET*, que codifica para un receptor tirosina quinasa. En un 3-4% de los pacientes se detectan mutaciones que provocan la pérdida del exón 14 durante el proceso de corte y empalme, o *splicing* del ARN. Las proteínas carentes de este exón se degradan en menor medida adquiriendo potencial oncogénico. Terapias dirigidas como tepotinib y capmatinib han demostrado su eficacia en este subgrupo de pacientes al inhibir la fosforilación del receptor MET [21].

1.1.4. Mutaciones en *KRAS.*

Recientemente, la medicina de precisión se ha trasladado también a los pacientes portadores de mutaciones en el gen *KRAS*, uno de los grupos moleculares más relevantes en CPNM, en los que la activación constitutiva de esta proteína debido a la imposibilidad de hidrolizar el GTP lidera la señalización celular aberrante [22]. Concretamente, la mutación p.(Gly12Cys) detectada hasta en un 13% de los pacientes predice la sensibilidad al tratamiento con sotorasib, molécula capaz de unirse de forma covalente al aminoácido 12Cys en la conformación inactiva de *KRAS* bloqueando la proteína en este estado [23].

1.1.5. Otras alteraciones.

Otras alteraciones minoritarias pero relevantes para aplicar la medicina de precisión son las mutaciones en el codón 600 del gen *BRAF* (2-3%.); los pacientes portadores se benefician del tratamiento combinado con dabrafenib (inhibidor de quinasas RAF) y trametinib (inhibidor de las quinasas MEK) [24]. Asimismo, los pacientes con reordenamientos de *ROS1* (1%) [25], *RET* (1%) [26] o genes *NTRK* (<1%) [27] se benefician del tratamiento dirigido con inhibidores de estas proteínas: crizotinib y entrectinib (ROS1), selpercatinib y pralsertinib (RET) y, larotrectinib y entrectinib (NTRK).

1.2. Mecanismos de resistencia a las terapias dirigidas.

A pesar de las buenas respuestas clínicas alcanzadas con tratamientos dirigidos, los tumores tienen la capacidad de adaptarse a la presión ejercida por el tratamiento desarrollando diversos mecanismos de resistencia que liderarán la progresión tumoral [28,29]. La caracterización molecular de la enfermedad en el momento de la progresión radiológica resulta esencial debido a la aprobación de tratamientos dirigidos capaces de superar determinados mecanismos de resistencia [30].

1.2.1. Modelos de aparición de los mecanismos de resistencia.

El CPNM se caracteriza por ser una enfermedad heterogénea en la que coexisten diversos clones tumorales con alteraciones moleculares diversas [31]. En ciertos pacientes es posible identificar mecanismos de resistencia de forma minoritaria en el momento del diagnóstico que, debido a la presión selectiva del tratamiento, proliferarán convirtiéndose en dominantes en la progresión (modelo de selección). En otros casos, estos mecanismos no se detectan al diagnóstico, sino que podrían adquirirse durante el tratamiento (modelo de adquisición) [32] (Figura 3). Consecuencia de las limitaciones espaciales del muestreo de las lesiones tisulares, es posible que la muestra estudiada no sea completamente representativa del tumor pudiendo existir el clon portador del mecanismo de resistencia en otra región de la lesión o incluso en alguna metástasis.

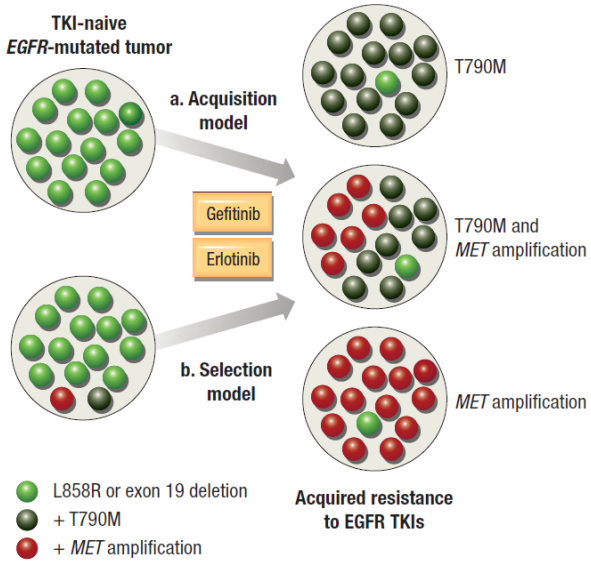

Figura 3. Modelos propuestos para la aparición de mecanismos de resistencia (Adaptada de Nguyen KS *et al*. 2009)

La presión selectiva del tratamiento dirigido, potenciará una plasticidad tumoral de la enfermedad con implicaciones clínicas [33]. En este sentido, un mejor entendimiento de los mecanismos de resistencia puede ofrecer una visión de la evolución clonal de la enfermedad durante el tratamiento para anticipar progresiones radiológicas y orientar líneas de tratamiento posteriores.

Con todo ello, además de los estudios moleculares al diagnóstico, la caracterización molecular de la enfermedad a la progresión es uno de los principales retos de los laboratorios de diagnóstico molecular ya que la identificación de determinados mecanismos de resistencia accionables mediante terapias dirigidas permite extender la medicina de precisión.

1.2.2. Mutación *EGFR*-Thr790Met.

El caso paradigmático de mecanismo de resistencia por su prevalencia y alternativas terapéuticas es la mutación c.2369C>T; p.(Thr790Met) en el gen *EGFR* descrita hasta en un 50-60% de los pacientes que progresan al tratamiento con EGFR-ITQs de 1ª y 2ª generación (erlotinib, gefitinib, afatinib y dacomitinib). Esta mutación, restablece la afinidad del EGFR mutado por el ATP a la vez que provoca alteraciones estéricas que afectarían a la unión de los ITQs [34]. La detección de esta mutación en el momento de la progresión radiológica permite al paciente iniciar una nueva línea de tratamiento dirigido con osimertinib, un EGFR-ITQ de 3ª generación capaz de unirse de forma irreversible al dominio tirosina quinasa del receptor EGFR mutado, tanto en ausencia como en presencia de la mutación de resistencia p.(Thr790Met) [35]. Por otro lado, la posterior aprobación de este fármaco como tratamiento de primera línea ha evidenciado los distintos mecanismos de resistencia que se desarrollan en este contexto respecto de su empleo como tratamiento de segunda línea tras la detección de p.(Thr790Met) [36].

1.2.3. Mutaciones en el dominio tirosina quinasa de *ALK*.

En el caso de las fusiones de *ALK*, la presencia de mutaciones puntuales en el dominio tirosina quinasa de ALK reordenado constituye el mecanismo de resistencia más relevante desde el punto de vista de la elección de nuevas estrategias terapéuticas. Estas mutaciones afectan a la unión de los ITQs a la proteína de fusión confiriendo distintos grados de resistencia para los ITQs aprobados. Los esfuerzos realizados en la caracterización de estas mutaciones y la evaluación del grado de sensibilidad y/o resistencia a los distintos fármacos aprobados permiten la elección de la siguiente línea de tratamiento basada en el perfil molecular de la enfermedad [37].

1.2.4. Otros mecanismos.

Los mecanismos de resistencia a las terapias dirigidas son dependientes del fármaco empleado y de las líneas de tratamiento previas, detectándose en frecuencias variables dependiendo de las series consultadas. Mecanismos de resistencia como la mutación *EGFR*-p.(Cys797Ser), la amplificación del gen *MET,* las alteraciones en los genes que regulan el ciclo celular y la transformación histológica han adquirido especial relevancia por su frecuencia y/o futuras opciones terapéuticas [28,36].

1.3. Muestras biológicas utilizadas en el diagnóstico molecular.

1.3.1 Biopsias tumorales y citologías.

En el CPNM, las muestras tumorales deben garantizar un diagnóstico patológico adecuado, así como preservar suficiente material para los estudios moleculares requeridos. Las biopsias tumorales y las citologías constituyen el estándar del diagnóstico molecular en la práctica clínica ya que el informe anatomopatológico permite garantizar que la muestra contiene suficiente número de células tumorales, así como un porcentaje tumoral adecuado para las técnicas analíticas. El manejo de estas muestras, responde a recomendaciones consolidadas para maximizar el aprovechamiento de la muestra y preservar la calidad de los ácidos nucleicos [16].

Sin embargo, los estudios moleculares deben ser capaces de superar los impedimentos intrínsecos del diagnóstico molecular en esta patología. Se estima que hasta en un 20-40% de los pacientes no es posible obtener muestras tisulares o citológicas [38,39]. El limitado tamaño de las muestras obtenidas compromete los estudios moleculares, ya que en enfermedad avanzada rara vez se dispone de muestras quirúrgicas [40]. El diagnóstico del CPNM es un proceso multietapa que puede provocar el agotamiento o escasez de la muestra y en último término la cantidad de ácidos nucleicos aislada sea limitante para realizar los estudios moleculares pertinentes.

Los ácidos nucleicos extraídos a partir de material tumoral fijado en formaldehído e incluido en parafina (FFPE) suelen ser de baja calidad. Durante los procesos de fijación e inclusión en parafina los ácidos nucleicos pueden fragmentarse excesivamente comprometiendo su amplificación por PCR. En este sentido, se han descrito tasas de fallo que pueden oscilar entre el 10-30% en función de la metodología empleada [41–43]. Además, en los procesos de fijación e inclusión los ácidos nucleicos sufren modificaciones en su estructura química, principalmente desaminación de citosinas (C-T) que puede dar lugar a falsos positivos por introducción de mutaciones artificiales [44–46].

Además de los estudios moleculares al diagnóstico, la creciente necesidad de abordar la caracterización molecular del tumor a la progresión para orientar nuevas estrategias terapéuticas requiere de la obtención de una nueva muestra tisular de aquella(s) lesión(es) en las que se haya objetivado la progresión radiológica. Sin embargo, dependiendo de su localización y del estado del paciente se estima que hasta en un 20% de los casos no es posible obtener una nueva muestra tumoral [47,48].

1.3.2 Biopsias líquidas.

En los últimos años ha surgido una nueva aproximación para el diagnóstico molecular que permite la caracterización molecular de la enfermedad en ausencia de una muestra tisular o citológica. La biopsia líquida se define como el estudio de componentes tumorales de distinta naturaleza que se liberan a sangre periférica y/u otros fluidos biológicos por necrosis o apoptosis de las células tumorales, por liberación activa o en forma de vesículas extracelulares [49] (Figura 4).

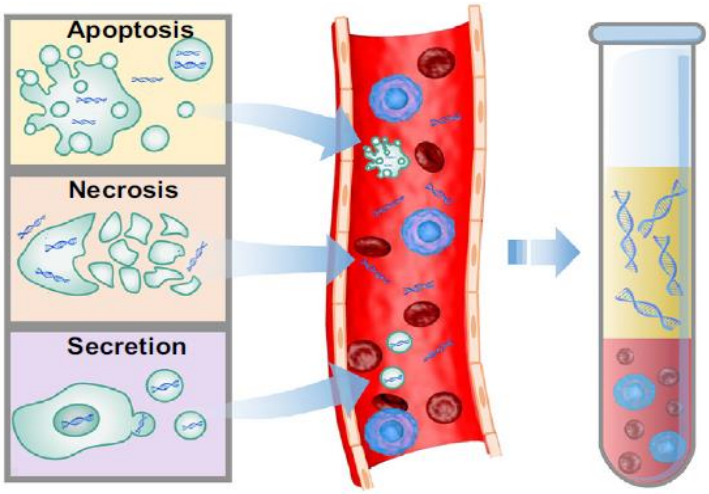

Figura 4. Mecanismos de liberación de ANtc (Adaptada de Luo H *et al*. 2021 [50])

Dentro de este abanico de material de origen tumoral, la detección de ácidos nucleicos tumorales circulantes (ANtc) ha tenido un mayor desarrollo y aplicación en la clínica. De hecho, los estudios de biopsia líquida constituyen una realidad asistencial en el CPNM avanzado como complemento a los estudios en muestras tumorales tisulares. Las guías clínicas y recomendaciones de diagnóstico enmarcan su uso en ausencia de muestra tisular adecuada para los estudios moleculares al diagnóstico y como primera aproximación para la caracterización molecular de la progresión radiológica al tratamiento con EGFR-ITQs de 1ª y 2ª generación [51–55].

La implementación de los estudios de biopsia líquida a la rutina asistencial del paciente con CPNM es especialmente relevante. Las biopsias líquidas constituyen una alternativa poco invasiva y menos costosa con tiempos de respuesta más rápidos [56]. Además, estos estudios permiten abordar la heterogeneidad tumoral al estudiar los ANtc liberados por todas las lesiones tumorales estando más representadas aquéllas con un comportamiento más agresivo [57]. Los estudios longitudinales de BL durante el seguimiento al tratamiento permiten evaluar la dinámica clonal de la enfermedad, y detectar mecanismos de resistencia antes de la progresión radiológica [58].

Sin embargo, la biopsia líquida es una aproximación indirecta al estudio molecular del tumor. Los ANtc suelen representar una pequeña proporción de todo el material genético circulante. Consecuentemente, la biopsia líquida se caracteriza por presentar una sensibilidad subóptima, siendo frecuente la no detección de alteraciones moleculares en plasma que sí se detectan en la correspondiente muestra de tejido tumoral [54,56].

La cantidad de ANtc es muy variable entre pacientes, siendo dependiente de múltiples factores como son la localización e irrigación de las lesiones tumorales, su tamaño, su tasa de proliferación, así como del estadio de la enfermedad [59]. Adicionalmente, la traslación de los estudios de biopsia líquida a la rutina clínica se ve comprometida por los requerimientos pre-analíticos. Es necesario controlar el tiempo entre la extracción de sangre y la separación del plasma debido a la corta vida media de los ANtc así como para evitar su dilución con material genético no tumoral procedente de las células sanguíneas [60].

Un control exhaustivo de las condiciones pre-analíticas y la puesta a punto de técnicas moleculares de elevada sensibilidad, como son la NGS o la PCR digital (PCRd), puede aumentar la tasa de detección de los estudios de biopsia líquida mejorando su aplicabilidad a la clínica [61,62].

1.4. Técnicas de diagnóstico molecular convencionales.

La incorporación a los laboratorios de técnicas relativamente sencillas y estandarizadas, como la secuenciación directa o método de *Sanger*, la PCR en tiempo real (RT-PCR), la inmunohistoquímica (IHQ) o la hibridación fluorescente *in situ* (FISH) han permitido la detección de alteraciones moleculares concretas en un solo gen (*single-test*). Estas técnicas han constituido el estándar del diagnóstico molecular en la era de la medicina de precisión [63].

1.4.1. Secuenciación directa o método de *Sanger.*

La amplificación por PCR de las regiones de interés seguida de la secuenciación mediante el método de *Sanger* o secuenciación directa, constituyó la primera estrategia para la detección de mutaciones somáticas a partir de muestras tisulares. Sin embargo, su límite de detección (LOD) cercano al 20% de frecuencia alélica puede generar falsos negativos en muestras con una baja celularidad tumoral. No obstante, al ser un método basado en la secuenciación del ADN permite la detección de mutaciones no descritas o poco frecuentes y es una técnica actualmente empleada para la confirmación y caracterización de mutaciones [64].

1.4.2. PCR en tiempo real (RT-PCR)

Para suplir las limitaciones de la secuenciación *Sanger* surgen aproximaciones de PCR en tiempo real (RT-PCR) que mediante el empleo de sondas y/o cebadores marcados con fluoróforos permiten detectar mutaciones con LOD cercanos al 5%. Estas aproximaciones son rápidas y de fácil interpretación. Sin embargo, únicamente permiten la detección de las variantes especificadas por el fabricante [64].

1.4.3. Inmunohistoquímica (IHQ).

La inmunohistoquímica (IHQ) se basa en la interacción entre anticuerpos específicos frente a determinados dominios de la proteína diana. Aplicada al diagnóstico molecular esta técnica permite, mediante la cuantificación de la intensidad de la expresión y de la localización proteica, detectar fusiones génicas [65]. Es una técnica económica y rápida que permite la detección de fusiones independientemente del gen acompañante. Sin embargo, la IHQ ha demostrado limitaciones en la detección de fusiones en determinados subtipos histológicos del CPNM y en muestras con amplificaciones génicas [66].

1.4.4. Hibridación fluorescente in situ (FISH).

Mediante la hibridación fluorescente in situ (FISH) es posible la detección de alteraciones cromosómicas estructurales como grandes deleciones, inversiones duplicaciones y translocaciones. Esta técnica se basa en la hibridación de grandes sondas de ADN marcadas fluorescentemente con cromosomas en interfase de la muestra tumoral. El análisis de la fluorescencia de cada sonda permite detectar alteraciones en la estructura del cromosoma. En el diagnóstico molecular del CPNM esta técnica se ha empleado principalmente para la detección de reordenamientos, destacando por no ser dependiente del gen acompañante. [65]. Sin embargo, la correcta interpretación de las señales se requiere amplia experiencia [66].

1.4.5. Limitaciones de las técnicas *single-test.*

Las técnicas de *single-test* o de gen único han sido ampliamente validadas para la detección de mutaciones en *EGFR* y de fusiones de *ALK* y *ROS1* entre otros, por lo que su uso en los laboratorios de diagnóstico molecular está ampliamente extendido [67]. No obstante, debido al creciente número de genes con relevancia clínica que es necesario estudiar, su aplicación de forma sucesiva puede implicar el agotamiento de la muestra [68]. En este escenario, el diagnóstico molecular basado en aproximaciones de gen único ha mostrado sus limitaciones siendo necesaria una continua actualización metodológica para satisfacer las crecientes demandas de la medicina de precisión.

1.5. Secuenciación masiva o *Next-Generation Sequencing* (NGS).

1.5.1. Concepto.

La NGS es una tecnología de secuenciación de ácidos nucleicos de alto rendimiento que permite el análisis de forma simultánea de diversas regiones del genoma. [69,70]. Es capaz de abordar el estudio de alteraciones moleculares de distinta naturaleza (mutaciones puntuales, pequeñas inserciones/deleciones, variación en el número de copias (VNC) y transcritos de fusión) a partir de pequeñas cantidades de ADN y/o ARN.

Mediante la NGS es posible abordar la secuenciación del genoma o el exoma completos; sin embargo, la estrategia de NGS más extendida en los laboratorios de diagnóstico molecular es el empleo de paneles de genes que incluyen un número variable de genes de interés para distintas neoplasias [71].

1.5.2. Tecnologías de secuenciación.

Las tecnologías de NGS de *Ion Torrent* e *Illumina* han tenido un mayor desarrollo en el ámbito del diagnóstico molecular. A grandes rasgos comparten una serie de pasos comunes [70]. En primer lugar, se realiza una selección de las regiones a estudiar, mediante amplificación por PCR (*Ampliseq, Ion Torrent*) o mediante captura con sondas específicas (*Illumina*). Seguidamente a estos fragmentos se les añaden secuencias conocidas de nucleótidos que actúan como adaptadores para amplificaciones posteriores o como *barcodes*/etiquetas que permiten asociar las secuencias generadas con la muestra correspondiente.

El siguiente paso consiste en la amplificación clonal de las librerías generadas, mediante PCR en emulsión (*Ion Torrent*) o mediante PCR puente sobre un soporte sólido (*Illumina*). En ambas estrategias el objetivo es producir un conjunto de moléculas clonales para generar suficiente señal en el proceso de secuenciación. En ambas tecnologías, la secuenciación es por síntesis ya que se detecta la adición del nucleótido a la cadena creciente de ADN mediante cambios de pH (*Ion Torrent*) o mediante fluorescencia (*Illumina*).

Las señales recogidas se convierten a datos de secuencias de nucleótidos que se alinean frente a la secuencia de referencia y se anotan las variantes detectadas. Finalmente es necesario evaluar el carácter patogénico, polimórfico o desconocido de estas variantes e integrar los resultados en el contexto clínico para la elaboración del informe de resultados.

1.5.3. Ventajas.

La principal ventaja de la NGS es que permite disponer de un perfil molecular del tumor más allá de las alteraciones identificadas por las técnicas convencionales. Además del estudio de alteraciones moleculares para las que existe un tratamiento dirigido aprobado, presenta el potencial de identificar otras alteraciones con potencial utilidad clínica [72,73].

El porcentaje de pacientes de CPNM portadores de alteraciones accionables es altamente dependiente del panel de genes empleado y de características clinicopatológicas de las cohortes; distintos estudios informan de porcentajes alrededor del 70% [74,75]. Por ello, ofrecer un perfil molecular de la enfermedad mediante NGS aumenta las opciones del paciente para recibir terapias dirigidas mientras que representa un respaldo a la investigación clínica traslacional [76].

La NGS ofrece una visión de la heterogeneidad intratumoral revelando relaciones de concurrencia y exclusividad mutacional que pueden tener implicaciones a nivel de respuesta a los tratamientos, especialmente en el caso de las terapias dirigidas [31,33,77]. Además, en la mayoría de las aproximaciones es posible conseguir este estudio con bajos requerimientos de muestra. Asimismo, tras ser implementada en la rutina asistencial, la NGS ha demostrado que puede ser una aproximación coste efectiva y con tiempos de respuesta aceptables [78,79].

1.5.4. Inconvenientes.

La NGS no está exenta de inconvenientes. La mayoría de los paneles de genes y reactivos empleados carecen de la marcado CE-IVD por lo que es necesaria su validación antes de su uso en rutina clínica. En este sentido, la inclusión de nuevos genes a un panel preestablecido, requiere de una nueva validación de la técnica. El análisis de resultados requiere experiencia y capacitación técnica para discriminar las variantes patogénicas de variantes de efecto desconocido y/o errores intrínsecos a la propia tecnología de secuenciación. Asimismo, integrar estos resultados en los informes de resultados representa un verdadero desafío para los laboratorios consecuencia de la falta de estandarización. Por último, la NGS genera una gran cantidad de datos que requieren capacidad computacional y de almacenamiento [78].

La implementación de la NGS en la rutina diagnóstica plantea una complejidad mayor que la asociada a un diagnóstico molecular basado en aproximaciones de gen único. Por ello, las recomendaciones exigen un sistema de gestión de la calidad que garantice la fiabilidad de los resultados y la competencia técnica de los laboratorios implicados [16]. En este sentido, la norma UNE-EN ISO 15189:2013 se ha consolidado como la más empleada por los laboratorios

clínicos para garantizar su competencia técnica en el desarrollo de pruebas específicas al cumplir con los requisitos técnicos y de gestión específicos. Mediante esta norma, el laboratorio dispone de las herramientas necesarias para garantizar la calidad de todas las fases del estudio molecular (Pre-analítica, analítica e interpretación/informes), la fiabilidad en los resultados obtenidos, la capacitación en la toma de decisiones clínicas, la evaluación y mejora continua de los procesos y la gestión de los recursos [80,81].

1.5.5. Recomendaciones para el empleo de NGS.

Las guías clínicas y las recomendaciones de diagnóstico molecular posicionan la NGS como técnica de elección para la tipificación molecular del CPNM avanzado debido al creciente abanico de terapias dirigidas [16,53,54,76,82,83]. Además, en este sentido, el consenso de la Sociedad Española de Anatomía Patológica y la Sociedad Española de Oncología Médica plantea la necesidad de ofrecer los estudios de NGS a todos los pacientes dentro del sistema nacional de salud para garantizar la equidad en el acceso a los nuevos tratamientos dirigidos [84].

1.6. Técnicas ultrasensibles en el diagnóstico molecular.

El diagnóstico molecular en CPNM puede beneficiarse del empleo de tecnologías ultrasensibles, especialmente a partir de muestras de biopsia líquida. En este contexto, la PCRd se ha postulado como una aproximación con un gran potencial debido a su capacidad para detectar mutaciones muy poco representadas sobre el ADN obtenido a partir de diversas muestras biológicas. Esta aproximación se fundamenta en la PCR convencional y el empleo de sondas marcadas con fluoróforos. La partición de la muestra, sobre un soporte sólido o en forma de emulsión, permite la generación de miles de reactores de PCR aislados conteniendo idealmente una única molécula molde. Tras la amplificación se produce la lectura de la fluorescencia en cada reactor de forma individual, cuantificándose de forma “digital” la presencia/ausencia de cada alelo [85] (Figura 5). Dependiendo del método, del tipo de muestra empleado y de las estrategias de validación, se han descrito LODs que alcanzan del 0.03% al 0.001% [86].

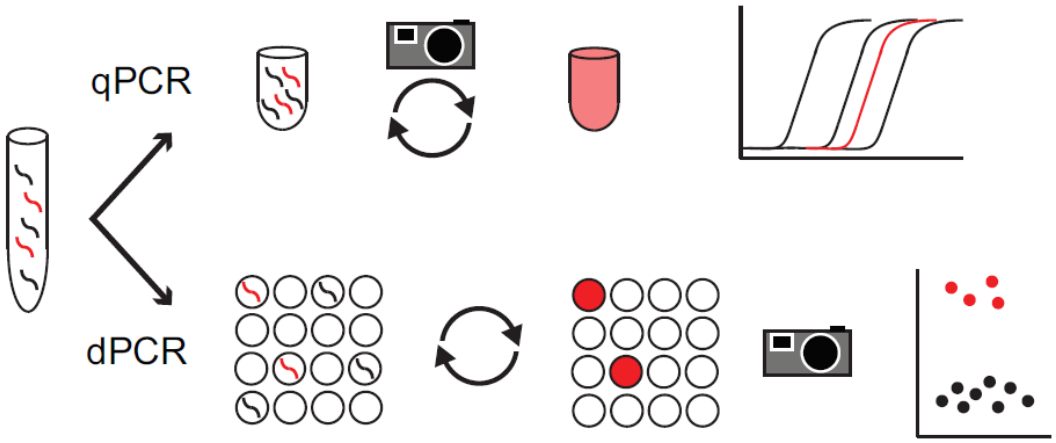

Figura 5. Comparación del fundamento de la RT-PCR (qPCR) y la PCRd (Adaptada de Salipante S *et al*., 2020)

Para la detección de mutaciones, los miles de lecturas de fluorescencia obtenidas en un ensayo de PCRd, respecto a la medición única de fluorescencia de los ensayos de RT-PCR, permiten que la señal del alelo normal no enmascare la fluorescencia del alelo mutado. El registro de todas estas lecturas de fluorescencia hace posible la cuantificación absoluta de las moléculas de partida [87]. Otra ventaja relevante es su resistencia a los inhibidores de la PCR; debido al registro digital de la fluorescencia, aquellas reacciones de PCR con una intensidad menor de fluorescencia (debido a una amplificación poco eficiente por la presencia de inhibidores) son igualmente detectadas y registradas como señal positiva.

A nivel técnico, la partición de la reacción de PCR previa a su amplificación y su carácter ultrasensible la convierten en una técnica muy susceptible a la contaminación. Un inconveniente respecto de las técnicas de RT-PCR, es la mayor complejidad del análisis de resultados y la falta de consenso para informar los resultados obtenidos [85]. Además, su empleo para el diagnóstico molecular en ADNtc representa un desafío por las limitaciones inherentes de la muestra, principalmente su fragmentación y escasa cantidad [87]. La traslación de esta aproximación a la rutina clínica requiere de su validación para establecer requerimientos mínimos de ADN y el LOD, entre otros.

2. CONTEXTUALIZACIÓN

Los avances en el entendimiento de las bases moleculares del cáncer y el desarrollo de terapias dirigidas frente a alteraciones moleculares accionables han promovido un cambio de paradigma hacia la medicina de precisión. Atender al creciente número de biomarcadores necesarios para guiar las decisiones terapéuticas en el CPNM avanzado constituye un importante desafío metodológico para los laboratorios responsables del diagnóstico molecular.

Las técnicas convencionales de diagnóstico han mostrado importantes limitaciones en diferentes contextos clínicos de la enfermedad. Consecuentemente existe la necesidad de implementar nuevas aproximaciones metodológicas que permitan satisfacer las necesidades actuales de la medicina de precisión.

En este sentido, tecnologías de alto rendimiento como la NGS podrían proporcionar el perfil molecular tumoral necesario para la toma de decisiones terapéuticas, así como garantizar la traslación de nuevos avances científicos a la clínica. Asimismo, aproximaciones ultrasensibles a partir de biopsias líquidas podrían ayudar a caracterizar la progresión de la enfermedad identificando mecanismos moleculares de resistencia, responsables de la evolución clonal de la enfermedad y necesarios para orientar nuevas líneas de tratamiento.

La relevancia del estudio de biomarcadores en el manejo clínico del CPNM avanzado exige que los laboratorios se enfrenten a la complejidad metodológica del diagnóstico molecular actual con buenas prácticas que aseguren la validez analítica y la validez clínica de las técnicas moleculares utilizadas. La traslación a la clínica de las tecnologías de alto rendimiento bajo sistemas de gestión de calidad permitiría garantizar la competencia técnica y, en último término, su utilidad clínica.

RESULTADOS

1. VALIDACIÓN TÉCNICA DE LOS ESTUDIOS DE NGS.

El primero de los artículos científicos que conforman esta Tesis Doctoral fue publicado en el año 2019 bajo el título "*Development, Implementation and Assessment of Molecular Diagnostics by Next Generation Sequencing in Personalized Treatment of Cancer: Experience of a Public Reference Healthcare Hospital" en la revista Cancers*. El objetivo principal de este trabajo fue realizar una validación técnica de la NGS como método de diagnóstico molecular.

Se analizaron 100 muestras de pacientes de CPNM mediante NGS con el panel *Oncomine Solid Tumor* (*ThermoFisher Scientific*), que permite la detección de mutaciones en 22 genes (*AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11* y *TP53*) y de transcritos de fusión de 4 oncogenes (*ALK, ROS1, RET* y *NTRK*). Paralelamente, las muestras fueron procesadas mediante las técnicas convencionales, consideradas de referencia, para la detección de mutaciones en *EGFR* (RT-PCR) y reordenamientos de *ALK* (IHQ/FISH) y *ROS1* (IHQ/FISH).

En muestras con suficiente cantidad de ADN y ARN, la tasa de resultados no válidos fue del 3.8%. Se obtuvo un promedio de 347.362 lecturas por muestra, una profundidad media de 3310X y un *on target* promedio de 92.6%. En los materiales de referencia, la NGS detectó todas las variantes presentes a baja frecuencia alélica (entre el 1-3% de VAF) siendo estos valores concordantes con los validados por el fabricante. Los estudios de concordancia de los resultados de NGS con las técnicas convencionales revelaron una sensibilidad diagnóstica de 92%, 100% y 100% y una especificidad diagnóstica de 100%, 99% y 100% para las alteraciones moleculares en EGFR, ALK y ROS1, respectivamente.

En este trabajo se evaluó el coste de los estudios de NGS y el tiempo de respuesta (TAT; *Turnaround time*), desde la recepción de la muestra hasta la emisión del informe de resultados. El análisis realizado en el año 2019, reveló un coste por muestra de la NGS ligeramente superior a las técnicas convencionales (421,23€ vs. 367,66€). La NGS presentó un TAT dos días superior respecto al de las técnicas convencionales. No obstante, la actualización en la automatización de los flujos de trabajo de la NGS ha reducido drásticamente el TAT y la evaluación de los costes requiere de actualizaciones periódicas.

En nuestra serie, los genes más frecuentemente mutados fueron *TP53* (49,0%), *KRAS* (31,0%), *EGFR* (13,0%), *BRAF* (11,0%) y *PIK3CA* (7,0%). Se identificaron alteraciones moleculares concurrentes hasta en un 35,0% de los pacientes. En un 58,0% de los pacientes reclutados se detectaron alteraciones accionables.

La validación técnica realizada consolida a la NGS como una aproximación capaz de superar las limitaciones del diagnóstico molecular del CPNM avanzado. Los resultados mostrados en este artículo forman parte de las actuaciones realizadas en la Unidad de Biología Molecular (UBM) para la acreditación de la técnica dentro del alcance de la Norma UNE-EN ISO 15189:2013 (Acreditación nº: 1302/LE2445) por la Entidad nacional de Acreditación (ENAC).

2. ESTUDIO DE LA EVOLUCIÓN CLONAL MEDIANTE NGS.

En la Tesis Doctoral se incluye el artículo "*Utility of Next-Generation Sequencing in the Reconstruction of Clonal Architecture in a Patient with an EGFR Mutated Advanced Non-Small Cell Lung Cancer: A Case Report*", publicado en la revista *Diagnostics.* Este trabajo refleja la utilidad de las tecnologías de alto rendimiento en el manejo clínico del paciente con CPNM avanzado, así como su potencial para ofrecer un mejor entendimiento de la evolución clonal. Los estudios de NGS se han realizado sobre sobre ADNtc en muestras de biopsia líquida con el panel *Oncomine Lung cfDNA Assay (ThermoFisher Scientific*) y sobre ADN obtenido a partir de muestras tumorales FFPE con el panel de 409 genes, *Ampliseq Comprehensive Cancer Panel* (*ThermoFisher Scientific*).

En este artículo se presenta el caso clínico de una mujer diagnosticada de CPNM con una deleción en el exón 19 de *EGFR* que fue tratada con un EGFR-ITQ en primera línea. En la progresión radiológica, el estudio molecular mediante RT-PCR sobre biopsia líquida identificó la mutación *EGFR*-Thr790Met como mecanismo de resistencia. Consecuencia del hallazgo molecular la paciente inició tratamiento con osimertinib. En la progresión a esta línea de tratamiento, la NGS en biopsia líquida permitió identificar mutaciones de pérdida de función en *TP53* y *RB1* cuya alta frecuencia alélica (VAF) era sugestiva de un posible carácter bialélico. Este evento molecular, prácticamente universal en el cáncer de pulmón de célula pequeña (CPCP), planteaba como hipótesis la transformación histológica como mecanismo de resistencia a osimertinib. La rebiopsia de una lesión hepática de nueva aparición, permitió confirmar nuestra hipótesis.

De forma retrospectiva, el estudio mediante NGS de la muestra de biopsia líquida obtenida en la progresión a la primera línea de tratamiento reveló la coexistencia de dos clones tumorales con mecanismos de resistencia; un clon con *EGFR*-Thr790Met y un clon portador de mutaciones inactivantes de *TP53* y *RB1*. El tratamiento con osimertinib actuó frente al clon portador de la mutación *EGFR*-Thr790Met y como consecuencia el clon *TP53* y *RB1* mutado, promovió la transformación histológica a CPCP.

La caracterización de la biopsia tumoral al diagnóstico mediante *Ampliseq Comprehensive Cancer Panel* reveló la presencia minoritaria del clon tumoral portador de mutaciones inactivantes de *TP53* y *RB1,* que posteriormente estaría implicado en ambas progresiones radiológicas. El estudio con este panel de la biopsia hepática con transformación histológica a CPCP reveló la presencia de las mutaciones de *TP53 y RB1,* así como la deleción del exón 19 en *EGFR,* todas ellas a una elevada frecuencia alélica (>70,0%).

Los resultados obtenidos muestran el impacto de la heterogeneidad molecular en el curso de la enfermedad y la respuesta a los tratamientos dirigidos. Su caracterización ha permitido identificar mecanismos de resistencia subyacentes que condicionan la evolución clonal.

3. APROXIMACIONES DE PCR ULTRASENSIBLES PARA LA DETECCIÓN DE *EGFR*-Thr790Met

La tercera publicación recogida en esta Tesis Doctoral "*Technical Validation and Clinical Implications of Ultrasensitive PCR Approaches for EGFR-Thr790Met Mutation Detection in Pretreatment FFPE Samples and in Liquid Biopsies from Non-Small Cell Lung Cancer Patients*" ha sido publicada en la revista *International Journal of Molecular Sciences*. El objetivo de este trabajo es la validación técnica de la PCR en tiempo real con ácido nucleico peptídico (PNA-Clamp) y de la PCR digital (ddPCR) como técnicas ultrasensibles para la detección de la mutación de resistencia *EGFR*-Thr790Met a partir de tejido tumoral FFPE y biopsias líquidas.

En la validación técnica se emplearon muestras de tejido tumoral FFPE previamente caracterizadas y material de referencia comercial. Se estableció el LOD del ensayo *PNA Clamp TaqMan* en 0,0996% y de la ddPCR en 0,1336%. La ddPCR mostró un LOD de 3 copias de ADN mutado/mL de plasma sobre ADNtc.

En una serie de 78 muestras de tejido tumoral FFPE ambas aproximaciones mostraron una concordancia del 94,2%. Respecto a la implicación clínica de la detección de *EGFR*-Thr790Met pretratamiento, en nuestra serie de 34 pacientes en estadio IV que iniciaron tratamiento con EGFR-ITQs de primera y segunda generación, la mutación *EGFR*-Thr790Met fue detectada en un 26,5% y se asoció una mayor supervivencia libre de progresión (SLP) (16,8 ± 7,8 vs. 11,5 ± 1,8 meses; p = 0,047).

En biopsias líquidas a la progresión, la ddPCR permitió aumentar la tasa de detección hasta el 46,0%, respecto del 32,4% obtenido con RT-PCR. Mediante ddPCR fue posible detectar la mutación *EGFR*-Thr790Met en líquido pleural y cefalorraquídeo, respectivamente. En

nuestra cohorte, la prevalencia del mecanismo de resistencia fue del 59,5% y la tasa de repuesta a osimertinib (90,0%) fue independiente del método y tipo de muestra sobre el que se detectó la mutación. Además, en biopsias líquidas obtenidas durante el seguimiento al tratamiento en pacientes que posteriormente desarrollaron *EGFR*-Thr790Met a la progresión, la ddPCR permitió anticipar la progresión radiológica hasta 7 meses.

Este trabajo refleja que el uso de técnicas ultrasensibles para el diagnóstico molecular a parir de tejido tumoral FFPE y biopsias líquidas es posible tras la validación técnica de los métodos. Según nuestros resultados, la mutación *EGFR*-Thr790Met se detecta en un 26,5% de los pacientes y se asocia a un mejor pronóstico. No obstante, los distintos métodos y estrategias de validación descritos en la literatura impiden alcanzar conclusiones sólidas. La validación técnica y clínica de la ddPCR para la detección de *EGFR*-Thr790Met en biopsia líquida ha permitido su acreditación por la norma UNE-EN ISO 15189:2013 por la ENAC.

4. UTILIDAD CLÍNICA DE LA NGS EN LA MEDICINA DE PRECISIÓN DEL CPNM.

El último artículo incluido en esta tesis doctoral, "*Impact of Molecular Testing by Next Generation Sequencing in the Clinical Management of Non–Small Cell Lung Cancer Patients in a Public Healthcare Hospital*" se encuentra en fase de revisión para su publicación en la revista *Cancers*. Este trabajo profundiza en el papel de la NGS para la caracterización molecular del CPNM y evalúa su utilidad clínica tras su acreditación con la norma UNE-EN ISO 15189:2013.

Se han reclutado 350 pacientes diagnosticados con CPNM (200 en estadio IV). El estudio combina el empleo del panel *Oncomine Solid Tumor* en 104 muestras y el panel *Oncomine Focus Assay (ThermoFisher Scientific*) en 246. Este panel permite la detección de mutaciones en 35 genes (*AKT1, ALK, AR, BRAF, CDK4, CTNNB1, DDR2, EGFR, ERBB2, ERBB3, ERBB4, ESR1, FGFR2, FGFR3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK1, JAK2, JAK3, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS, PDGFRA, PIK3CA, RAF1, RET, ROS1* y *SMO*), variación en el número de copias (VNC) de 19 genes (*ALK, AR, BRAF, CCND1, CDK4, CDK6, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, FGFR4, KIT, KRAS, MET, MYC, MYCN, PDGFRA* y *PIK3CA*) y transcritos de fusión de 23 oncogenes (*ABL1, AKT3, ALK, AXL, BRAF, EGFR, ERBB2, ERG, ETV1, ETV4, ETV5, FGFR1, FGFR2, FGFR3, MET, NTRK1, NTRK2, NTRK3, PDGFRA, PPARG, RAF1, RET y ROS1*).

En nuestra cohorte, el 54,3% de los pacientes presentaron una única alteración molecular, 22,3% presentaron alteraciones concurrentes y en un 23.4% no se identificaron alteraciones. Los genes *TP53* (51,0%), *KRAS* (26,6%), *EGFR* (12,9%), *BRAF* (6,9%) y *PIK3CA*

(5,4%) fueron los más frecuentemente mutados. Los transcritos de fusión, que involucraron a los genes *ALK* (4,0%), *MET* (3,6%) y *ROS1* (1,4%) fueron los más frecuentes. Respecto a la VNC, las amplificaciones de *EGFR* (2,4%) y *MYC* (2,0%) fueron las más prevalentes.

La aplicación de la NGS ha permitido identificar perfiles moleculares dependientes del sexo y el hábito tabáquico. Las mutaciones en *EGFR* ($p < 0,001$), *ERBB2* ($p = 0,013$) y las fusiones de *ALK* ($p = 0,049$) fueron más frecuentemente detectadas en mujeres mientras que las alteraciones de *TP53* se asociaron con varones ($p = 0,045$). Respecto al hábito tabáquico, las mutaciones de *KRAS* fueron frecuentemente detectadas en fumadores o ex fumadores mientras que las mutaciones de *EGFR* ($p < 0,001$), *ERBB2* ($p =0,029$) así como las fusiones de *ALK* ($p < 0,001$), *ROS1* ($p =0,030$), *RET* ($p = 0,012$), y *MET*-Ex14 ($p < 0,001$) se asociaron a pacientes nunca fumadores. En este sentido, los pacientes exfumadores con mutación de *EGFR* presentaron un hábito tabáquico significativamente menor (15 vs. 35 años/paquete, $p = 0,03$).

El carácter multiplexado de la NGS ha permitido revelar relaciones de concurrencia y exclusividad mutacional. Las mutaciones en *KRAS* fueron altamente excluyentes con las mutaciones en *EGFR* ($p < 0,001$), *TP53* ($p = 0,01$) y las fusiones de *ALK* ($p = 0,01$). Las mutaciones de *EGFR* y las amplificaciones del citado gen frecuentemente concurrieron en nuestra serie de pacientes ($p < 0,01$). Asimismo, las amplificaciones de *KRAS* y *CCND1* se asociaron en nuestra cohorte ($p = 0,01$).

El estudio molecular mediante NGS ha permitido identificar alteraciones accionables en un 65,7% de los pacientes. De ellos, un 54,4% fueron candidatos al tratamiento con terapias aprobadas mientras que un 45,6% podría ser reclutado en ensayos clínicos con terapias dirigidas experimentales en fase I o fase II. Como consecuencia de los perfiles moleculares anteriormente descritos, el impacto de la medicina de precisión fue altamente dependiente del sexo y el hábito tabáquico. Un 80,5% de las mujeres presentaba alteraciones accionables respecto del 56,7% de los varones ($p < 0,001$). El porcentaje de pacientes candidatos a terapias dirigidas fue significativamente más alto en pacientes no fumadores (87,7%) que en pacientes ex fumadores (59,8%) o fumadores (61,4%).

Desde que la NGS se estableciera como la técnica de elección para el diagnóstico molecular del CPNM avanzado en nuestro centro, el 36,4% de los pacientes ha recibido al menos una línea de tratamiento con terapias dirigidas en base al perfil molecular.

El análisis de SLP a la primera línea de tratamiento reveló diferencias estadísticamente significativas. Los pacientes que recibieron terapia dirigida [33 (22,6%)] mostraron una mayor SLP que los pacientes tratados con quimioterapia [69 (47,3%)] (13.4 meses (95% CI, 10,2-16,6)

vs. 5,2 meses (95% CI, 4,2-6,2) (p =0,001). Los pacientes tratados con esquemas basados en inmunoterapias [44 (30,1%)] alcanzaron una mayor SLP [7.8 meses (95% CI, 3,5-12,1)] que los tratados con quimioterapia (p = 0,011).

La SG fue diferente en función de las terapias administradas. (p < 0,001). Los pacientes que únicamente fueron tratados con esquemas de quimioterapia mostraron peor SG [8,8 meses (95% CI, 4,5-13,1)]. Los pacientes tratados con al menos una línea de tratamiento con terapia dirigida, [HR: 0.3 (95% CI, 0,2-0,6)], inmunoterapias [HR: 0.2 (95% CI, 0,1-0,4)], o ambas [HR: 0.2 (95% CI, 0,1-0,6)], presentaron un riesgo de muerte menor frente al grupo comparador tratados únicamente con quimioterapia.

Este trabajo ha mostrado distintos perfiles moleculares en función de características clínico-patológicas con gran impacto en la aplicación de la medicina de precisión. Asimismo, los resultados obtenidos muestran la utilidad clínica de la NGS acreditada por la norma UNE-EN ISO 15189:2013 al expandir la aplicación de la medicina de precisión en nuestro centro.

CONCLUSIONES

1. Las métricas de calidad obtenidas durante la validación técnica de la NGS muestran el gran rendimiento de esta tecnología sobre ácidos nucleicos obtenidos a partir de muestras tumorales FFPE. Esta metodología es capaz de detectar alteraciones somáticas a baja VAF (<5%) y muestra una gran concordancia con las técnicas convencionales de gen único. La validación técnica realizada ha permitido la acreditación de la técnica de NGS bajo la norma UNE-EN ISO 15189:2013.
2. En un 76,6% de los pacientes de nuestra serie, la NGS ha permitido la detección de al menos una alteración molecular. Los genes más frecuentemente mutados son: *TP53* (51,0%), *KRAS* (26,6%) y *EGFR* (12,9%).
3. Las frecuencias de las alteraciones moleculares difieren significativamente en función del sexo y el hábito tabáquico. Como consecuencia, las alteraciones moleculares accionables son significativamente más frecuentes en mujeres y en pacientes no fumadores.
4. La tecnología de NGS como técnica de elección para el diagnóstico molecular del CPNM avanzado ha permitido que un 36,4% de los pacientes en estadio IV de nuestra serie se beneficien de tratamientos dirigidos.
5. En nuestra serie, las terapias dirigidas han conseguido mejorar los resultados de SLP y SG.
6. La validación técnica de aproximaciones de PCR ultrasensibles permite la detección pretratamiento de *EGFR*-Thr790Met sobre ADN obtenido a partir de muestras tumorales FFPE. La mutación *EGFR*-Thr790Met se ha identificado en un 26,5% de los pacientes de CPNM en estadio IV de nuestra serie, asociándose a un mejor pronóstico.
7. La aproximación de ddPCR ha aumentado la tasa de detección de *EGFR*-Thr790Met a partir de biopsias líquidas. Asimismo ha permitido su detección temprana en biopsias líquidas, anticipándose a la progresión radiológica. Consecuentemente, se ha acreditado bajo la norma UNE-EN ISO 15189:2013.
8. La caracterización molecular de la enfermedad mediante NGS tanto a partir de tejido tumoral FFPE como a partir de biopsias líquidas permite trazar la evolución clonal de la enfermedad en respuesta al tratamiento.
9. La validación técnica de las tecnologías de alto rendimiento y su traslación a la clínica bajo un sistema de gestión de calidad ha permitido demostrar su utilidad clínica.

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Development, Implementation and Assessment of Molecular Diagnostics by Next Generation Sequencing in Personalized Treatment of Cancer: Experience of a Public Reference Healthcare Hospital

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Abstract: The establishment of precision medicine in cancer patients requires the study of several biomarkers. Single-gene testing approaches are limited by sample availability and turnaround time. Next generation sequencing (NGS) provides an alternative for detecting genetic alterations in several genes with low sample requirements. Here we show the implementation to routine diagnostics of a NGS assay under International Organization for Standardization (UNE-EN ISO 15189:2013) accreditation. For this purpose, 106 non-small cell lung cancer (NSCLC) and 102 metastatic colorectal cancer (mCRC) specimens were selected for NGS analysis with Oncomine Solid Tumor (ThermoFisher). In NSCLC the most prevalently mutated gene was *TP53* (49%), followed by *KRAS* (31%) and *EGFR* (13%); in mCRC, *TP53* (50%), *KRAS* (48%) and *PIK3CA* (16%) were the most frequently mutated genes. Moreover, NGS identified actionable genetic alterations in 58% of NSCLC patients, and 49% of mCRC patients did not harbor primary resistance mechanisms to anti-EGFR treatment. Validation with conventional approaches showed an overall agreement >90%. Turnaround time and cost analysis revealed that NGS implementation is feasible in the public healthcare context. Therefore, NGS is a multiplexed molecular diagnostic tool able to overcome the limitations of current molecular diagnosis in advanced cancer, allowing an improved and economically sustainable molecular profiling.

Keywords: next generation sequencing; non-small cell lung cancer; metastatic colorectal cancer; molecular diagnostics; UNE-EN ISO 15189 accreditation

1. Introduction

Cancer is a complex and heterogeneous disease with considerable variation in histological and biological features. Understanding the role of genetic alterations involved in cancer development has led to its reclassification into different molecular subtypes that reflect biological behavior and may lead to further effective therapeutic targets to achieve improved outcome [1,2]. For this purpose, single gene testing approaches are traditionally used to identify individual alterations, currently targeted

with approved drugs. However, comprehensive molecular characterization of tumors is hampered by the limited amount of cytology samples and/or formalin-fixed paraffin-embedded (FFPE) tissue biopsies, and by the turnaround time to assess multiple targetable genes and high economic costs. Moreover, the routine methods do not board the mutational co-occurrences, so they do not detect other alterations which in many cases are responsible for disease progression.

The knowledge of a tumor's genetic profile is crucial to improve clinical-decision making in the patient management. Consequently, laboratories must integrate high-throughput sequencing technologies in routine molecular diagnostics [3]. These allow the simultaneous testing of multiple genetic alterations (point mutations, insertions, deletions, copy number variations and translocations) and quantify molecular subclones by procedures that provide accurate, reliable and cost-effective results. In this sense, next-generation sequencing (NGS) has overcome the cited challenges, posing an attractive alternative to traditional molecular diagnostic testing for cancer [4–6]. In fact, the College of American Pathologists has suggested the use of expanded panels in its latest guideline [7–9] and both the National Comprehensive Cancer Network (NCCN) and the European Society for Medical Oncology (ESMO) have proposed broader molecular profiling to identify rare driver mutations in non-small cell lung cancer (NSCLC) and metastatic colorectal cancer (mCRC) patients, for which effective drugs are already available or under development in clinical trials [10–13]. However, given the implications of NGS studies on the treatment of cancer patients, the establishment of an internal quality management system is strongly recommended. In this regard, the UNE-EN ISO 15189:2013 accreditation has been recognized as the international standard for quality management systems for all fields in clinical laboratories [14].

The purpose of the current study is to evaluate the integration of NGS technology in a routine clinical setting. We describe the mutational profile of two highly prevalent cancers (advanced NSCLC and mCRC) and analyze its diagnostic potential to characterize molecular heterogeneity and to increase the therapeutic opportunities with targeted therapies; we assess NGS technology at a technical and economical level; and we describe our experience in clinical practice of an NGS pipeline for cancer molecular diagnostics in the UNE-EN ISO 15189:2013 accreditation scope.

2. Results

2.1. Performance Evaluation of Next Generation Sequencing

2.1.1. Next Generation Sequencing Quality Assessment

NGS assay was able to detect the seven low frequency variants (between 1–3%) present in two reference materials used as positive controls. The variant allele frequency (VAF) detected was consistent to the data obtained by digital droplet PCR (ddPCR) assays except for *EGFR* p.Leu858Arg and *KRAS* p.Gly13Asp mutations in which NGS VAF was slightly higher (5% versus 3% and 4% versus 3%, respectively).

Quality control analysis revealed excellent performance of the NGS panels (Figure S1). The median of total reads per sample was 347,362 with a median read depth of 3310 reads per amplicon. Uniformity was 97.1% on average and the average "on-target" reads per sample was 92.6%. Moreover, 96.6% of targeted bases showed ≥500 × read depth.

On the other hand, NGS showed an invalid test rate of 3.8% (8/208 FFPE specimens). Six NSCLC samples and two mCRC failed due to low sequencing quality metrics (total reads <100,000). These samples were subsequently excluded from the study.

2.1.2. Comparison of Next Generation Sequencing with Conventional Methods

For *ROS1* rearrangements and *NRAS* mutations the overall agreement (OA) was 100% (Table 1). Regarding *EGFR*, one specimen showed the p.Thr790Met mutation by conventional methodology which was not reported by NGS (OA; 99.0%). Instead, NGS and Sanger Sequencing (SS) revealed

a synonymous change in homozygosis in 787 codon p.(Gln787Gln). NGS technology allowed the detection of an *ALK* rearrangement not detected by immunohistochemistry (IHQ) or fluorescence in situ hybridization (FISH) (OA: 99.0%). In *KRAS*, seven specimens gave discordant results when comparing with Real Time (RT)-qPCR assay (OA: 94.7%). *BRAF* pVal600Glu mutation was detected by High Resolution Melting (HRM) in nine out of eleven NGS *BRAF* p.Val600Glu mutated samples (OA: 96.4%).

Table 1. Comparison of NGS results with conventional methods.

Conventional Methods Result				
Gene	**Mutation/Fusion Detected**	**Not Detected**	**Parameter**	**Agreement**
EGFR-NGS Result				
Mutation Detected	13	0	PPA	92.9%
Not Detected	1	86	NPA	100%
			OA	99.0%
KRAS-NGS Result				
Mutation Detected	74	5	PPA	97.4%
Not Detected	2	50	NPA	90.9%
			OA	94.7%
NRAS-NGS Result				
Mutation Detected	3	0	PPA	100%
Not Detected	0	54	NPA	100%
			OA	100%
BRAF-NGS Result				
Mutation Detected	9	2	PPA	100%
Not Detected	0	45	NPA	95.8%
			OA	96.4%
ALK Fusions-NGS Result				
Fusion Detected	4	1	PPA	100%
Not Detected	0	95	NPA	99.0%
			OA	99.0%
ROS1 Fusions-NGS Result				
Fusion Detected	1	0	PPA	100%
Not Detected	0	99	NPA	100%
			OA	100%

NGS—Next Generation Sequencing; PPA—Positive percent agreement; NPA—Negative percent agreement; OA—Overall agreement.

2.1.3. Turnaround Time (TAT) and Cost Comparison

In order to compare NGS with conventional methodologies under theoretical conditions, we calculated the turnaround time and cost for three mandatory testing genes in NSCLC (*EGFR*, *ALK* and *ROS1*) and mCRC (*KRAS*, *NRAS* and *BRAF*). Starting in both cases from FFPE tissue blocks we were able to prepare libraries, sequence eight NSCLC or ten mCRC samples, and analyze data in five working days. Conventional methodologies for molecular testing of *EGFR*, *ALK* and *ROS1* resulted approximately in three days while testing *KRAS*, *NRAS* and *BRAF* in mCRC resulted approximately in four working days. However, if *KRAS* is positive, TAT is reduced to three days. TAT and cost comparison between NGS and conventional methods is shown in Table 2.

Table 2. Turnaround time and cost comparison between NGS and conventional methods.

Analysis	System	Hands-on Time, min (h)	Time Duration, min (h)	Costs (€)
NGS Analysis (8 NSCLC samples (DNA + RNA))				
DNA and RNA isolation	Manual	90 (1.5)	1140 (19.0)	166.96
Quantification and sample dilution	Qubit	30 (0.5)	30 (0.5)	13.68
Library preparation DNA	Veriti Thermal Cycler	120 (2.0)	1440 (24.0)	984.00
Library preparation RNA	Veriti Thermal Cycler			1214.72
Emulsion PCR	One Touch	20 (0.3)	480 (8.0)	124.16
Enrichment	One Touch ES	10 (0.2)	30 (0.5)	23.70
Sequencing	PGM System	10 (0.2)	240 (4.0)	607.00
Data Processing and analysis	Ion Reporter	160 (2.6)	180 (3.0)	-
Laboratory personnel costs †			-	235.60
Total Cost		-	-	3369.84
Cost per sample		-	-	421.23
Working days		440 (7.3)	5 days	-
Conventional Molecular Analysis (8 NSCLC samples (DNA))				
DNA isolation	Manual	60 (1.0)	1140 (19.0)	84.16
Quantification and sample dilution	Qubit	15 (0.25)	30 (0.5)	5.04
EGFR (Exon 18, 19, 20 and 21)	RT-qPCR	20 (0.3)	120 (2.0)	1391.50
ALK- Rearrangements	IHQ	10 (0.2)	960 (16.0)	672.00
ROS1-Rearrangements	IHQ	10 (0.2)	960 (16.0)	672.00
Sanger sequencing	SS	30 (0.5)	480 (8.0)	40.00
Data Processing and analysis		30 (0.5)	30 (0.5)	-
Laboratory personnel costs †			-	76.55
Total Cost		-	-	2941.27
Cost per sample				367.66
Working days		175 (2.9)	3 days	-
NGS Analysis (10 mCRC samples (DNA))				
DNA isolation	Manual	60 (1.0)	1140 (19.0)	105.20
Quantification and sample dilution	Qubit	15 (0.25)	30 (0.5)	6.30
Library preparation DNA	Veriti Thermal Cycler	120 (2.0)	1440 (24.0)	1230.00
Emulsion PCR	One Touch	20 (0.4)	480 (8.0)	77.60
Enrichment	One Touch ES	10 (0.2)	30 (0.5)	14.80
Sequencing	PGM System	10 (0.2)	240 (4.0)	379.40
Data Processing and analysis	Ion Reporter	160 (2.6)	180 (3.0)	-
Laboratory personnel costs †			-	219.85
Total Cost		-	-	2033.15
Cost per sample		-	-	203.32
Working days		395 (6.6)	5 days	-
Conventional Molecular Analysis (10 mCRC samples (DNA))				
DNA isolation	Manual	60 (1.0)	1140 (19.0)	105.20
Quantification and sample dilution	Qubit	15 (0.25)	30 (0.5)	6.30
KRAS (Exon 2,3 and 4)	RT-qPCR	20 (0.3)	150 (2.5)	1530.60
NRAS (Exon 2,3 and 4)	IHQ	20 (0.3)	150 (2.5)	1391.50
BRAF (Codon 600)	IHQ	30 (0.5)	120 (2.0)	1001.80
Sanger sequencing	SS	30 (0.5)	480 (8.0)	50.00
Data Processing and analysis		30 (0.5)	30 (0.5)	-
Laboratory personnel costs †		-	-	87.05
Total Cost		-	-	4172.47
Cost per sample		-	-	417.25
Working days		250 (3.4)	4 days *	-

NSCLC—Non-small cell lung cancer; mCRC—metastatic colorectal cancer; NGS—Next-generation sequencing; PGM—Personal Genome Machine; RT-qPCR—Real-Time quantitative polymerase chain reaction; SS—Sanger sequencing; IHQ—Immunohistochemistry; HRM—High resolution melting; - Non applicable. † Laboratory personnel costs—Cost is calculated based on the time required by the technician/physician in each analysis step (Hands-on time). * If *KRAS* is mutated global time duration has been estimated in 3 days.

2.1.4. Clinical Laboratory Accreditation

This thorough validation consisting of reference material analysis, quality control metrics assessment, experimental validation with conventional methodologies and TAT and cost comparison

allowed us to include the assay within the scope of the recently granted UNE-EN ISO 15189:2013 accreditation (Entidad Nacional de Acreditación, ENAC, Nº1302/LE2445). Moreover, this NGS assay was externally validated within the European Genetics Quality Network (EMQN) External Quality Assessment Scheme for Oncogene Panel Testing, obtaining satisfactory results in 2017 and 2018 editions [15].

2.2. Next Generation Sequencing Results in the Routine Setting

2.2.1. Pathogenic Alterations Detected by Next Generation Sequencing

Sequencing analysis identified on average 1.45 non-synonymous and non-polymorphic variants per sample (291/200) (Figure 1). After filtering, a total of 168 different non-synonymous and non-polymorphic variants were detected, of which 149 were classified as somatic alterations previously reported and 19 were variants of uncertain significance (VUS).

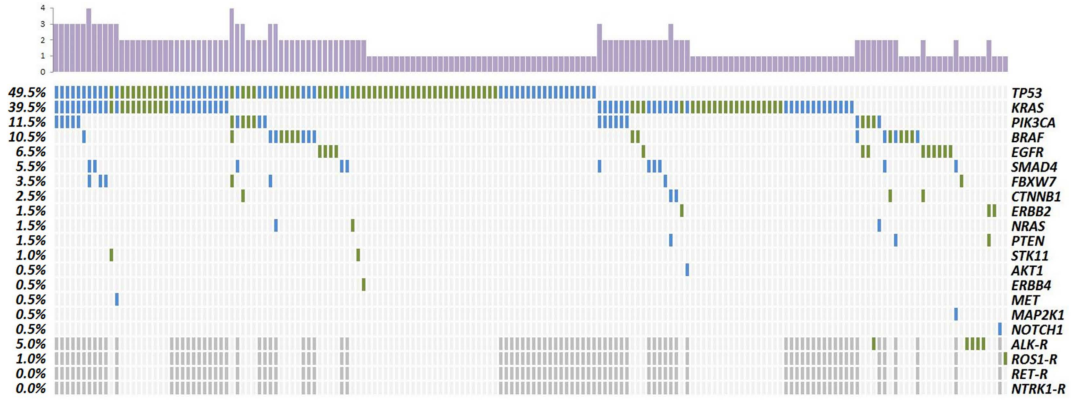

Figure 1. Distribution of gene alterations in NSCLC (green) and mCRC patients (blue). Column chart in the upper part represents the total number of mutations for each sample. Left column indicates the percentage of samples with specific gene alteration. Dark grey—Not tested. R—Rearrangements.

In the NSCLC cohort, the most prevalently mutated gene was *TP53* (49%) followed by *KRAS* (31%), *EGFR* (13%), *BRAF* (11%) and *PIK3CA* (7%). Rearrangements were found in *ALK* and *ROS1* (5% and 1%, respectively). In the entire group, 9% of patients did not carry any somatic mutation; 56% harbored one somatic mutation and 35% two or more (Figure 1, Table S1).

In mCRC patients, only 17% did not harbor any mutation, 33% carried one somatic mutation and 50% harbored two or more. Half of the patients carried mutations in *TP53* (50%). Mutations in *KRAS* were the second most prevalent (48%), specifically mutations in codon 12 accounted for 37%. We also detected pathogenic variants in codon 13 (5%), codon 146 (5%), codon 117 (1%) and the uncommon codon 19 mutation (p.Leu19Phe) found in concomitancy with a codon 146 mutation. Pathogenic variants in *PIK3CA* supposed 16% and mutations in *SMAD4* were detected in 11% of patients. Regarding *BRAF*, eight samples harbored the classical p.Val600Glu and two showed mutations outside this hotspot. *NRAS* mutated samples (2%) harbored the hotspot p.Gln61Arg mutation (Figure 1, Table S2).

2.2.2. Concurrent Molecular Alterations

Thirty-five NSCLC patients harbored co-occurring mutations. One patient carried an indel in *EGFR* exon 19 accompanied by *KRAS* codon 12 mutation. Three *EGFR* mutations and one exon 19 deletion appeared in concurrency with *TP53* mutations. Two *EGFR* mutated patients also presented *PIK3CA* mutations; and one harbored a double *EGFR* codon 18 mutation in concurrency with *CTNNB1* mutation. Among the 31 *KRAS* mutated samples, two were in concurrency with *BRAF* mutations; one carried also a mutation in *ERBB2* and one sample harbored double *KRAS* mutations. One sample

carrying a *NRAS* mutation also presented a *TP53* frameshift mutation. *PIK3CA* mutations were found to be concurrent with *EGFR*, *BRAF*, *TP53* mutations or *ALK* fusion. We identified two samples with three concurrent mutations. In one sample we identified mutations in *PIK3CA*, *CTNNB1* and *TP53*. Another patient carried mutations in *KRAS*, *STK11* and *TP53*. Moreover, one sample harbored four concurrent mutations in *PIK3CA*, *BRAF*, *FBXW7* and *TP53* (Figure 2, Table S1).

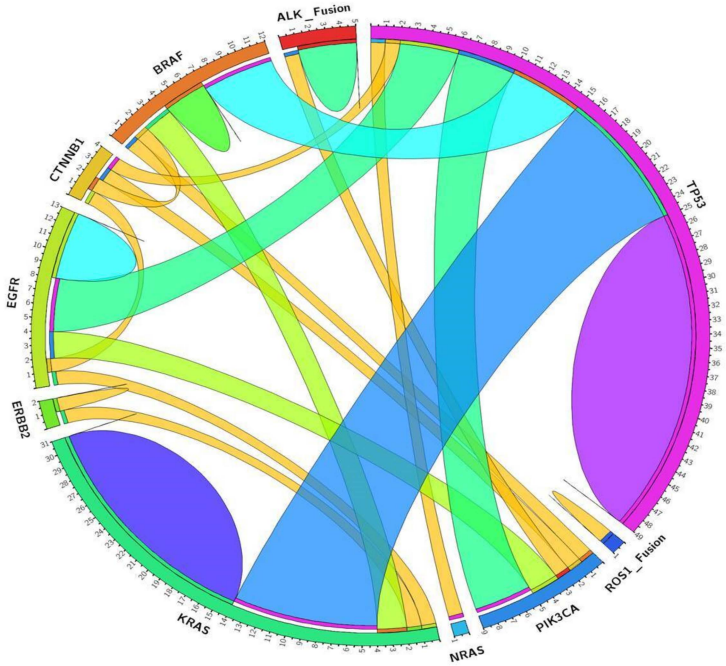

Figure 2. Circos diagram. Associations among the most prevalently mutated genes in NSCLC patients.

Fifty mCRC patients harbored concurrent mutations. *KRAS* mutations were found to be concomitant with *TP53* (n = 11), *PIK3CA* (n = 5), *SMAD4* (n = 3) and *FBXW7* (n = 1), *CTNNB1* (n = 1) and *AKT* (n = 1). *BRAF* p.Val600Glu mutation was found in concurrency with *TP53* (n = 3), *PTEN* (n = 1), *PIK3CA* (n = 1) and *SMAD4* (n = 1). *NRAS* and *PIK3CA* were concomitant in one sample. Fifteen patients carried three concurrent mutations, *KRAS-PIK3CA-TP53* being the most frequent combination (n = 5). Interestingly, one patient harbored concurrent mutations in *KRAS*, *BRAF* and *TP53* and other carried mutations in *NRAS*, *BRAF* and *TP53*. One patient carried four concurrent mutations in *KRAS*, *SMAD4*, *FBXW7* and *TP53* (Figure 3, Table S2).

2.2.3. Clinically Relevant Genetic Variants

NGS identified actionable genomic alterations in 58% of NSCLC patients (Figure 4). The most prevalent changes detected in *EGFR* were exon 21 mutations (6%) followed by exon 19 alterations (5%). Codon 12 was the most frequently mutated in *KRAS* (23%) followed by codon 13 (3%) and codon 61 (2%). In *NRAS* only codon 61 was found mutated (1%). Regarding *BRAF*, three out eleven detected mutations occurred on the hotspot Val600. In regard to *PIK3CA*, codon 542 mutations were the most frequent (2%) followed by mutations in codons 545 and 1047 (1% for both). Duplication in exon 20 (p.Ala771_Met774dup; 2%) and the hotspot mutation p.Arg784His (1%) were found in *ERBB2*. Four patients showed fusions between *ALK* and *EML4*, in all of them, the rearrangement involved exon 20 of *ALK*, in three patients with exon 6 of *EML4* and in other with exon 13. One patient showed a fusion of *ALK* with an unknown partner. Finally, one patient presented a fusion between *ROS1* (exon 35) and *CD74* (exon 6).

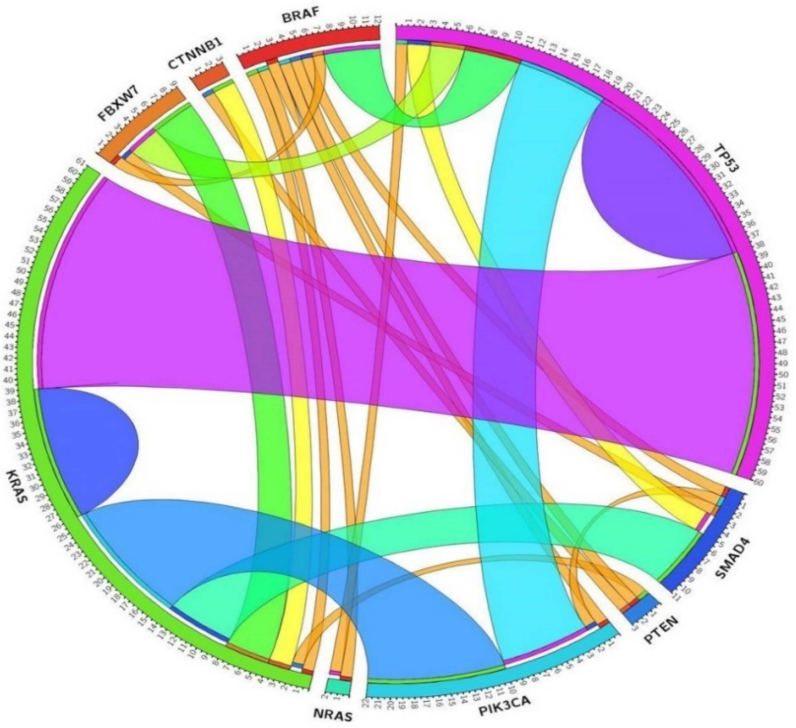

Figure 3. Circos diagram. Associations among the most prevalently mutated genes in mCRC patients.

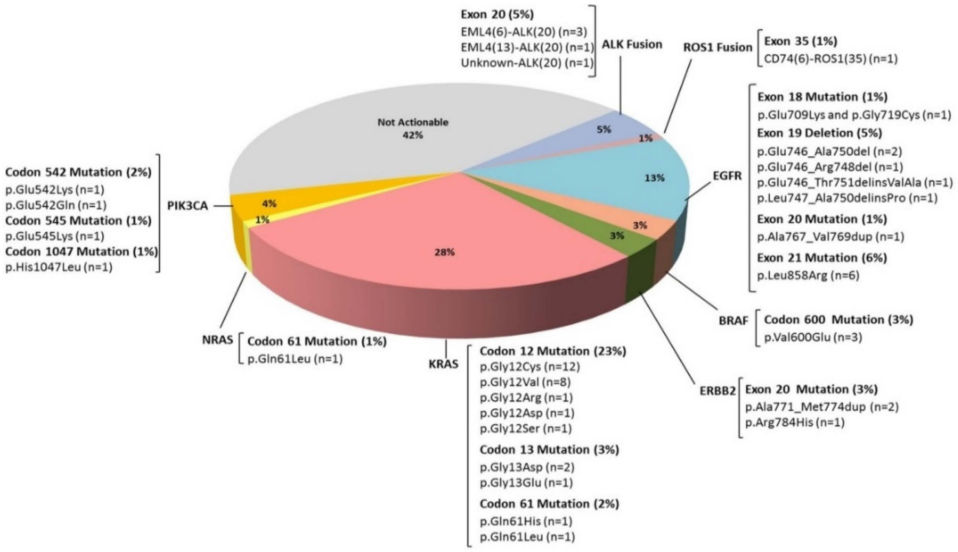

Figure 4. Percentage of NSCLC patients with actionable alterations detected by NGS. Fifty-eight percent of patients included in the study were susceptible to being treated with targeted drugs approved in advanced cancers or in clinical trials.

Regarding targeted therapy in mCRC patients, 50% of patients harbored *RAS* mutations as a primary resistance mechanism to anti-EGFR therapies. Additionally, in the *RAS* wild type patients, NGS identified eight *BRAF* V600E mutated patients, five *PIK3CA* mutated patients and one patient harboring p.Lys57Asn in *MAP2K1* gene (Figure 5).

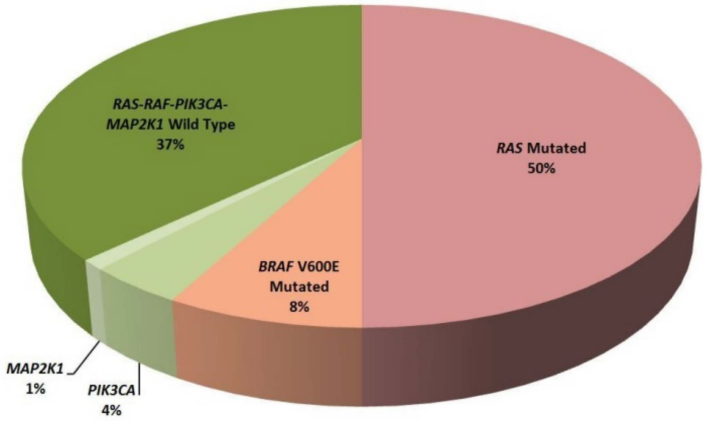

Figure 5. Classification of mCRC patients according to clinically relevant alterations detected by NGS.

3. Discussion

NGS has emerged as a promising strategy to achieve precision medicine [16,17]. These approaches are able to identify multiple cancer genes simultaneously with low sample requirement, reducing sequencing costs and molecular diagnostics turn-around time. However, the integration a high-throughput technology into clinical routine practice of a public health system represents a major challenge. Laboratories performing clinically-relevant tests must improve their quality and competence [18] and for this purpose, accreditation and participation in External Quality Assessment (EQA) programs are strongly recommended [19].

In this study, we show that NGS technology is able to efficiently amplify and sequence multiple genes using only 10 ng of DNA or RNA obtained from FFPE samples. The quality metrics analysis revealed excellent read depth and coverage for all the targeted regions, allowing confident somatic variant detection [20]. These results technically validate the NGS assay and grant the identification of low VAF variants. The invalid test rate obtained (3.8%) is concordant with previously reported studies in FFPE samples [21,22].

In particular, these panels were able to confidently detect variants at <5% VAF, which can be relevant, in challenging, low tumor percentage FFPE samples. Therefore, and taking into account that current recommendations of NGS studies in FFPE samples propose not reporting variants with a VAF lower than 5% [23,24] (unless they have an important therapeutic or prognostic impact) we established this value as a threshold for reporting variants.

Moreover, we found an excellent correlation between NGS and single-gene conventional methods. The analysis of discordant results revealed that NGS is a more robust method compared with conventional approaches. Concerning *EGFR* mutations, one patient reported as positive with Cobas® assay and negative by NGS also carried a homozygous and synonymous variant near codon 790 (p.Gln787Gln). However, since both NGS and SS did not detect this mutation, we hypothesize that the synonymous variant could affect primer or probe hybridization of the Cobas® assay, resulting in a false positive detection of the p.Thr790Met mutation. In *KRAS* testing, we found seven discordant cases. Two samples resulted positive by RT-qPCR assay but were not detected by NGS. These samples were re-tested using a new lot of the AmoyDx assay, providing then concordant results with the NGS assay. Among the five negative samples for *KRAS* mutations by RT-qPCR, NGS reported mutations at low VAF in two cases (3.9% and 5.0%, respectively). In theory, these VAFs should be detected by the AmoyDx assay, which has a limit of detection (LOD) of 1–2%, established by using cell line DNA. However, we suspect this LOD could be higher when using highly degraded DNA obtained from FFPE samples. In the three remaining cases, NGS revealed *KRAS* mutations at high VAF (12%, 12% and 20%), which could be confirmed by SS. Moreover, these samples were re-tested by a technician in another institution, showing concordant results with the NGS assay. For *BRAF* p.Val600Glu mutation, NGS revealed two mutated samples with VAF of 4% and 5%, not detected by HRM (LOD = 10%). Regarding

fusion transcripts, an OncoNetwork collaborative research study was able to detect *EML4*/*ALK* fusion up to 1% dilution [25]. In our cohort, we detected five fusions by NGS, of which one was not detected by IHQ nor FISH (53 nuclei counts). Our results are in agreement with Velizheva et al., who conclude that targeted NGS is a more robust and reliable method for fusion detection, especially in borderline cases, compared to single target assays such as FISH [26]. Taken together, NGS has proven to be a valid alternative to conventional molecular testing in terms of diagnostic accuracy [27].

TAT and economic costs are essential for NGS implementation in routine molecular diagnostics in a public healthcare hospital. Here, we found a great economic benefit in the employment of NGS technology versus conventional methodologies when it came to wild type *KRAS* mCRC patients. In *KRAS* mutated patients this benefit is not observed, however, a complete NGS test is achieved with a €30 difference per sample, therefore being an economically sustainable approach. In NSCLC, the extra cost associated with NGS studies (€51.5/patient) can be assumed based on the ability to identify actionable alterations with significant impact on patients' outcome.

The NGS approach described in this study requires a manual library and template preparation (emulsion PCR, enrichment and chip loading). Consequently, hands-on time is clearly higher than conventional studies in both NSCLC and mCRC samples. However, the development of new automatized devices for library and template preparation has drastically reduced hands-on time to approximately 1 hour, making NGS implementation in terms of technical staff much easier. Global time duration of NGS studies has also been higher than conventional approaches in NSCLC (5 versus 3 working days) and in mCRC (5 versus 3–4 working days depending on *KRAS* mutational status). This delay in molecular studies should not be an important limitation of NGS implementation because of its ability to identify clinically relevant alterations beyond the routinely tested genes. Moreover, the coexistence of both strategies may allow the choice of a faster conventional strategy when needed, especially in patients whose clinical situation requires a molecular result in a short period of time.

The implementation of this workflow in diagnostics routine was our first experience with NGS which allowed us to acquire a great expertise in amplicon-based NGS approaches. This has permitted us improve and optimized the process by implementing more complex gene panels (such as Oncomine Focus Assay, able to detect hotspot mutations in 35 genes, copy number variation in 19 genes and fusion transcripts of 23 driver genes) and automatizing the process (IonChef Instrument). Moreover, the development of new and faster sequencers (Ion S5 Instrument) is able to reduce global time duration to 4 days. However, it is important to acknowledge that NGS is economically sustainable when the appropriate number of samples is studied in the same experiment. In this sense, and according to the number of samples received for NGS studies, we are reporting NGS results under routine laboratory conditions in approximately 10–15 working days, as recommended [28–30].

The major advantage of the NGS approach is to provide information about potential therapeutic targets to improve clinical outcomes of patients with advanced cancer. Multiple biomarker testing has become a major challenge for molecular diagnostic laboratories because of the increasing number of approved targeted therapies and clinical trials. In this scenario NGS has been postulated as a technology with clinical applicability able to provide an exhaustive molecular profiling, deciphering tumoral heterogeneity that can in certain cases have a prognostic value and/or explain treatment resistance [31].

The mutation prevalence identified in our study for NSCLC and mCRC samples is concordant with previously published studies [32–34]. Exhaustive molecular profiling can provide relevant information for a patient's clinical management. In our study, 58% of NSCLC patients harbored a potential clinically-actionable alteration, confirming the applicability of these studies for candidate selection. Regarding mCRC, in *RAS* wild type patients (n = 50) NGS identified four patients with mutated *PIK3CA*. Response to anti-EGFR treatment in these patients is still controversial [35] although *RAS-RAF* and *PIK3CA* wild-type patients seem to have better responses [36]. Moreover, one patient harbored the p.Lys57Asn mutation in *MAP2K1* that has been described as a primary resistance

mechanism to anti-EGFR treatment [37]. Taken together NGS identified 49% of patients without resistance mechanisms to this targeted therapy.

Concurrent mutations have been detected in 35% of NSCLC patients and in 50% of mCRC patients revealing tumor biology complexity. Although there are no well-established molecular prognostic factors neither in NSCLC nor mCRC certain passenger mutations may be associated with an adverse prognosis. In this sense, *TP53* [38] or *STK11* in concomitancy with *KRAS* mutations [39] have been associated with a worse prognosis in NSCLC. In mCRC, *TP53* [40] or *SMAD4* [41] mutations have been related to a worse response to anti-EGFR therapy and *FBXW7* [42] has recently been described as a strong worse prognostic factor.

4. Materials and Methods

4.1. Patients and Samples

The study included a series of 106 advanced NSCLC (stages III–IV) and 102 mCRC (stage IV) patients diagnosed in the Department of Medical Oncology at the University Hospital La Fe (Valencia, Spain) from 2015 to 2017. The epidemiological, clinical and pathological features of these patients are summarized in Table 3. All patients showed their agreement by signing the informed consent elaborated in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki [43] and the study was approved by the Hospital Ethics Committee (2015/0713; 16 February 2016, 2015/0096; 15 July 2016 2017/0070 29 March 2017), Tissue samples were examined in the Department of Pathology and only those with at least 150 total cells and 20% of tumor content were considered valid for molecular analysis. Two reference standard DNA samples provided by the European Molecular Genetics Quality Network (EMQN) were also used as positive controls.

Table 3. Epidemiological and clinical-pathological characteristics of the patients included.

NSCLC Patients (*n* = 100)		**mCRC Patients (*n* = 100)**	
Variable	*n*	Variable	*n*
Age (mean ±SD)	65.18 ± 10.66	Age (mean ±SD)	64.91 ± 10.82
Age, years		Age, years	
<60	30	<60	34
≥60	70	≥60	66
Gender		Gender	
Male	65	Male	63
Female	35	Female	37
Anatomic site		Anatomic site	
Primary tumor	85	Primary tumor	85
Regional lymph nodes	5	Liver	7
Brain	4	Lung	4
Liver	2	Peritoneum	2
Others	4	Others	2
Histologic NSCLC type		Histologic mCRC type	
Adenocarcinoma	87	Adenocarcinoma	100
Squamous Cell Carcinoma	3		
NOS	10		
Smoking status		Tumor Location	
Non-smoker	21	Sigmoid Colon	31
Ex-smoker	45	Rectum	26
Current-smoker	34	Right (ascending) colon	14
		Left (descending) colon	9
		Transverse colon	6
		Splenic flexure	5
		Cecum	3
		Unknown	6

NSCLC—Non-small cell lung cancer; mCRC—metastatic colorectal cancer; NOS—Not Otherwise Specified.

4.2. DNA and RNA Preparation

Genomic DNA was isolated from three 5 μm thick FFPE sections using Deparaffinization Solution and the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany). RNA was extracted from three 15 μm thick FFPE sections employing the RecoverAllTM Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). DNA and RNA concentration was assessed using Qubit 3.0 fluorometer with DNA HS or RNA HS Assay Kit (ThermoFisher Scientific).

4.3. Molecular Analysis by Next Generation Sequencing

Molecular analysis was performed at the Molecular Biology Unit (University Hospital la Fe) using *Conformité Européenne*-In vitro diagnostic (CE-IVD) approved kits and workflows.

4.3.1. Next Generation Sequencing Panels

Oncomine Solid Tumor DNA kit (OST-DNA; ThermoFisher Scientific) was used for mutation detection in 22 genes involved in colon and lung cancer (*AKT1* (NM_001014431.1), *ALK* (NM_004304.4), *BRAF* (NM_004333.4), *CTNNB1* (NM_001904.3), *DDR2* (NM_006182.2), *EGFR* (NM_005228.3), *ERBB2* (NM_004448.3), *ERBB4* (NM_005235.2), *FBXW7* (NM_033632.3), *FGFR1* (NM_001174067.1), *FGFR2* (NM_022970.3), *FGFR3* (NM_001163213.1), *KRAS* (NM_033360.3), *MAP2K1* (NM_002755.3), *MET* (NM_001127500.1), *NOTCH1* (NM_017617.3), *NRAS* (NM_002524.4), *PIK3CA* (NM_006218.2), *PTEN* (NM_000314.4), *SMAD4* (NM_005359.5), *STK11* (NM_000455.4), *TP53* (NM_000546.5)). The design includes 92 amplicons. For RNA sequencing of NSCLC samples, we used Oncomine Solid Tumor Fusion Transcript kit (OST-RNA; ThermoFisher Scientific), that allows the detection of fusion transcripts involving *ALK*, *RET*, *ROS1* and *NTRK1* genes with 85 amplicons. All NGS studies were conducted with the Ion Torrent Personal Genome Machine (PGM) technology (ThermoFisher Scientific).

4.3.2. Ion Torrent Library Preparation

For DNA libraries preparation, multiplex PCR was performed on 10 ng of DNA. After primer digestion and barcode ligation, library fragments were purified with Agencourt® AMPure® XP (Beckman Coulter, Brea, CA, USA). Finally, quantification and dilution (100 pM) of the amplified libraries was performed using the Ion Library Equalizer Kit (ThermoFisher Scientific) as described by the manufacturer.

RNA libraries preparation included a previous cDNA synthesis step from 10 ng of RNA using the SuperScript kit VILO cDNA synthesis kit (ThermoFisher Scientific). In this case, a multiplex PCR amplification of cDNA was performed. Library quantification was carried out by qPCR, inferring the concentration from a standard curve generated with Ion Library Quantification Kit (ThermoFisher Scientific). RNA libraries were diluted to a concentration of 100 pM.

In NSCLC, DNA and RNA libraries from eight patients were combined in a 4:1 proportion, generating the library pool. In mCRC samples, 10 DNA libraries were combined in equal proportion.

4.3.3. Clonal Amplification and DNA Sequencing

The library pool was clonally amplified in an emulsion PCR reaction using Ion Sphere Particles (ISPs) in the One Touch 2 Instrument. Subsequently, template-positive ISPs were enriched using the Ion One Touch ES with the Ion PGM Hi-Q OT2 kit following manufacturer´s protocol. Enriched template-positive ISPs were subjected to sequencing on the Ion Torrent Personal Genome Machine (PGM) on a 318v2 Ion Chip using Ion PGM Sequencing Hi-Q kit (all kits from ThermoFisher Scientific).

4.3.4. Base Calling, Variant Annotation and Prediction Tools Analysis

Raw data processing and alignment to the hg19 human reference genome was performed with Torrent Suite v5.6. Aligned sequences (Binary Alignment Map (BAM) files) were automatically transferred to the Ion Reporter Software (v5.6) to perform variant calling/annotation by using

commercial workflows. Intronic variants and synonymous changes were filtered out. Variants with low total read depth (<500 total) and/or low variant read depth (<20 reads) were excluded. Additionally, Variants were visually examined using the Integrative Genomics Viewer (IGV) software (v.2.4). Subsequently, sequence variation databases such as Catalogue of Somatic Mutations in Cancer (COSMIC) [44], VarSome [45], The 1000 Genomes Project [46] and Single Nucleotide Polymorphism Database (dbSNPs) [47] were used to assess the pathogenicity of the detected variants. In variants with unknown significance, prediction tools like Provean [48], Sorting intolerant from tolerant (SIFT) [49] and PolyPhen-2 [50] were used in order to predict the effect of the amino acid substitution on the protein structure and function.

4.4. Experimental Verification

Verification instead of a full validation analysis was performed according to our national accreditation body (ENAC; Entidad Nacional de Acreditación), since the OST-DNA and OST-RNA kits are CE-IVD approved. The performance of NGS testing was extensively evaluated on different aspects. Firstly, we used well-characterized reference material to assess the presence or absence of somatic variants (point mutation and small insertions/deletions) and their allele frequencies. Secondly, we considered the pre-analytical conditions and assessed the quality of NGS analysis on determining FFPE samples as start material, allowing us to establish the sequencing quality metrics. Thirdly, diagnostic sensitivity and specificity were determined experimentally by comparing it with conventional methods for routinely tested alterations; additionally, clinical reporting was adapted according to international diagnostic standards and professional guidelines. Finally, to ensure a consistent high standard of performance, it was essential to establish an EQA program to monitor the quality of NGS testing in clinical practice and to propose corrective actions when needed.

4.4.1. Low Frequency Variant Detection

To evaluate the performance of the NGS assay for low frequency variant (<5%) detection we used reference materials provided by the European Molecular Genetics Quality Network (EMQN) in the External Quality Assessment Scheme for Oncogene Panel Testing (2017 and 2018). One of the reference materials used harbored the *EGFR* hotspot mutation p.Leu858Arg (VAF:3%), the *EGFR* deletion p.Glu746_Ala750del (VAF:2%) and the resistance hotspot mutation p.Thr790Met (VAF:1%) and the other harbored the following low frequency variants: *EGFR* p.Leu858Arg (VAF:3%), p.Thr790Met (VAF:2%), *KRAS* p.Gly13Asp (VAF:3%) and *PIK3CA* p.His1047Arg (VAF:3%). All described variants had previously been validated by ddPCR.

4.4.2. Next Generation Sequencing Metrics

The number of reads, mean depth, "on-target" reads and uniformity were the parameters used as quality control check points for further sample analysis. A total number of reads higher than 100,000 together with "on-target" and uniformity values >80% were required for each DNA library and 20,000 total reads for each RNA library.

4.4.3. Assessment of the Diagnostic Sensitivity and Specificity of the NGS Assay

Detected missense mutations in *EGFR*, *NRAS*, *KRAS* and *BRAF* genes, as well as *ALK* and *ROS1* genes rearrangements were tested by conventional methods. *EGFR* mutations were validated by Cobas® *EGFR* Mutation Test v2 (CE-IVD) (Roche Diagnostics, Basel, Switzerland); *NRAS* and *KRAS* mutations were confirmed by Real Time (RT)-qPCR using AmoyDx® *KRAS* Mutation Detection Kit and AmoyDx® *NRAS* Mutation Detection Kit (AmoyDx, Xiamen, China); *BRAF* mutations were validated by High Resolution Melting (HRM) as previously described [51]. *ALK* and *ROS1* rearrangements were studied by immunohistochemistry (IHQ) employing VENTANA *ALK* (D5F3) CDx Assay (Roche Diagnostics, Basel, Switzerland) and IHQ *ROS1* Clon D4D6 (Cell Signaling Technology, Danvers, MA, USA), respectively. Positive IHQ assays were confirmed by fluorescence in situ hybridization (FISH)

employing Vysis *ALK* Break Apart FISH Probe Kit (Abbott Laboratories, Chicago, Illinois, USA) and Vysis 6q22 *ROS1* Break Apart FISH Probe Kit (Abbott Laboratories), respectively.

4.4.4. External Quality Assessment (EQA) Program

To ensure a consistent high standard of performance, this assay was externally validated by the participation in the EMQN External Quality Assessment Scheme for Oncogene Panel Testing (2017/2018).

4.5. Statistics

Quantitative variables were summarized by their mean and standard deviation, and categorical variables by absolute frequencies.

5. Conclusions

NGS is a technology able to assess multiple genetic biomarkers that has demonstrated a great concordance with conventional single target assays, providing an exhaustive molecular profiling of clinically relevant alterations at reasonable costs and turnaround times. The implementation of NGS in the diagnostic routine under the scope of UNE-EN ISO 15189:2013 accreditation has provided relevant information for patients' clinical management, improving the molecular diagnostic in our center.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/8/1196/s1, Figure S1: NGS quality metrics, Table S1: Pathogenic variants detected in NSCLC patients, Table S2: Pathogenic variants detected in mCRC patients.

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Supplementary Materials: Development Implementation and Assessment of Molecular Diagnostics by Next Generation Sequencing in Personalized Treatment of Cancer: Experience of a Public Reference Healthcare Hospital.

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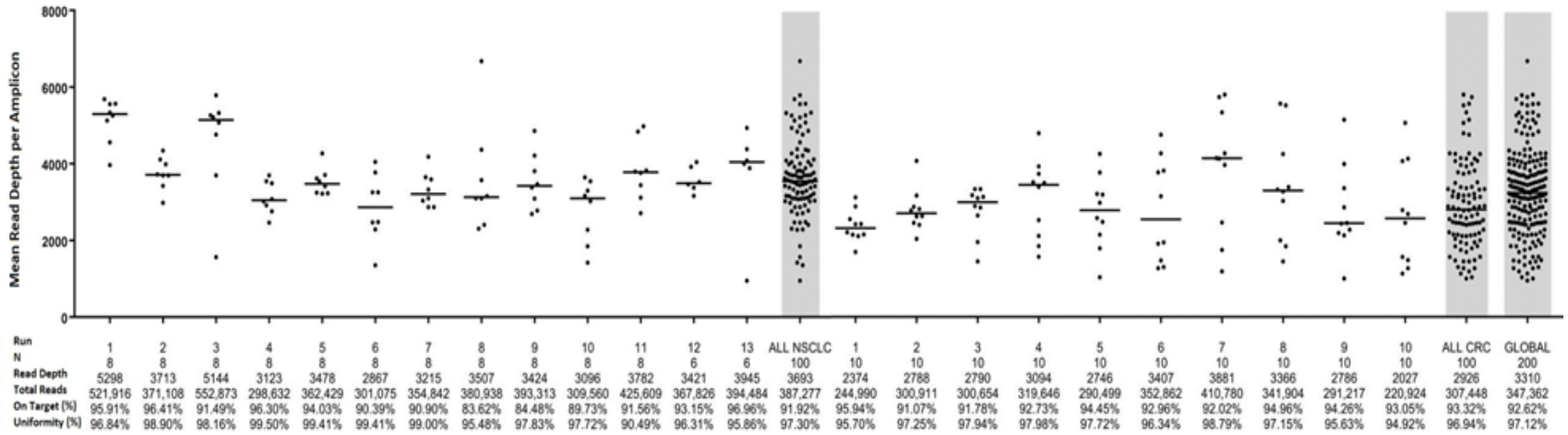

Figure S1. NGS quality metrics. N- Number of samples analyzed per run; Read Depth- Represents the mean value of reads of each amplicon; Total Reads-Represent mean total reads per samples; "On target"-Represents the mean % of reads over the sequences of interest; Uniformity-Represents the mean % of uniformity coverage.

Table S1. Pathogenic variants detected in NSCLC patients.

Patient	Chr Position	Gene	NG/NM	Fusion	Mutation Type	Exon	Amino Acid Change	CDS	Total Coverage	Variant Coverage	VAF
NSCLC1	chr3:178952090	*PIK3CA*	NM_006218.2		missense	21	p.Gly1049Arg	c.3145G>C	3995	773	19 %
	chr7:55259515	*EGFR*	NM_005228.3		missense	21	p.Leu858Arg	c.2573T>G	3959	800	20 %
NSCLC2	chr7:55242462	*EGFR*	NM_005228.3		nonframeshift deletion	19	p.Glu746_Thr751delinsVA	c.2237_2253delinsTGCT	3996	2318	58 %
NSCLC3	chr7:55242464	*EGFR*	NM_005228.3		nonframeshift deletion	19	p.Glu746_Ala750del	c.2235_2249del	3535	107	3 %
NSCLC4	chr7:55259515	*EGFR*	NM_005228.3		missense	21	p.Leu858Arg	c.2573T>G	3523	390	11 %
NSCLC5	chr3:178952085	*PIK3CA*	NM_006218.2		missense	21	p.His1047Leu	c.3140A>T	3994	3052	76 %
	chr17:7578190	*TP53*	NM_000546.5		missense	6	p.Tyr220Cys	c.659A>G	3999	2653	66 %
NSCLC7	chr2:42522656	*ALK*	NG_009445.1	EML4 - ALK		6|20					-
NSCLC8	chr17:7577094	*TP53*	NM_000546.5		missense	8	p.Arg282Trp	c.844C>T	1396	237	17 %
NSCLC9	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	3993	1375	34 %
	chr17:7578442	*TP53*	NM_000546.5		missense	5	p.Tyr163Cys	c.488A>G	1128	668	59 %
NSCLC10	chr17:7578457	*TP53*	NM_000546.5		missense	5	p.Arg158Pro	c.473G>C	1657	1365	82 %
NSCLC11	chr12:25380275	*KRAS*	NM_033360.3		missense	3	p.Gln61His	c.183A>T	3996	1568	39 %
NSCLC12	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3329	788	24 %
NSCLC13	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Asp	c.35G>A	3996	1738	43 %
	chr17:7578530	*TP53*	NM_000546.5		missense	5	p.Phe134Val	c.400T>G	2745	743	27 %
NSCLC14	chr17:7577534	*TP53*	NM_000546.5		missense	7	p.Arg249Ser	c.747G>T	3215	535	17 %
	chr17:7578406	*TP53*	NM_000546.5		missense	5	p.Arg175Leu	c.524G>T	2127	211	10 %
NSCLC15	chr12:25398281	*KRAS*	NM_033360.3		missense	2	p.Gly13Asp	c.38G>A	3998	1275	32 %
	chr17:7578468	*TP53*	NM_000546.5		frameshift deletion	5	p.Gly154fs	c.461_461del	1119	228	20 %
NSCLC16	chr2:42522656	*ALK*	NG_009445.1	EML4 - ALK		6|20					-
NSCLC17	chr3:41266113	*CTNNB1*	NM_001904.3		missense	3	p.Ser37Cys	c.110C>G	1781	298	17 %
	chr7:55241677	*EGFR*	NM_005228.3		missense	18	p.Glu709Lys	c.2125G>A	2721	1101	40 %
	chr7:55241707	*EGFR*	NM_005228.3		missense	18	p.Gly719Cys	c.2155G>T	2709	1100	41 %
NSCLC18	chr17:7577535	*TP53*	NM_000546.5		missense	7	p.Arg249Thr	c.746G>C	3997	1583	40 %
NSCLC19	chr7:55259515	*EGFR*	NM_005228.3		missense	21	p.Leu858Arg	c.2573T>G	3637	248	7 %
	chr17:7578269	*TP53*	NM_000546.5		missense	6	p.Leu194Phe	c.580C>T	2652	228	9 %
NSCLC20	chr12:25398281	*KRAS*	NM_033360.3		missense	2	p.Gly13Asp	c.38G>A	3513	599	17 %
	chr17:7578461	*TP53*	NM_000546.5		missense	5	p.Val157Phe	c.469G>T	3379	849	25 %
NSCLC22	chr6:117645578	*ROS1*	NG_033929.1	EZR - ROS1		10|34					-
NSCLC23	chr7:140453136	*BRAF*	NM_004333.4		missense	15	p.Val600Glu	c.1799T>A	3997	128	3 %
NSCLC24	chr7:140453136	*BRAF*	NM_004333.4		missense	15	p.Val600Glu	c.1799T>A	3993	205	5 %
	chr12:25380275	*KRAS*	NM_033360.3		missense	3	p.Gln61His	c.183A>C	3996	525	13 %
NSCLC25	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3994	248	6 %
NSCLC26	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	3988	305	8 %
	chr17:7578535	*TP53*	NM_000546.5		missense	5	p.Lys132Arg	c.395A>G	3098	176	6 %
NSCLC27	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	3880	504	13 %
NSCLC28	chr7:140481402	*BRAF*	NM_004333.4		missense	11	p.Gly469Val	c.1406G>T	3956	1038	26 %
NSCLC29	chr17:7577538	*TP53*	NM_000546.5		missense	7	p.Arg248Leu	c.743G>T	3958	669	17 %
NSCLC30	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	2386	357	15 %
NSCLC31	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	2688	738	27 %
	chr17:7577538	*TP53*	NM_000546.5		missense	7	p.Arg248Leu	c.743G>T	3878	629	16 %

	chr19:1221313	*STK11*	NM_000455.4		frameshift deletion	6	p.Pro281fs	c.837_837del	1984	567	29 %
NSCLC32	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	3905	976	25 %
NSCLC33	chr17:7578469	*TP53*	NM_000546.5		missense	5	p.Gly154Val	c.461G>T	1336	469	35 %
NSCLC34	chr17:7574003	*TP53*	NM_000546.5		nonsense	10	p.Arg342Ter	c.1024C>T	3695	763	21 %
	chr17:7577114	*TP53*	NM_000546.5		frameshift insertion	8	p.Cys275fs	c.823_824insT	2190	87	4 %
NSCLC35	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3999	2387	60 %
	chr17:7577120	*TP53*	NM_000546.5		missense	8	p.Arg273Leu	c.818G>T	1726	900	52 %
NSCLC36	chr17:7578403	*TP53*	NM_000546.5		missense	5	p.Cys176Tyr	c.527G>A	2573	1840	72 %
NSCLC38	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	2941	254	9 %
	chr3:41266134	*CTNNB1*	NM_001904.3		missense	3	p.Pro44Leu	c.131C>T	2260	789	35 %
NSCLC39	chr3:178936091	*PIK3CA*	NM_006218.2		missense	10	p.Glu545Lys	c.1633G>A	3887	550	14 %
	chr17:7579355	*TP53*	NM_000546.5		missense	4	p.Leu111Arg	c.332T>G	2014	785	39 %
NSCLC40	chr7:140453134	*BRAF*	NM_004333.4		missense	15	p.Lys601Glu	c.1801A>G	1452	397	27 %
NSCLC41	chr17:7577046	*TP53*	NM_000546.5		nonsense	8	p.Glu298Ter	c.892G>T	1550	145	9 %
NSCLC42	chr17:7579358	*TP53*	NM_000546.5		missense	4	p.Arg110Leu	c.329G>T	552	130	24 %
NSCLC43	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	2833	170	6 %
NSCLC44	chr17:7578526	*TP53*	NM_000546.5		missense	5	p.Cys135Tyr	c.404G>A	1405	638	45 %
NSCLC45	chr17:7578208	*TP53*	NM_000546.5		missense	6	p.His214Arg	c.641A>G	3215	1476	46 %
NSCLC46	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	3996	896	22 %
NSCLC48	chr3:178936082	*PIK3CA*	NM_006218.2		missense	10	p.Glu542Gln	c.1624G>C	3664	282	8 %
	chr17:7578461	*TP53*	NM_000546.5		missense	5	p.Val157Phe	c.469G>T	2290	225	10 %
NSCLC49	chr17:7578395	*TP53*	NM_000546.5		missense	5	p.His179Tyr	c.535C>T	3965	845	21 %
NSCLC50	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3991	1456	36 %
	chr17:7577528	*TP53*	NM_000546.5		nonframeshift deletion	7	p.Arg249del	c.745_747delAGG	3912	1240	32 %
NSCLC51	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3728	888	24 %
	chr17:7578517	*TP53*	NM_000546.5		missense	5	p.Ala138Val	c.413C>T	2464	486	20 %
NSCLC52	chr7:140453154	*BRAF*	NM_004333.4		missense	15	p.Asp594Val	c.1781A>T	1493	64	4 %
	chr12:25398281	*KRAS*	NM_033360.3		missense	2	p.Gly13Asp	c.38G>A	1946	98	5 %
NSCLC53	chr10:89717712	*PTEN*	NM_000314.4		missense	7	p.Pro246Leu	c.737C>T	3977	422	11 %
	chr17:37880981	*ERBB2*	NM_004448.3		duplication	20	p.Ala771_Met774dup	c.2311_2322dup	2146	556	26 %
NSCLC55	chr17:7577022	*TP53*	NM_000546.5		nonsense	8	p.Arg306Ter	c.916C>T	1091	300	28 %
NSCLC56	chr4:153247289	*FBXW7*	NM_033632.3		missense	10	p.Arg505Ser	c.1513C>A	3996	121	3 %
NSCLC57	chr7:55242478	*EGFR*	NM_005228.3		frameshift deletion		p.Glu747_Ala750delinsP	c.2238_2248delinsGC	3559	172	5 %
	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3438	2740	80 %
NSCLC58	chr7:140481411	*BRAF*	NM_004333.4		missense	11	p.Gly466Val	c.1397G>T	2459	453	18 %
	chr17:7579882	*TP53*	NM_000546.5		nonsense	2	p.Glu11Ter	c.31G>T	3442	630	18 %
NSCLC59	chr7:55242468	*EGFR*	NM_005228.3		nonframeshift deletion	19	p.Glu746_Arg748del	c.2236_2244del	1647	1101	67 %
	chr17:7577105	*TP53*	NM_000546.5		missense	8	p.Pro278Arg	c.833C>G	524	84	16 %
NSCLC60	chr2:42522656	*ALK*	NG_009445.1	EML4|ALK		13|20					-
NSCLC61	chr7:140453136	*BRAF*	NM_004333.4		missense	15	p.Val600Glu	c.1799T>A	3920	227	6 %
	chr17:7577517	*TP53*	NM_000546.5		missense	7	p.Ile255Asn	c.764T>A	1850	104	6 %
NSCLC64	chr1:115256529	*NRAS*	NM_002524.4		missense	3	p.Gln61Leu	c.182A>T	3987	500	13 %
	chr17:7577035	*TP53*	NM_000546.5		frameshift deletion	8	p.Pro301fs	c.902_902delC	1416	89	6 %
	chr3:178952146	*PIK3CA*	NM_006218.2		missense	21	p.Leu1067Phe	c.3201G>C	3997	365	9 %
NSCLC65	chr4:153249460	*FBXW7*	NM_033632.3		missense	9	p.Asp440His	c.1318G>C	3996	263	7 %
	chr7:140481411	*BRAF*	NM_004333.4		missense	11	p.Gly466Ala	c.1397G>C	2599	651	25 %

	chr17:7578441	*TP53*	NM_000546.5		nonsense	5	p.Tyr163Ter	c.489C>A	1795	444	25 %
NSCLC66	chr2:42522656	*ALK*	NG_009445.1	EML4 - ALK		6|20					-
NSCLC67	chr17:7578452	*TP53*	NM_000546.5		frameshift deletion	5	p.Met160fs	c.477_477delC	3261	579	18 %
NSCLC68	chr7:55259515	*EGFR*	NM_005228.3		missense	21	p.Leu858Arg	c.2573T>G	3973	113	3 %
	chr17:7578430	*TP53*	NM_000546.5		frameshift deletion	5	p.Gln167fs	c.499_499delC	3289	170	5 %
NSCLC69	chr17:7578280	*TP53*	NM_000546.5		missense	6	p.Pro190Leu	c.569C>T	1334	114	9 %
NSCLC70	chr12:25398280	*KRAS*	NM_033360.3		missense	2	p.Gly13Glu	c.38_39delinsAA	3982	1797	45 %
NSCLC71	chr17:7577099	*TP53*	NM_000546.5		missense	8	p.Arg280Thr	c.839G>C	1332	461	35 %
NSCLC72	chr17:7577539	*TP53*	NM_000546.5		missense	7	p.Arg248Trp	c.742C>T	1676	616	37 %
NSCLC73	chr17:7577108	*TP53*	NM_000546.5		missense	8	p.Cys277Phe	c.830G>T	1465	473	32 %
NSCLC74	chr7:55259515	*EGFR*	NM_005228.3		missense	21	p.Leu858Arg	c.2573T>G	523	167	32 %
NSCLC75	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3998	641	16 %
NSCLC76	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3994	2031	51 %
	chr17:7577534	*TP53*	NM_000546.5		missense	7	p.Arg249Ser	c.747G>C	1786	642	36 %
NSCLC78	chr12:25380285	*KRAS*	NM_033360.3		missense	3	p.Thr58Ile	c.173C>T	4000	98	2 %
	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3994	1132	28 %
NSCLC79	chr7:140481411	*BRAF*	NM_004333.4		missense	11	p.Gly466Val	c.1397G>T	3999	1994	50 %
	chr17:7577103	*TP53*	NM_000546.5		frameshift insertion	8	p.Gly279fs	c.834_835insT	2670	1151	43 %
NSCLC80	chr2:212578301	*ERBB4*	NM_005235.2		missense	8	p.Gly319Val	c.956G>T	2793	1008	36 %
	chr17:7578457	*TP53*	NM_000546.5		missense	5	p.Arg158Leu	c.473G>T	1036	784	76 %
NSCLC81	chr17:37880981	*ERBB2*	NM_004448.3		duplication	20	p.Ala771_Met774dup	c.2311_2322dup	1467	200	14 %
NSCLC82	chr7:140481411	*BRAF*	NM_004333.4		missense	11	p.Gly466Val	c.1397G>T	3993	343	9 %
	chr17:7578442	*TP53*	NM_000546.5		missense	5	p.Tyr163Cys	c.488A>G	3860	238	6 %
NSCLC83	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Arg	c.34G>C	3993	777	19 %
NSCLC84	chr17:7578469	*TP53*	NM_000546.5		missense	5	p.Gly154Val	c.461G>T	1480	351	24 %
NSCLC85	chr17:7578392	*TP53*	NM_000546.5		nonsense	5	p.Glu180Ter	c.538G>T	1666	534	32 %
	chr19:1220487	*STK11*	NM_000455.4		missense	4	p.Asp194Tyr	c.580G>T	749	209	28 %
NSCLC86	chr17:7577090	*TP53*	NM_000546.5		missense	8	p.Arg283Pro	c.848G>C	1148	51	4 %
NSCLC87	chr17:7578431	*TP53*	NM_000546.5		nonsense	5	p.Gln167Ter	c.499C>T	1896	880	46 %
NSCLC88	chr3:41266098	*CTNNB1*	NM_001904.3		missense	3	p.Asp32Val	c.95A>T	513	20	4 %
	chr7:140453155	*BRAF*	NM_004333.4		missense	15	p.Asp594Asn	c.1780G>A	798	187	23 %
NSCLC89	chr3:178936082	*PIK3CA*	NM_006218.2		missense	10	p.Glu542Lys	c.1624 G>A	710	16	2 %
	chr7:55242464	*EGFR*	NM_005228.3		nonframeshift deletion	19	p.Glu746_Ala750del	c.2235_2249del	1283	242	19 %
NSCLC90	chr12:25398284	*KRAS*	NM_033360.3		missense	2	p.Gly12Val	c.35G>T	3993	589	15 %
NSCLC91	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3996	157	4 %
	chr17:37881022	*ERBB2*	NM_004448.3		missense	20	p.Arg784His	c.2351G>A	4000	147	4 %
NSCLC92	chr7:55259515	*EGFR*	NM_005228.3		missense	21	p.Leu858Arg	c.2573T>G	3989	409	10 %
	chr17:7578469	*TP53*	NM_000546.5		missense	5	p.Gly154Val	c.461G>T	2903	199	7 %
NSCLC93	chr7:55248998	*EGFR*	NM_005228.3		duplication	20	p.Ala767_Val769dup	c.2300_2308dup	2758	1009	37 %
NSCLC94	chr12:25380276	*KRAS*	NM_033360.3		missense	3	p.Gln61Leu	c.182A>T	3998	535	13 %
NSCLC96	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Ser	c.34G>A	1552	287	18 %
NSCLC97	chr2:42522656	*ALK*	NG_009445.1	ALK Fusion							-
	chr3:178936082	*PIK3CA*	NM_006218.2		missense	9	p.Glu542Lys	c.1624G>A	1994	136	7 %
NSCLC98	chr17:7578394	*TP53*	NM_000546.5		missense	5	p.His179Arg	c.536A>G	1769	198	11 %
NSCLC99	chr12:25398285	*KRAS*	NM_033360.3		missense	2	p.Gly12Cys	c.34G>T	3659	509	14 %
NSCLC100	chr17:7578457	*TP53*	NM_000546.5		missense	5	p.Arg158Leu	c.473G>T	3835	2682	70 %

CDS- Coding sequence nomenclature; Total Coverage- Total reads in the genomic region; Variant Coverage- Total variant reads; VAF- Variant allele frequency (%).

Table S2. Pathogenic variants detected in mCRC patients.

Patient	Chr Position	Gene	NG/NM	Mutation Type	Exon	Amino Acid Change	CDS	Total Coverage	Variant Coverage	VAF
CRC1	chr17:7577547	*TP53*	NM_000546.5	Missense	7	p.Gly245Val	c.734G>T	1686	166	10 %
CRC3	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	4777	1603	34 %
	chr18:48591826	*SMAD4*	NM_005359.5	Missense	9	p.Glu330Gly	c.989A>G	3709	1556	42 %
CRC4	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Cys	c.34G>T	2505	547	22 %
	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3142	705	22 %
CRC5	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Cys	c.34G>T	3997	1126	28 %
	chr17:7578382	*TP53*	NM_000546.5	Nonsense	5	p.Ser183Ter	c.548C>G	2085	993	48 %
CRC6	chr17:7578392	*TP53*	NM_000546.5	Deletion	5	p. Arg174_His179del	c.520_537del	1490	730	49 %
	chr17:7578269	*TP53*	NM_000546.5	Missense	6	p.Leu194Phe	c.580C>T	1846	49	3 %
CRC7	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	1544	236	15 %
CRC8	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	3994	1.358	34 %
CRC9	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	2733	1.613	59 %
CRC10	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3689	165	4 %
	chr17:7577120	*TP53*	NM_000546.5	Missense	8	p.Arg273His	c.818G>A	1433	166	12 %
CRC11	chr17:7578212	*TP53*	NM_000546.5	Nonsense	6	p.Arg213Ter	c.637C>T	2045	382	19 %
CRC12	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Cys	c.34G>T	3997	1.126	28 %
	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	2268	58	3 %
	chr17:7577121	*TP53*	NM_000546.5	Missense	8	p.Arg273Cys	c.817C>T	2150	163	8 %
CRC13	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Ala	c.35G>C	2938	762	26 %
	chr17:7578395	*TP53*	NM_000546.5	Missense	5	p.His179Tyr	c.535C>T	2444	1.040	43 %
	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	2870	207	7 %
CRC14	chr3:178952090	*PIK3CA*	NM_006218.2	Missense	20	p.His1049Arg	c.3145G>C	3123	226	7 %
	chr17:7578406	*TP53*	NM_000546.5	Missense	5	p.Arg175His	c.524G>A	2765	144	5 %
CRC15	chr1:115256529	*NRAS*	NM_002524.4	Missense	3	p.Gln61Arg	c.182A>G	1773	105	6 %
	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	1847	107	6 %
CRC16	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	2154	356	17 %
CRC18	chr12:25378647	*KRAS*	NM_033360.3	Missense	4	p.Lys117Asn	c.351A>T	3897	1.888	48 %
CRC19	chr17:7577538	*TP53*	NM_000546.5	Missense	7	p.Arg248Gln	c.743G>A	3984	330	8 %
	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	3993	710	18 %
CRC20	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3930	100	3 %
	chr17:7577538	*TP53*	NM_000546.5	Missense	7	p.Arg248Gln	c.743G>A	3974	923	23 %
CRC21	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	3987	1.237	31 %
	chr17:7577022	*TP53*	NM_000546.5	Nonsense	8	p.Arg306Ter	c.916C>T	1148	515	45 %
	chr3:178936095	*PIK3CA*	NM_006218.2	Missense	9	p.Gln546Arg	c.1637A>G	3410	310	9 %
CRC22	chr18:48603032	*SMAD4*	NM_005359.5	Nonsense	11	p.Arg445Ter	c.1333C>T	2530	169	7 %
	chr17:7578403	*TP53*	NM_000546.5	Missense	5	p.Cys176Tyr	c.527G>A	553	110	20 %
CRC23	chr3:178952085	*PIK3CA*	NM_006218.2	Missense	20	p.His1047Arg	c.3140A>G	1384	286	21 %
	chr17:7577539	*TP53*	NM_000546.5	Missense	7	p.Arg248Trp	c.742C>T	1811	260	14 %
CRC24	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	2192	314	14 %
	chr4:153249348	*FBXW7*	NM_033632.3	Missense	8	p.Arg465His	c.1394G>A	3283	765	23 %

	chr17:7577547	*TP53*	NM_000546.5	Missense	7	p.Gly245Asp	c.734G>A	1304	252	19 %
	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Ser	c.34G>A	3997	1.822	46 %
CRC27	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3934	334	8 %
	chr17:7577551	*TP53*	NM_000546.5	Missense	7	p.Gly244Ser	c.730G>A	4000	1.632	41 %
CRC28	chr17:7577559	*TP53*	NM_000546.5	Missense	7	p.Ser241Phe	c.722C>T	2123	587	28 %
CRC30	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	3486	346	10 %
	chr17:7577094	*TP53*	NM_000546.5	Missense	8	p.Arg282Trp	c.844C>T	580	220	38 %
CRC31	chr12:25378562	*KRAS*	NM_033360.3	Missense	4	p.Ala146Thr	c.436G>A	4000	1.003	25 %
	chr12:25398262	*KRAS*	NM_033360.3	Missense	2	p.Leu19Phe	c.57G>T	3175	683	22 %
	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	2595	994	38 %
CRC32	chr3:178936091	*PIK3CA*	NM_006218.2	Missense	9	p.Glu545Lys	c.1633G>A	2186	514	24 %
	chr17:7578388	*TP53*	NM_000546.5	Missense	5	p.Arg181His	c.542G>A	1668	541	32 %
	chr17:7578461	*TP53*	NM_000546.5	Missense	5	p.Val157Leu	c.469G>C	1665	1.038	62 %
CRC33	chr12:25398282	*KRAS*	NM_033360.3	Missense	2	p.Gly13Cys	c.37G>T	3997	1.037	26 %
CRC34	chr17:7577022	*TP53*	NM_000546.5	Nonsense	8	p.Arg306Ter	c.916C>T	926	279	30 %
CRC35	chr17:7577120	*TP53*	NM_000546.5	Missense	8	p.Arg273His	c.818G>A	520	260	50 %
CRC36	chr17:7577538	*TP53*	NM_000546.5	Missense	7	p.Arg248Gln	c.743G>A	1240	567	45 %
CRC38	chr12:25378561	*KRAS*	NM_033360.3	Missense	4	p.Ala146Val	c.437C>T	3100	352	11 %
	chr17:7577538	*TP53*	NM_000546.5	Missense	7	p.Arg248Gln	c.743G>A	1387	201	14 %
CRC39	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	3088	429	14 %
	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3759	218	6 %
	chr7:140453148	*BRAF*	NM_004333.4	Missense	15	p.Gly596Val	c.1787G>T	3915	647	17 %
CRC40	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	3979	606	15 %
	chr17:7578406	*TP53*	NM_000546.5	Missense	5	p.Arg175His	c.524G>A	820	220	27 %
CRC41	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	3309	756	23 %
CRC42	chr4:153249348	*FBXW7*	NM_033632.3	Missense	8	p.Arg465His	c.1394G>A	2213	624	28 %
	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Ser	c.34G>A	2981	246	8 %
CRC43	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	3972	1.210	30 %
	chr18:48591892	*SMAD4*	NM_005359.5	Missense	9	p.Gly352Val	c.1055G>T	3997	377	9 %
CRC44	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Ser	c.34G>A	5555	2.817	51 %
	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3813	790	21 %
CRC45	chr17:7577081	*TP53*	NM_000546.5	Missense	8	p.Glu286Gly	c.857A>G	1722	843	49 %
CRC46	chr14:105246551	*AKT*	NM_0001014431.1	Missense	4	p.Glu17Lys	c.49G>A	700	21	3 %
	chr12:25399291	*KRAS*	NM_033360.3	Missense	2	p.Gly13Asp	c.38G>A	4000	243	6 %
	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	5059	476	9 %
CRC47	chr3:178936095	*PIK3CA*	NM_006218.2	Missense	9	p.Gln546Arg	c.1637A>G	4078	623	15 %
	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361Cys	c.1081C>T	3348	551	16 %
CRC49	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	2185	1.198	55 %
	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Phe	c.34_35delGGinsTT	3707	1.380	37 %
CRC51	chr7:116411923	*MET*	NM_001127500.1	Missense	14	p.Arg988Cys	c.2962C>T	2473	893	36 %
	chr17:7578406	*TP53*	NM_000546.5	Missense	5	p.Arg175His	c.524G>A	2473	893	36 %
CRC53	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	3972	1.210	30 %
	chr3:178952085	*PIK3CA*	NM_006218.2	Missense	20	p.His1047Arg	c.3140A>G	3270	1.921	59 %
CRC54	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	4000	1.985	50 %
	chr17:7577539	*TP53*	NM_000546.5	Missense	7	p.Arg248Trp	c.742C>T	1219	352	29 %
CRC55	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	2713	447	16 %

	chr3:178936091	*PIK3CA*	NM_006218.2	Missense	9	p.Glu545Lys	c.1633G>A	3519	557	16 %
CRC57	chr17:7577094	*TP53*	NM_000546.5	Missense	8	p.Arg282Trp	c.844C>T	2827	2.227	79 %
	chr4:153249446	*FBXW7*	NM_033632.3	Missense	11	p.Ser582Leu	c.1745C>T	6903	1.631	24 %
CRC58	chr12:25378562	*KRAS*	NM_033360.3	Missense	4	p.Ala146Val	c.437C>T	3503	1.575	45 %
	chr17:7578263	*TP53*	NM_000546.5	Nonsense	6	p.Arg196Ter	c.586C>T	2290	771	34 %
CRC59	chr17:7577538	*TP53*	NM_000546.5	Missense	7	p.Arg248Gln	c.743G>A	2905	652	22 %
	chr4:153249348	*FBXW7*	NM_033632.3	Missense	8	p.Arg465His	c.1394G>A	4444	1.148	26 %
CRC60	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	5064	1.715	34 %
	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361Cys	c.1081C>T	5582	313	6 %
	chr17:7578406	*TP53*	NM_000546.5	Missense	5	p.Arg175His	c.524G>A	2198	1.071	49 %
	chr3:41266136	*CTNNB1*	NM_001904.3	Missense	3	p.Ser45Pro	c.133T>C	1961	812	41 %
CRC61	chr12:25378562	*KRAS*	NM_033360.3	Missense	4	p.Ala146Thr	c.436G>A	3232	653	20 %
	chr10:89717769	*PTEN*	NM_000314.4	Deletion	7	p.Lys267fs	c.795del	3202	917	29 %
	chr10:89720852	*PTEN*	NM_000314.4	Missense	8	p.Arg335Ter	c.1003C>T	1932	505	26 %
CRC62	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	6260	908	15 %
CRC63	chr17:7578406	*TP53*	NM_000546.5	Missense	5	p.Arg175His	c.524G>A	2317	710	31 %
CRC64	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	7456	2.076	28 %
CRC65	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	8743	3.635	42 %
	chr17:7577509	*TP53*	NM_000546.5	Missense	7	p.Glu258Lys	c.772G>A	2227	1.475	66 %
CRC67	chr12:25399291	*KRAS*	NM_033360.3	Missense	2	p.Gly13Asp	c.38G>A	1043	120	12 %
CRC68	chr17:7578550	*TP53*	NM_000546.5	Missense	5	p.Ser127Phe	c.380C>T	893	444	50 %
CRC69	chr17:7577120	*TP53*	NM_000546.5	Missense	8	p.Arg273His	c.818G>A	512	236	46 %
CRC70	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	4342	1.861	43 %
	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361Cys	c.1081C>T	2151	455	21 %
CRC71	chr17:7574018	*TP53*	NM_000546.5	Missense	10	p.Arg337Cys	c.1009C>T	503	65	13 %
	chr17:7578437	*TP53*	NM_000546.5	Nonsense	5	p.Gln165Ter	c.493C>T	503	65	13 %
CRC72	chr12:25378561	*KRAS*	NM_033360.3	Missense	4	p.Ala146Val	c.437C>T	3852	1.551	40 %
	chr17:7578406	*TP53*	NM_000546.5	Missense	5	p.Arg175His	c.524G>A	900	219	24 %
CRC73	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	1926	572	30 %
	chr10:89711907	*PTEN*	NM_000314.4	Missense	6	p.Tyr177His	c.529T>C	1756	556	32 %
CRC75	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	3670	1.084	30 %
	chr17:577548	*TP53*	NM_000546.5	Missense	7	p.Gly245Ser	c.733G>A	3999	523	13 %
	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	2207	619	28 %
CRC76	chr17:7577580	*TP53*	NM_000546.5	Missense	7	p.Tyr234Cys	c.701A>G	3916	1.013	26 %
	chr17:7578388	*TP53*	NM_000546.5	Missense	5	p.Arg181Pro	c.542G>C	2798	493	18 %
CRC79	chr3:41266137	*CTNNB1*	NM_001904.3	Missense	3	p.Ser45Phe	c.134C>T	2738	1.684	62 %
	chr12:25399291	*KRAS*	NM_033360.3	Missense	2	p.Gly13Asp	c.38G>A	5040	1.716	34 %
CRC80	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361His	c.1082G>A	5362	1.763	33 %
	chr17:7577538	*TP53*	NM_000546.5	Missense	7	p.Arg248Gln	c.743G>A	10196	3.395	33 %
	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	3843	656	17 %
CRC81	chr18:48591901	*SMAD4*	NM_005359.5	Missense	9	p.Asp355Val	c.1064A>T	2709	266	10 %
	chr17:7578212	*TP53*	NM_000546.5	Nonsense	6	p.Arg213Ter	c.637C>T	2974	272	9 %
	chr7:140453132	*BRAF*	NM_004333.4	Missense	15	p.Lys601Asn	c.1803A>C	1289	161	12 %
CRC84	chr1:115256529	*NRAS*	NM_002524.4	Missense	3	p.Gln61Arg	c.182A>G	2955	327	11 %
	chr17:7577124	*TP53*	NM_000546.5	Missense	8	p.Val272Leu	c.814G>T	1156	147	13 %
CRC82	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	2514	1.051	42 %

CRC85	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	2078	291	14 %
	chr3:178936082	*PIK3CA*	NM_006218.2	Missense	9	p.Gly542Lys	c.1624 G>A	3964	849	21 %
CRC86	chr15:66727455	*MAP2K1*	NM_002755.3	Missense	2	p.Lys57Asn	c.171G>C	1408	169	12 %
	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361His	c.1082G>A	3722	599	16 %
CRC87	chr12:25399291	*KRAS*	NM_033360.3	Missense	2	p.Gly13Asp	c.38G>A	4564	500	11 %
	chr17:7577094	*TP53*	NM_000546.5	Missense	8	p.Arg282Trp	c.844C>T	876	134	15 %
CRC88	chr4:153258983	*FBXW7*	NM_033632.3	Nonsense	4	p.Arg278Ter	c.832C>T	5498	547	10 %
	chr12:25398285	*KRAS*	NM_033360.3	Missense	2	p.Gly12Ser	c.34G>A	6281	1.442	23 %
	chr17:7577563	*TP53*	NM_000546.5	Missense	7	p.Asn239Ile	c.716A>T	3477	557	16 %
	chr17:7577563	*TP53*	NM_000546.5	Missense	7	p.Ser240Gly	c.718A>G	3477	557	16 %
CRC89	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Ser	c.34G>A	8221	1.366	17 %
CRC90	chr17:7578272	*TP53*	NM_000546.5	Missense	6	p.His193Tyr	c.577C>T	1911	233	12 %
CRC91	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	3104	686	22 %
	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361Cys	c.1081C>T	2253	460	20 %
CRC92	chr7:140453136	*BRAF*	NM_004333.4	Missense	15	p.Val600Glu	c.1799T>A	2077	142	7 %
	chr17:7577120	*TP53*	NM_000546.5	Missense	8	p.Arg273His	c.818G>A	2077	142	7 %
CRC94	chr17:7578529	*TP53*	NM_000546.5	Missense	5	p.Phe134Cys	c.401T>G	3395	1.329	39 %
CRC95	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Asp	c.35G>A	1386	495	36 %
	chr17:7577094	*TP53*	NM_000546.5	Missense	8	p.Arg282Trp	c.844C>T	821	226	28 %
CRC97	chr9:139399362	*NOTCH*	NM_017617.3	Missense	26	p.Arg1594Gln	c.4781G>A	560	252	45 %
CRC98	chr17:7577140	*TP53*	NM_000546.5	Frameshift	8	p.Gly266fs	c.797delG	905	445	49 %
CRC99	chr12:25398284	*KRAS*	NM_033360.3	Missense	2	p.Gly12Val	c.35G>T	3984	1.423	36 %
	chr17:7577094	*TP53*	NM_000546.5	Missense	8	p.Arg282Trp	c.844C>T	1758	875	50 %
CRC100	chr18:48591918	*SMAD4*	NM_005359.5	Missense	9	p.Arg361His	c.1082G>A	2325	745	32 %
	chr17:7578266	*TP53*	NM_000546.5	Missense	6	p.Ile195Phe	c.583A>T	689	219	32 %

CDS- Coding sequence nomenclature; Total Coverage- Total reads in the genomic region; Variant Coverage- Total variant reads; VAF- Variant allele frequency (%).

diagnostics

MDPI

Case Report

Utility of Next-Generation Sequencing in the Reconstruction of Clonal Architecture in a Patient with an *EGFR* Mutated Advanced Non-Small Cell Lung Cancer: A Case Report

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Abstract: EGFR tyrosine kinase inhibitors (EGFR-TKIs) have revolutionized the treatment of non-small cell lung cancer (NSCLC) patients with activating *EGFR* mutations. However, targeted therapies impose a strong selective pressure against the coexisting tumor populations that lead to the emergence of resistant clones. Molecular characterization of the disease is essential for the clinical management of the patient, both at diagnosis and after progression. Next-generation sequencing (NGS) has been established as a technique capable of providing clinically useful molecular profiling of the disease in tissue samples and in non-invasive liquid biopsy samples (LB). Here, we describe a case report of a patient with metastatic NSCLC harboring *EGFR* mutation who developed two independent resistance mechanisms (*EGFR*-T790M and *TP53* + *RB1* mutations) to dacomitinib. Osimertinib given as a second-line treatment eliminated the *EGFR*-T790M population and simultaneously consolidated the proliferation of the *TP53* + *RB1* clone that eventually led to the histologic transformation to small-cell lung cancer (SCLC). Comprehensive NGS profiling revealed the presence of the *TP53* + *RB1* clone in the pretreatment biopsy, while *EGFR*-T790M was only detected after progression on dacomitinib. Implementation of NGS studies in routine molecular diagnosis of tissue and LB samples provides a more comprehensive view of the clonal architecture of the disease in order to guide therapeutic decision-making.

Keywords: non-small cell lung cancer; precision medicine; *EGFR* mutations; resistance mechanisms; molecular diagnostics; next-generation sequencing; liquid biopsy

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1. Introduction

The development of targeted drugs against specific molecular aberrations has prompted the expansion of precision medicine in oncology [1]. Non-small cell lung cancer (NSCLC) patients harboring activating epidermal growth factor receptor (*EGFR*) mutations benefit from tyrosine kinase inhibitors (TKIs) with remarkable responses [2]. Despite the initial high response rate, resistance mechanisms will emerge as a consequence of the selective pressure of EGFR-TKI therapy against the multiple coexisting tumor populations [3].

The p.(Thr790Met) mutation in exon 20 of the *EGFR* gene is the major resistance mechanism to first- and second-generation EGFR-TKIs, being detected in 50–60% of patients with progressive disease [4]. Its detection has become a major challenge for the molecular diagnostics laboratories since the approval of Osimertinib, a third-generation EGFR-TKI, which has overcome this resistance mechanism by being highly effective in patients harboring p.(Thr790Met) [5]. However, various *EGFR* point mutations, tyrosine kinase receptors amplification, cell-cycle gene alterations, and lineage plasticity have been associated with Osimertinib resistance [6–8].

As a consequence of several treatment lines, the proliferation of certain tumor clones with individual molecular characteristics promotes a clonal architecture of the disease with clinical implications in terms of the emergence of resistant clones [9]. Based on the multiple resistance mechanisms to targeted therapies, comprehensive and dynamic molecular characterization of the disease is essential to provide the most updated information for patients' clinical management [10].

In this sense, despite their limited sensitivity, liquid biopsies are a non-invasive approach to dealing with tumor heterogeneity and clonal architecture [11,12]. Circulating tumor nucleic acids released from both the primary tumor and metastatic sites to the systemic circulation represent the main cancer-derived material, and consequently, serially obtained samples may provide real-time information on the clonal composition of the disease [13,14].

To efficiently characterize tumors at the molecular level, single-gene approaches are being replaced by next-generation sequencing (NGS) because of its ability to simultaneously assess for diverse molecular alterations (point mutations, insertions, deletions, copy number variations and translocations) in a group of relevant genes [15,16]. This technique is able to board the tumor heterogeneity by quantifying tumor clones that harbor non-routinely assessed molecular alterations that could be responsible for future progression. Its application to both formalin-fixed paraffin-embedded (FFPE) samples and circulating tumor nucleic acids (ctNA) in liquid biopsies studies converts it into an essential tool for molecular diagnostics [17,18].

We herein report the utility of NGS studies in deciphering the clonal architecture of the disease in a patient with NSCLC exhibiting p.(Thr790Met) at progression to a second-generation EGFR-TKI, followed by a small-cell lung cancer (SCLC) transformation as a resistance mechanism to Osimertinib administered as a second-line treatment.

2. Case Report

A 68-year-old woman who quit smoking 15 years ago (30 packs/year) was diagnosed with stage-IV NSCLC adenocarcinoma (T3N3M1) in November 2014. Positron emission tomography–computed tomography (PET-TC) identified a 3 cm pulmonary mass in the upper left lobe. Adenopathies were detected in the aortopulmonary window and subcarinal and paratracheal lymph nodes. Bone metastases were identified in the left iliac crest, left femoral head and left scapula.

A biopsy specimen with 25% of tumor content was obtained through CT-guided bronchoscopy. A deletion in exon 19 of the *EGFR* gene was detected through Real Time PCR (Cobas® EGFR Mutation Test v2 CE-IVD. Roche Diagnostics, Basel, Switzerland). Sanger Sequencing of exon 19 was conducted to confirm and characterize the mutation (*EGFR*-LRG_304t1: c.2235_2249del; p.(Glu746_Ala750del)).

In December 2014, the patient started first-line dacomitinib treatment (45 mg/day), a second-generation irreversible epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), within a clinical trial (NCT01774721). CT evaluation scan in February 2015 indicated a partial response which was maintained as a stable disease until new pulmonary nodes were detected in a CT scan in October 2016 (Progression Free Survival (PFS): 22.2 months).

Due to disease progression, a liquid biopsy study was conducted through real-time PCR (Cobas® EGFR Mutation Test v2 CE-IVD. Roche Diagnostics, Basel, Switzerland). This molecular study identified a deletion in exon 19 in concomitancy with the

p.(Thr790Met) point mutation in exon 20, a well-known resistance mechanism to first and second-generation EGFR-TKI and a predictive biomarker of third-generation EGFR-TKI treatment benefit.

Consequently, Osimertinib (80 mg/day) was administered as second-line treatment, achieving a partial response maintained until the emergence of new bone and hepatic metastases in September 2017 (Osimertinib-PFS: 9.6 months). In order to characterize the resistance mechanism to second-line treatment and to explore new therapeutic approaches, a NGS study with Oncomine Lung cfDNA Assay (ThermoFisher Scientific, Waltham, MA, USA) was performed in liquid biopsy.

This study revealed the founder deletion in exon 19 of the *EGFR* gene found: c.2235_2249del; p.(Glu746_Ala750del) in a variant allele frequency (VAF) of 58%. Moreover, this mutation was found in concomitancy with a *TP53* deletion LRG_321t1: c.529_546del; p.(Pro177_Cys182del) in a VAF of 68%, while no evidence of p.(Thr790Met) was found in this sample.

The detection of a high VAF *TP53* mutation suggested a biallelic inactivation of this gene, which in concomitancy with the biallelic loss of *RB1* has been described as a virtually universal molecular event in SCLC [19]. In this sense, histologic transformation became a potential resistance mechanism to Osimertinib.

Since sequencing of the *RB1* gene is not included in Oncomine Lung cfDNA Assay, a Guardant360®CDx study was requested. This study added a truncating *RB1* mutation in a VAF of 68% [LRG_517t1: c.2047del; p.(Leu683Phefs*13)] to the known molecular profile. Taking together these results, liquid biopsy studies provided solid evidence of the resistance mechanism to second-line Osimertinib treatment in this patient.

Histologic characterization of a biopsy obtained from a novel hepatic metastasis confirmed the histologic transformation to SCLC. Subsequently patient received a third-line Cisplatin + Etoposide treatment with an initial partial response. Progressive disease with the emergence of a new adrenal gland and bone metastases was detected in the fourth month of treatment. The patient died in March 2018.

To further characterize diagnosis and SCLC-transformed biopsies, high throughput molecular techniques were conducted. Firstly, both samples were analyzed to search the *EGFR*-T790M clone with digital droplet PCR (ddPCR) and a PNA-Clamp TaqMan Assay with a limit of detection (LOD) of 0.1336% and 0.0996%, respectively. Neither of the assays could detect this variant in either of the samples.

Moreover, to provide a broader molecular profile, both pretreatment and SCLC-transformed biopsies were retrospectively analyzed with Ampliseq Comprehensive Cancer Panel, a 409 gene NGS panel (ThermoFisher Scientific, Waltham, MA, USA). At progression, among other variants with unknown significance (VUS), SCLC transformed metastasis harbored *EGFR* (73%), *RB1* (96%) and *TP53* variants (90%). Interestingly, this NGS study revealed the presence of the *RB1* and *TP53* variants in the NSCLC pretreatment sample at low VAFs (3% and 2.5%, respectively) in concomitancy with the *EGFR* variant (20%) (Figure 1, Table 1).

Table 1. Variant allele frequencies of the *EGFR*, *TP53* and *RB1* variants detected in NGS studies.

Moment	*EGFR* Exon 19	*EGFR* p.(Thr790)	*TP53* Exon 5	*RB1* p.(Leu683)
NSCLC FFPE sample	p.(Glu746_Ala750del) 20%	NMD	p.(Pro177_Cys182del) 2.5%	p.(Leu683Phefs*13) 3%
Disease progression on dacomitinib (LB)	p.(Glu746_Ala750del) 7.9%	p.(Thr790Met) 3%	p.(Pro177_Cys182del) 2.5%	NI
Disease progression on osimertinib (LB)	p.(Glu746_Ala750del) 58%	NMD	p.(Pro177_Cys182del) 68%	NI
SCLC FFPE sample	p.(Glu746_Ala750del) 73%	NMD	p.(Pro177_Cys182del) 90%	p.(Leu683Phefs*13) 96%

NSCLC: Non-small cell lung cancer; FFPE: formalin-fixed paraffin-embedded; SCLC: Small cell lung cancer; NMD: No Mutation Detected; LB: Liquid Biopsy; NI: Not Included.

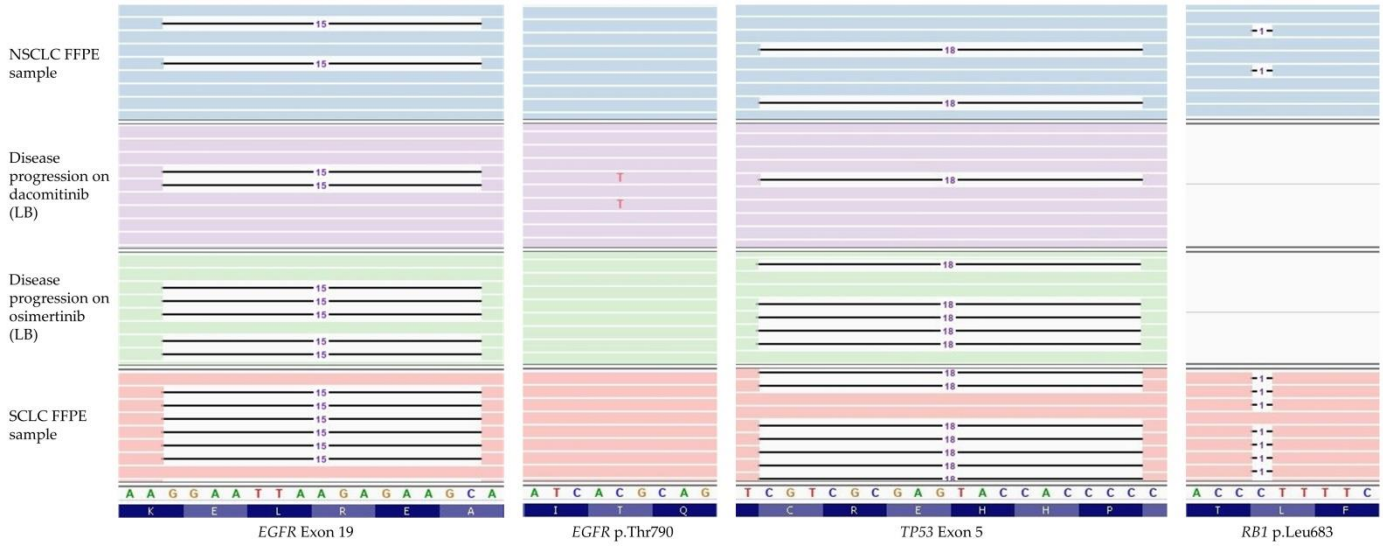

Figure 1. Integrative Genomics Viewer (IGV) browser visualization of the NGS results in the genomic positions, which became relevant for clinical management. For each sample and gene, colored bars represent the reads aligned along the reference genome (the *RB1* gene was only covered in the NGS studies of FFPE samples). Mismatched nucleotides are labeled. Black horizontal lines represent the nucleotides that have been deleted.

In order to recreate the clonal evolution of the disease, a liquid biopsy sample obtained after progression on dacomitinib was retrospectively analyzed with Oncomine Lung cfDNA Assay (ThermoFisher Scientific, Waltham, MA, USA). Apart from previously detected *EGFR* exon 19 deletion (VAF 7.9%) and *EGFR* p.(Thr790Met) mutations (VAF 3%), the *TP53* deletion was also detected in a VAF of 2.5% (Figure 2).

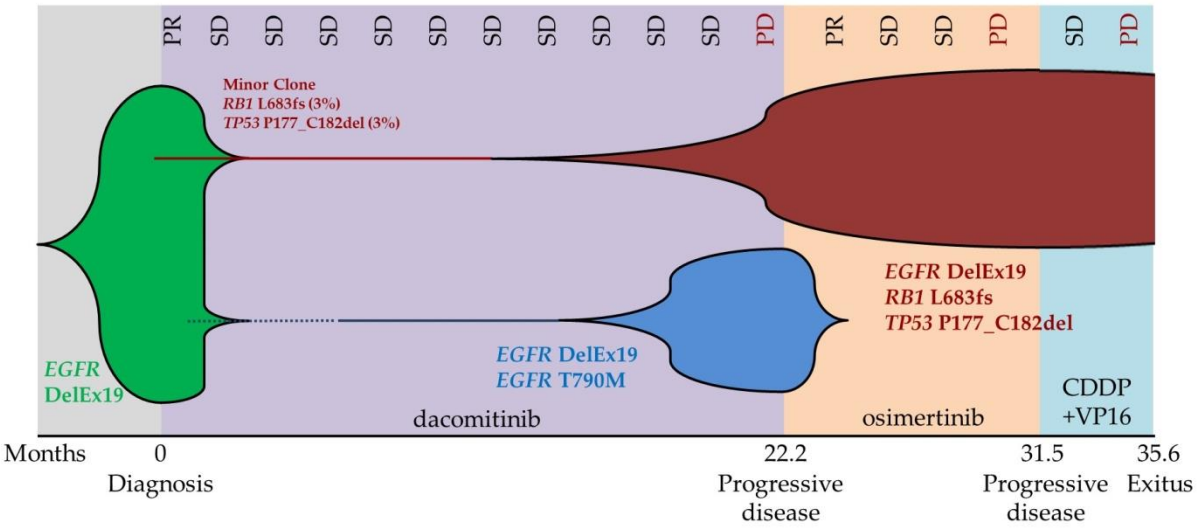

Figure 2. Clonal architecture of the disease was inferred through NGS analysis of FFPE and LB samples. The bottom horizontal axis represents the time from diagnosis and the subsequent treatment lines. The upper horizontal axis includes the results of the CT scans. PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; CDDP + VP16: Cisplatin + Etoposide.

3. Discussion

In the last decade, NSCLC has become a paradigm of precision medicine in advanced cancer [20]. The development of drugs targeting specific molecular alterations has demonstrated a huge impact on patient clinical management [21]. Especially in *EGFR* mutated

patients, tumor heterogeneity directly influences treatment response because of the evolution of resistance clones during tumor expansion [22].

Elucidating tumor heterogeneity may be crucial to infer clonal evolution to delay resistance or identify the best therapeutic approach after this event. For this purpose, molecular diagnostics laboratories must integrate cutting-edge approaches into their clinical routine.

In our patient, liquid biopsy samples provided clinically relevant information for treatment decisions when progressive disease to both EGFR-TKI treatment lines was revealed. The detection of *EGFR* p.(Thr790Met) after progression on dacomitinib provided a second line of treatment with a targeted drug. Subsequently, when the patient had disease progression on Osimertinib, a liquid biopsy study through NGS provided solid evidence of histological transformation as the resistance mechanism, which was subsequently confirmed in a new hepatic biopsy.

Moreover, in this case, retrospective NGS studies provided a more exhaustive and dynamic molecular profile, deciphering the tumor's clonal architecture. In the pretreatment FFPE sample, NGS comprehensive panel revealed the presence of concomitant low-VAF *RB1* c.2047del; p.(Leu683Phefs*13) and *TP53* c.529_546del; p.(Pro177_Cys182del) mutations together with the dominant *EGFR* c.2235_2249del; p.(Glu746_Ala750del) mutation. The concomitancy of *RB1* and *TP53* in *EGFR* mutated patients has been described as a molecular signature that confers a higher risk of lineage transformation to SCLC [23,24]. This resistance mechanism has been described in approximately 10% of patients with disease progression after first- or second-generation EGFR-TKIs treatment, while its frequency as a resistance mechanism to Osimertinib as second-line treatment has been reported in up to 4–15% of patients.

Interestingly, the retrospective NGS study after progression on dacomitinib detected the *EGFR* p.(Thr790Met) and the *TP53* p.(Pro177_Cys182del) at similar VAFs. We hypothesized that the strong selective pressure enhanced the proliferation of two resistant tumor clones, which led to treatment failure; the *TP53* + *RB1* mutated clone, which was already present in the diagnostic FFPE sample, and the *EGFR* p.(Thr790Met) clone, which was not detected in the pretreatment FFPE sample by either NGS or ultrasensitive techniques (ddPCR and PNA Clamp TaqMan Assay).

The emergence of the *EGFR* p.(Thr790Met) clone could be explained by the acquisition model, which sustains that this variant is acquired as a response to the strong selective pressure imposed by first- and second-generation EGFR-TKI. However, we should not discard the selection model being this clone in an extremely low abundance or even being absent in tumor region which was biopsied [25].

After progression on Osimertinib, liquid biopsy studies detected the deletion in exon 19 of *EGFR* together with the *TP53* and *RB1* mutations. This molecular profile was also detected in the SCLC-transformed biopsy, which confirmed the biallelic loss of *RB1* (VAF: 96%) and *TP53* (VAF: 94%) and the permanence of the *EGFR* variant (VAF: 73%). Osimertinib should have eradicated the tumor clone harboring *EGFR* p.(Thr790Met) and, on the other hand, prompted the development of the clone with biallelic loss of *TP53* and *RB1,* which led to the histologic transformation to SCLC.

This clinical case reflects the remarkable heterogeneity of *EGFR* mutation-positive tumors, which may include minor clones harboring potential resistance mechanisms. Targeted treatment with EGFR-TKIs imposes a strong selective pressure on tumor cells prompting the clonal evolution of tumor populations. In this subgroup of high-risk patients, further research is needed to enhance the application of precision medicine. Although no targeted therapies have been approved for *TP53* and *RB1* mutations, combination treatment strategies at treatment onset have been reported in small cohorts or subgroup analyses of clinical trials. Combination therapies of EGFR-TKIs and chemotherapy, antiangiogenic agents or immunotherapy may be valuable options in the coming years in certain *EGFR* mutated patients [26–28].

High throughput molecular approaches in both FFPE and liquid biopsy samples allow a comprehensive, quantitative and dynamic understanding of genomic heterogeneity.

Implementation of these techniques in the clinical routine may reflect the multiple adaptive changes in response to treatment and lead to a personalized molecular-guided treatment decision to delay or react against the radiological progression.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Human Rights, the Conference of Helsinki, and approved by the Hospital Ethics Committee (2015/0713; 16 February 2016 and 2017/0070; 29 March 2017).

Informed Consent Statement: The patient has shown their agreement to participate in molecular studies by signing the informed consent elaborated in accordance with the recommendations of the Declaration of Human Rights and the Conference of Helsinki. Informed written consent for publication was not requested since no data from the manuscript can potentially and clearly identify the patient.

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Technical Validation and Clinical Implications of Ultrasensitive PCR Approaches for *EGFR*-Thr790Met Mutation Detection in Pretreatment FFPE Samples and in Liquid Biopsies from Non-Small Cell Lung Cancer Patients

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Abstract: In pretreatment tumor samples of *EGFR*-mutated non-small cell lung cancer (NSCLC) patients, *EGFR*-Thr790Met mutation has been detected in a variable prevalence by different ultrasensitive assays with controversial prognostic value. Furthermore, its detection in liquid biopsy (LB) samples remains challenging, being hampered by the shortage of circulating tumor DNA (ctDNA). Here, we describe the technical validation and clinical implications of a real-time PCR with peptide nucleic acid (PNA-Clamp) and digital droplet PCR (ddPCR) for *EGFR*-Thr790Met detection in diagnosis FFPE samples and in LB. Limit of blank (LOB) and limit of detection (LOD) were established by analyzing negative and low variant allele frequency (VAF) FFPE and LB specimens. In a cohort of 78 FFPE samples, both techniques showed an overall agreement (OA) of 94.20%. *EGFR*-Thr790Met was detected in 26.47% of cases and was associated with better progression-free survival (PFS) (16.83 ± 7.76 vs. 11.47 ± 1.83 months; $p = 0.047$). In LB, ddPCR was implemented in routine diagnostics under UNE-EN ISO 15189:2013 accreditation, increasing the detection rate of 32.43% by conventional methods up to 45.95%. During follow-up, ddPCR detected *EGFR*-Thr790Met up to 7 months before radiological progression. Extensively validated ultrasensitive assays might decipher the utility of pretreatment *EGFR*-Thr790Met and improve its detection rate in LB studies, even anticipating radiological progression.

Keywords: non-small cell lung cancer; molecular biology; *EGFR* p.(Thr790Met) mutation; ultrasensitive assays; prognostic and predictive biomarkers

1. Introduction

EGFR activating mutations define a specific molecular subset of non-small cell lung cancer (NSCLC) patients. In-frame deletions in exon 19 and the point mutation p.(Leu858Arg) in exon 21 constitute approximately 90% of all *EGFR* activating mutations

found in NSCLC [1]. These mutations confer sensitivity to treatment with tyrosine kinase inhibitors (EGFR-TKIs) [2]. Despite the high response rate, the vast majority of patients will develop resistance to EGFR-TKI treatment and experience disease progression. In patients treated with first- or second-generation EGFR-TKIs, the most common resistance mechanism is the *EGFR* point mutation c.2369C>T; p.(Thr790Met) in exon 20, which is detected in 50–60% of patients with progressive disease [3].

The mechanism by which this mutation emerges remains unclear, although two hypotheses have been proposed [4]. The acquisition model suggests that *EGFR*-Thr790Met mutation emerges during EGFR-TKI treatment [5]. However, *EGFR*-Thr790Met mutation is detected by conventional methods in 1–8% of EGFR-TKI-naïve patients which suggests a selection model of a minor clone harboring this mutation [6,7].

Tumor heterogeneity is a well-known event in cancer with critical impact in diagnosis and cancer treatment [8,9]. The pretreatment presence of tumor cells clones harboring *EGFR*-Thr790Met mutation may have been underestimated because of the limit of detection (LOD) of the conventional *EGFR* testing methods. These methods, currently based on real-time PCR, such as Taqman PCR or Scorpion Amplification Refractory Mutation System (SARMS), reach a LOD of 1–5% mutant alleles [10]. New ultrasensitive approaches are able to identify specific mutations with a LOD even down to 0.01%, but also require more technical training, experience and validation efforts to obtain reproducible and transferable results. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), mutant allele enrichment PCR techniques (PNA-LNA, PCR Clamp, etc.), COLD-PCR methods or digital PCR approaches have been developed for ultrasensitive mutation detection in FFPE samples [11–14]. However, using these techniques, a detection rate of *EGFR*-Thr790Met ranging from 17% to 80% has been reported and, as a consequence, the emergence model and its clinical impact remains unclear [15–22].

Nevertheless, in patients with progressive disease with first- or second-generation EGFR-TKIs, *EGFR*-Thr790Met is a well-established predictive biomarker of osimertinib treatment benefit [23]. Since obtaining a second tumor biopsy for molecular testing is not feasible in the majority of patients, liquid biopsy (LB) constitutes a non-invasive approach to analyze circulating tumor DNA (ctDNA) in blood and other body fluids. However, due to the low amount of ctDNA in peripheral blood, current evidence shows that LB studies show suboptimal sensitivity, with tumor-detected and ctDNA-undetected cases being relatively common [24].

Ultrasensitive approaches such as digital droplet PCR (ddPCR) have shown promising results as a new diagnostic strategy able to increase the detection rate of *EGFR*-Thr790Met in LB studies [25]. However, due to the relevance of its detection, validation studies are needed before implementing this technique to routine molecular diagnosis. Moreover, monitoring tumor mutations through serially obtained liquid biopsies is a highly promising approach for patients' clinical management. These studies have demonstrated the ability to detect *EGFR*-Thr790Met before radiological progression [26,27] and its use as a new standard of tumor progression is currently being researched [28].

In this work, we report the in-house validation of two ultrasensitive PCR methods for pretreatment *EGFR*-Thr790Met detection in order to establish the prevalence of this mutation and to assess its prognostic value. Moreover, we also report the diagnostic performance of ddPCR for *EGFR*-Thr790Met detection in LB at treatment failure and during EGFR-TKI treatment follow-up.

2. Results

2.1. Dilution Bank Preparation

DNA from the H1975 cell line and DNA from healthy donors were processed by ddPCR in three independent experiments to detect the *EGFR*-Thr790Met mutation before preparing the dilution bank. H1975 was found to harbor this mutation at a VAF of 80.50

± 0.63% while DNA from healthy donors was confirmed as negative. A dilution bank with an increasing quantity of wild type DNA was prepared as described in the Materials and Methods section. These aliquots were tested in two independent experiments to ensure the VAF. The observed VAF showed a high and significant correlation with the theoretical VAFs ($R^2 = 0.998$, Pearson correlation test; r = 0.999 and *p*-value < 0.01) Figure 1.

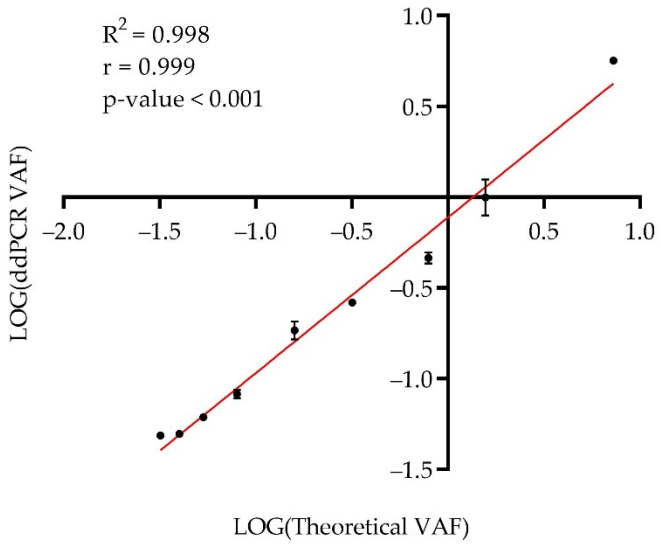

Figure 1. Correlation between theoretical VAF (%) and observed VAF (%) by ddPCR. Observed values are the mean of two independent experiments with error bars representing the standard deviation. Logarithmic transformation of both variables was applied to enhance visualization.

2.2. Limit of Blank (LOB) and Limit of Detection (LOD) Establishment

The study of wild type FFPE samples established the LOB of the PNA Clamp TaqMan assay as 0.0364% while the LOB of the ddPCR assay was set to 0.0957%. Low-positivity samples were included in the validation procedure. We established a LOD of 0.0996% for PNA Clamp TaqMan assay and 0.1336% for ddPCR assay. For both assays, between LOB and LOD values, we established an "uncertainty zone" in which we cannot truly assess *EGFR*-Thr790Met presence. Regarding ddPCR validation in LB samples, LOB was established in 1.6 mutant copies/mL of plasma and LOD was set at 3.0 copies/mL of plasma.

2.3. PNA Clamp TaqMan Assay and ddPCR Results:Comparison

A total of 78 FFPE diagnostic samples were studied by ddPCR and PNA Clamp assays. In PNA Clamp TaqMan assays, seven samples showed a VAF between LOB and LOD (uncertain result) (7/78; 8.97%) while in ddPCR assays, two different samples gave an uncertain result (2/78; 2.56%). Consequently, excluding these samples, a total of 69 samples gave a reliable result by both methodologies (69/78; 88.46%). In both approaches, 18 samples were positive while 47 were classified as not detected. Only four samples were positive in the ddPCR assay and not detectable with PNA Clamp TaqMan assay (overall agreement, OA: 94.20% (Figure 2). These data suggest that both methods are useful for the detection of *EGFR*-Thr790Met mutation in FFPE samples.

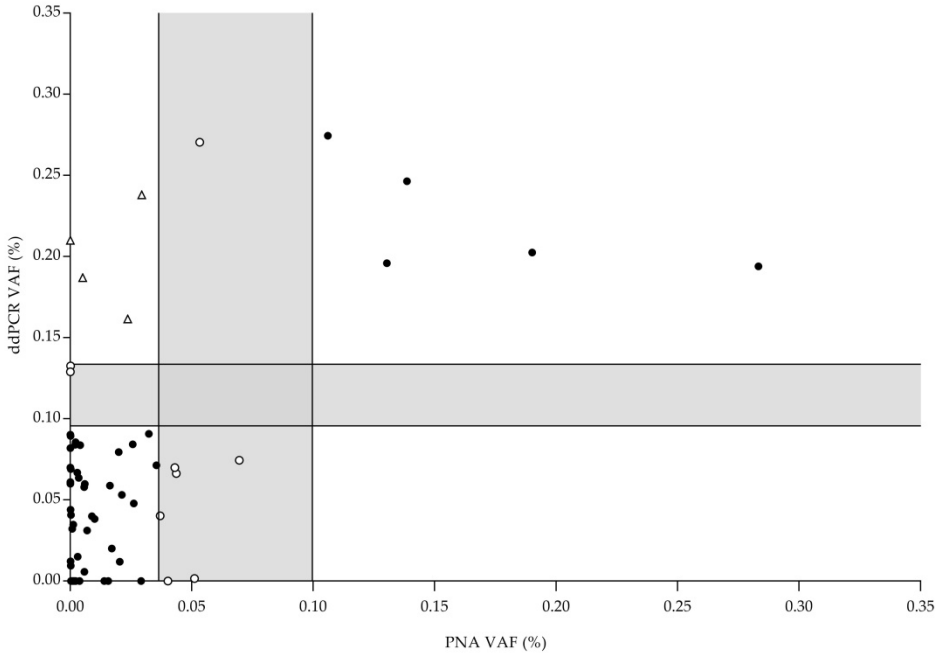

Figure 2. ddPCR and PNA Clamp TaqMan assays results for 78 FFPE specimens. Each symbol represents one sample whose position is determined by PNA Clamp TaqMan assay result (*X*-axis) and ddPCR assay result (*Y*-axis). Gray bars represent uncertainty zone of each methodology which is limited by LOB and LOD values. (●) Samples with concordant results, (○) samples in uncertainty area and (Δ) samples with discordant results. Positive samples with VAF higher than 0.3% are not depicted.

2.4. EGFR-Thr790Met Prevalence in Pretreated Stage IV NSCLC Association with Patients' Characteristics

Thirty-four stage IV NSCLC patients with *EGFR*-Thr790Met genotype determined by ultrasensitive methods were selected to assess the relationship between this mutation and patients' clinical–pathological characteristics. Nine patients (9/34; 26.47%) showed *EGFR*-Thr790Met pretreatment while the other 25 did not show this mutation (25/34; 73.53%). No statistically significant associations were found between pretreatment *EGFR*-Thr790Met mutation detection and clinical–pathological characteristics of patients. However, *EGFR*-Thr790Met was more frequent in male patients (66.7% vs. 33.3%), in patients without bone metastasis (88.9% vs. 11.1%) and in older patients (77.8% vs. 22.2%). Clinicopathological characteristics of patients according to *EGFR*-Thr790Met genotype are shown in Table 1.

Table 1. Patient's characteristics according to the presence of *EGFR*-Thr790Met.

Characteristics	T790M Negative (*n* = 25) *n* (%)	T790M Positive (*n* = 9) *n* (%)	*p*-Value
Sex			0.139
Male	9 (36.0%)	6 (66.7%)	
Female	16 (64.0%)	3 (33.3%)	
Age (years)			0.240
<65	13 (52.0%)	2 (22.2%)	
≥65	12 (48.0%)	7 (77.8%)	
Smoking status			0.254
Never smoker	17 (68.0%)	4 (44.4%)	
(Former) smoker	8 (32.0%)	5 (55.6%)	

CNS metastasis			1.000
No	18 (72.0%)	7 (77.8%)	
Yes	7 (28.0%)	2 (22.2%)	
Bone metastasis			0.214
No	15 (60.0%)	8 (88.9%)	
Yes	10 (40.0%)	1 (11.1%)	
Type of *EGFR* mutation *			1.000
Deletion exon 19	15 (62.5%)	5 (55.6%)	
Leu858Arg	9 (37.5%)	4 (44.4%)	
EGFR-TKI Treatment			0.216
Erlotinib	15 (60.0%)	3 (33.3%)	
Gefitinib	4 (16.0%)	4 (44.4%)	
Afatinib	3 (12.0%)	0 (0.0%)	
Dacomitinib	1 (4.0%)	0 (0.0%)	
Erlotinib–Gefitinib	1 (4.0%)	1 (11.1%)	
Erlotinib + Ramucirumab	1 (4.0%)	0 (0.0%)	
Erlotinib + Bevacizumab	0 (0.0%)	1 (11.1%)	

CNS: Central nervous system. *EGFR*: Epidermal growth factor receptor. * Patient with p.(Leu858Arg) and concomitant p.(Ser768Ile) was excluded from the analysis.

2.5. EGFR-Thr790Met Pretreatment Status and Progression-Free Survival (PFS)

At the time of the analysis, all patients progressed on first-line treatment with EGFR-TKIs (one patient censored). Median PFS was significantly higher in the *EGFR*-Thr790Met-positive patients (16.83 ± 7.76 months) than in negative patients (11.47 ± 1.83 months) (p= 0.047; HR 2.45, 95% CI: 0.99–6.08) (Figure 3).

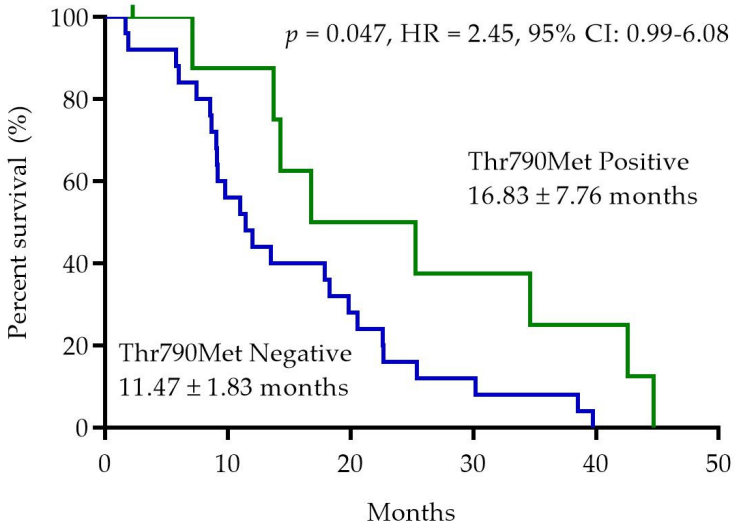

Figure 3. Duration of progression-free survival according to consensus *EGFR*-Thr790Met pretreatment status determined by PNA Clamp TaqMan Assay and ddPCR. HR: Hazard ratio, CI: Confidence interval.

2.6. EGFR-Thr790Met Detection in LB at the Time of Disease Progression

Among the patients recruited in the LB cohort, we detected *EGFR*-Thr790Met in 12 samples (12/37; 32.43%) using Cobas® EGFR Mutation Test v2 (CE-IVD). However, using ddPCR we were able to detect this mutation in five additional samples (17/37; 45.95%). ddPCR showed a sensitivity of 100%, a specificity of 80% and an OA of 86.49%. The five

discordant samples showed an average of 27.40 (range: 5.02–86.11) mutant copies/mL of plasma. Mutant alleles were below the LOD of the Cobas® assay (100 mutant copies/mL of plasma) in all discordant samples.

In two of the 20 *EGFR*-Thr790Met-negative patients, LB studies were expanded to other body fluids and ddPCR assay was able to detect *EGFR*-Thr790Met in pleural and cerebrospinal fluid, respectively.

Among the remaining 18 patients, nine underwent bronchoscopy to obtain a second tumor biopsy. Molecular studies were performed in seven patients (no malignant cells were detected in two biopsies), with *EGFR*-Thr790Met detected in three cases by conventional methods. Taken together, these results show that the global *EGFR*-Thr790Met resistance mechanism's prevalence in our cohort was 22/37 (59.46%) (Figure 4).

Regarding clinical implications of *EGFR*-Thr790Met, all 22 patients showing *EGFR*-Thr790Met started osimertinib as a second-line treatment with a response rate of 18/20 (90.00%; two patients with incomplete clinical data). Interestingly, in the two patients with progressive disease with osimertinib, *EGFR*-Thr790Met was detected by both the Cobas® and ddPCR method in plasma obtained from peripheral blood.

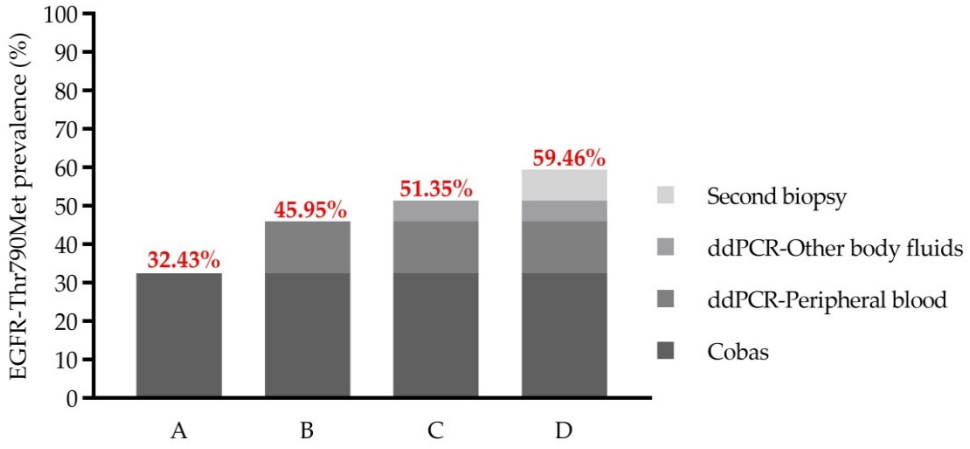

Figure 4. Increase in EGFR-Thr790Met prevalence at the time of disease progression according to different testing strategies. A: Cobas®, B: ddPCR—peripheral blood, C: ddPCR—peripheral blood + ddPCR—other body fluids and D: Strategy C + second tissue biopsies. Prevalence obtained with each strategy is depicted.

2.7. Early Detection of EGFR-Thr790Met in LB by ddPCR during Treatment Follow-Up

Serially obtained liquid biopsies during EGFR-TKI treatment of two patients with *EGFR*-Thr790Met detected in progressive disease were prospectively analyzed by ddPCR. In both cases, ddPCR was able to anticipate radiological progression by detecting 20.0 and 37.5 mutant copies/mL of plasma 7.6 and 6.6 months in advance, respectively. An increase in plasma *EGFR*-Thr790Met abundance was observed three months later, that reached its maximum at radiological progression with 343.0 and 256.0 mutant copies/mL of plasma for patients A and B, respectively (Figure 5).

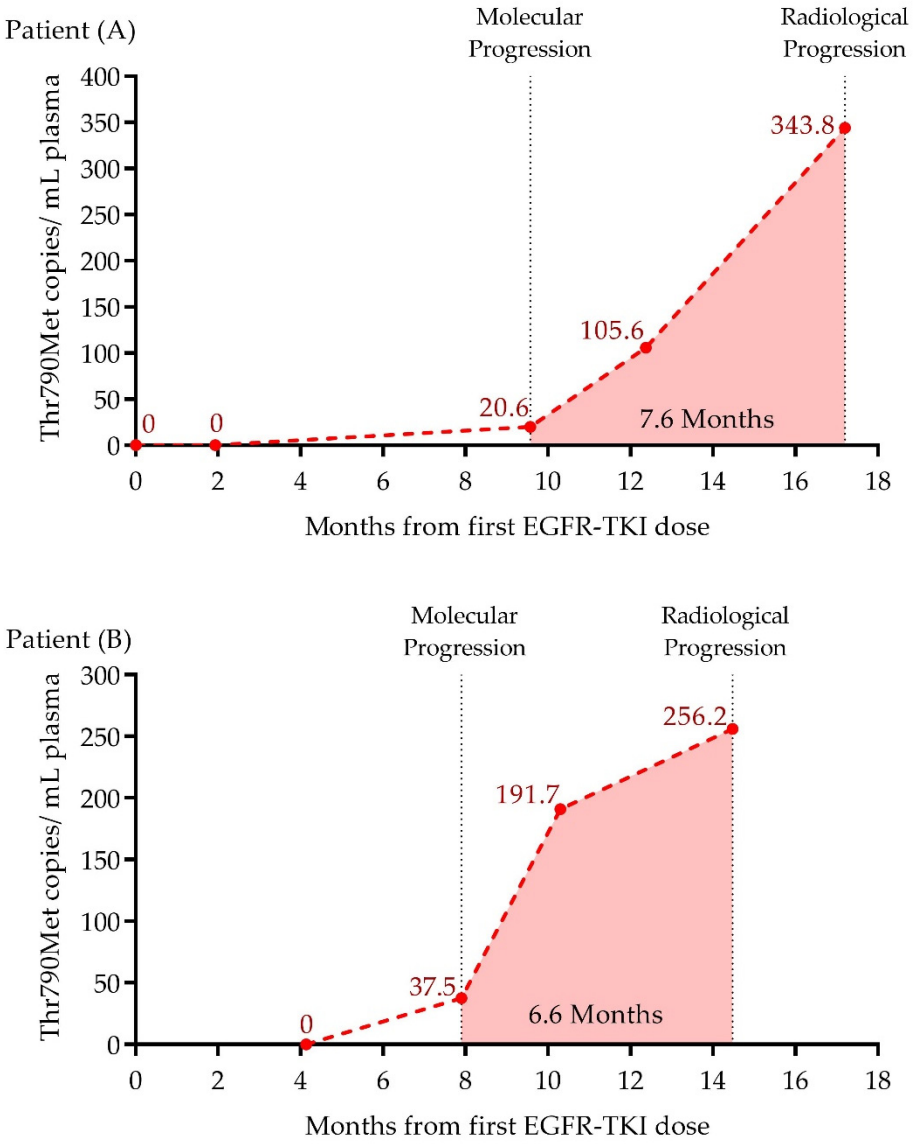

Figure 5. Dynamics of *EGFR*-Thr790Met mutation detected by ddPCR during follow-up in NSCLC patients treated with EGFR-TKI. Patients A and B (**A**,**B**). Red dots represent the *EGFR*-Thr790Met copies/mL of plasma detected in each sample. Molecular progression and radiological progression are depicted. Light red filled area comprises the months between these moments.

3. Discussion

EGFR-mutated NSCLC patients benefit from targeted therapy with EGFR-TKI. Acquisition and selection models have been proposed to explain the emergence of *EGFR*-Thr790Met resistance mutation, however, no consensus has been reached due to methodological challenges and, as a consequence, its pretreatment clinical utility remains unknown. In contrast, this mutation plays a crucial role in progression, since positive patients are eligible for a second-line targeted treatment with osimertinib. In this scenario, LB is positioned as a non-invasive alternative to second tissue biopsy studies for molecular characterization of progressive disease. Implementation of ultrasensitive assays in clinical routine LB studies could identify patients with circulating *EGFR*-Thr790Met alleles in low abundance, minimizing second biopsies and even anticipating radiological progression.

Using ddPCR and a PNA Clamp TaqMan assay, we have detected the presence of pretreatment *EGFR*-Thr790Met mutation in 26.5% of stage IV NSCLC patients. In the literature, several groups have reported variable detection rates due to the series of patients included, the diversity of ultrasensitive assays and the different approaches developed for their technical validation.

Regarding ddPCR, Watanabe et al. and Vendrell et al. detected EGFR-Thr790Met mutation in 79.9% and 66.0% of pretreatment FFPE cases, respectively [16,17]. In contrast, Beau-Faller et al. and Matsumoto et al. reported a lower detection rate using ddPCR (8% and 42.4%, respectively) [29,30]. Interestingly, Tatematsu et al. reported a detection rate of 40% in a group of 20 frozen tumor samples, and although there was a limited number of patients, they suggested that the study of non-FFPE samples may have reduced its false positive rate [22]. In this sense, Lettig et al. established a detection rate of 17% in a cohort of 114 patients using a ddPCR assay, detecting an increase in false positive droplets when processing DNA from FFPE samples [15]. Moreover, Rosell et al. and Costa et al. reported a detection rate of 35.0% and 65.3%, respectively, using a PNA Clamp assay previously validated with DNA from cell lines [20,21].

The variability in the reported frequency of pretreatment *EGFR*-Thr790Met suggests that its detection may be compromised by the quality of FFPE DNA. Fixation and paraffin-embedding processes produce a highly fragmented and chemically modified DNA that can lead to artificial mutation calls in ultrasensitive assays because of the low LOD of these techniques [31,32].

For this reason, in this work we focused on the implementation and validation of two ultrasensitive assays for *EGFR*-Thr790Met testing in FFPE samples. First of all, we decided to use DNA obtained from FFPE samples and commercial FFPE-like samples in order to establish the LOB and LOD. In these samples, DNA quantity and quality were as compromised as in clinical samples, making the established LOB and LOD more precise and transferable. Moreover, the requirement of agreement between ddPCR and PNA Clamp TaqMan assay constitutes a stricter genotyping strategy than in most of the published studies. Consequently, the prevalence of *EGFR*-Thr790Met mutation established in our work is lower than in most of the previously reported studies using ultrasensitive methods [16,17,21,30,33].

The clinical significance of the pretreatment low frequency of *EGFR*-Thr790Met mutation has not been determined. Our findings are consistent with Fujita et al. who reported a longer PFS in *EGFR*-Thr790Met-positive patients (10 vs. 8 months, $p = 0.44$) [33] and also with Vendrell et al. and Lettig et al. who have described a better prognosis in pretreatment *EGFR*-Thr790Met-positive patients (29.2 vs. 11 months, $p = 0.009$ and HR = 0.40, $p = 0.04$, respectively) [15,16]. However, several studies reported a significantly shorter PFS in pretreatment positive patients (Su et al., 6.7 vs. 10.2 months $p < 0.05$; Lee et al., 6.3 vs. 11.5 months $p < 0.001$; Rosell et al., 12 vs. 18 months $p = 0.05$; Costa et al., 9.8 vs. 15.8 months $p = 0.0185$; Maheswaran et al., 7.7 vs. 11.5 months $p < 0.001$ and Matsumoto et al., 6.9 vs. 13.8 months $p < 0.001$) [18–21,30,34]. This observation is also supported by Ma et al. and Ding et al. who concluded in two meta-analyses that *EGFR*-Thr790Met confers a worse prognosis in EGFR-TKI-naïve patients (HR = 2.21 and HR = 1.95, respectively) [35,36]. Moreover Beau-Faller et al. recently reported a significantly shorter PFS only in patients harboring this mutation in a VAF > 1%, suggesting that abundance of this resistant clone could also influence its prognostic value [29].

Interestingly, Chmielecki et al. reported in a preclinical study a slower growth of cultured tumor cells harboring *EGFR*-Thr790Met mutation [37]. This observation is reflected in clinical studies which describe an indolent progression and better outcome in patients who develop *EGFR*-Thr790Met mutation at disease progression [38,39], possibly because other resistance mechanisms involve more complex genetic changes [40]. However, heterogeneity of *EGFR* mutant tumors may include minor clones harboring diverse resistance mechanisms, leading to a complex evolutionary model of TKI-resistant clones [41].

The heterogeneity of the samples analyzed and the different methods employed to detect this mutation in previously reported studies prevent us drawing solid conclusions.

Extensively validated and standardized ultrasensitive studies are needed to establish a consensus about pretreatment *EGFR*-Thr790Met prevalence and its clinical impact. Moreover, since the approval of osimertinib as a first-line treatment in *EGFR*-mutated NSCLC patients, pretreatment *EGFR*-Thr790Met arouses great interest. Its detection could lead to the application of a more personalized medicine in this subgroup of patients [42].

Regarding LB studies, ctDNA quantity varies between patients and over time, being influenced by tumor location, treatment and cell proliferation rates [43]. For this reason, ultrasensitive approaches such as ddPCR are extremely promising. Cobas® EGFR Mutation Test v2 is a CE-IVD marked method for detecting EGFR mutations in NSCLC patients with a LOD of 100 mutant copies/mL of plasma for *EGFR*-Thr790Met mutation. However, in our work, the ddPCR assay with a LOD of 3 mutant copies/mL of plasma led to the detection of *EGFR*-Thr790Met in 13.51% of positive patients apart from those detected with Cobas. The technical validation of ddPCR for *EGFR*-Thr790Met detection in LB samples reported in this work led to its implementation in routine diagnostics under UNE-EN ISO 15189:2013 accreditation.

Moreover, ddPCR studies have allowed the detection of *EGFR*-Thr790Met prior to radiological progression. Although longitudinal monitoring in LB is not included in clinical guidelines, the clinical utility of early *EGFR*-Thr790Met detection (molecular progression) arouses great interest as an early radiological progression marker [28].

In conclusion, the determination of *EGFR*-Thr790Met by ultrasensitive assays in pretreatment FFPE biopsies is feasible only with extensive validation studies that ensure the correct genotyping of NSCLC patients. Our work reveals the presence of pretreatment *EGFR*-Thr790Met in 26.5% of stage IV NSCLC patients. However, the clinical significance of pretreatment *EGFR*-Thr790Met mutation remains unresolved and needs to be assessed in a larger cohort. Moreover, we report that implementation and technical validation of ddPCR studies for *EGFR*-Thr790Met detection in LB are able to identify a higher number of positive patients and even anticipate radiological progression.

4. Materials and Methods

4.1. Patients

One thousand one hundred and six patients were recruited via the Medical Oncology Department at the Hospital Universitario y Politécnico La Fe in Valencia (Spain) from January 2010 to September 2019. One hundred and forty-seven patients had NSCLC positive for activating *EGFR* mutations (13.3%). Only 78 of 147 patients had sufficient formalin fixed paraffin embedded tissue (FFPE) for the ultrasensitive *EGFR*-Thr790Met mutation testing. Of these, 34 met the eligibility criteria for inclusion in the pretreatment cohort: (1) diagnosed with advanced NSCLC and (2) treated in first line with first- or second-generation EGFR-TKI. A flow chart of patient enrollment is shown in Figure 6. The main characteristics of these patients are shown in Table 2.

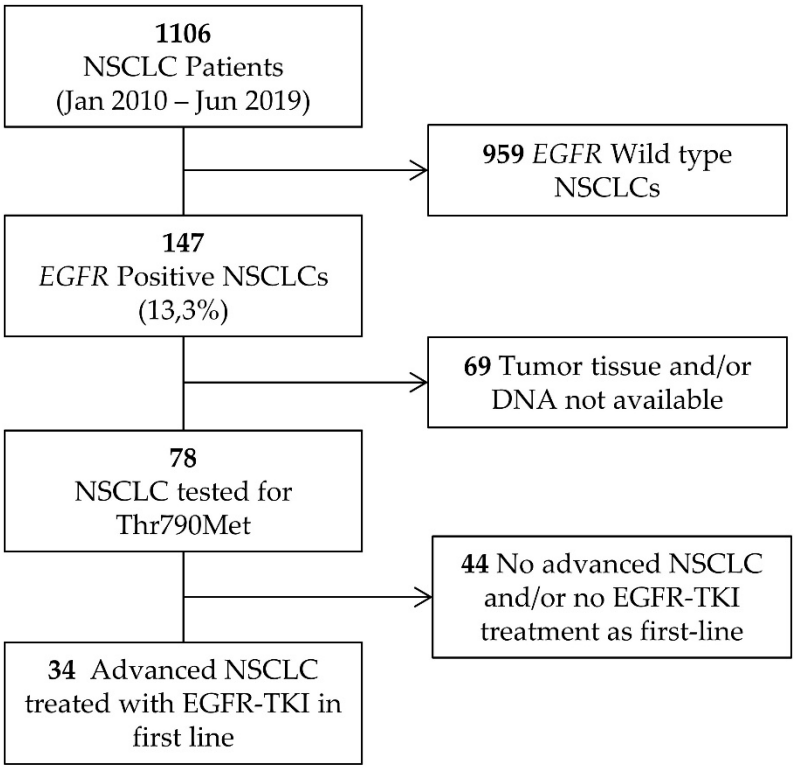

Figure 6. Flow chart showing the patients included in the pretreatment cohort. NSCLC: Non-small cell lung cancer. EGFR-TKI: Epidermal growth factor receptor tyrosine kinase inhibitor.

Table 2. Epidemiological and clinical–pathological characteristics of the patients included in the pretreatment cohort.

Characteristics	*n*	%
Sex		
Male	15	44.12%
Female	19	55.88%
Age (years)		
Mean	65.47	
Range	32–85	
Smoking status		
Never smoker	21	61.76%
Former smoker	6	17.65%
Current smoker	7	20.59%
Histologic type		
Adenocarcinoma	33	97.06%
Squamous	1	2.94%
CNS metastasis		
Yes	9	26.47%
No	25	73.53%
Bone metastasis		
Yes	11	32.35%
No	23	67.65%
Type of *EGFR* mutation		
Deletion 19	20	58.82%
Leu858Arg	13	38.24%
Leu858Arg/Ser768Ile	1	2.94%

EGFR-TKI Treatment		
Erlotinib	18	52.94%
Gefitinib	8	23.53%
Afatinib	3	8.82%
Dacomitinib	1	2.94%
Erlotinib–Gefitinib	2	5.88%
Erlotinib + Ramucirumab	1	2.94%
Erlotinib + Bevacizumab	1	2.94%

EGFR-TKI: Epidermal growth factor receptor tyrosine kinase inhibitor.

In the liquid biopsy cohort, 37 *EGFR*-positive NSCLC patients were recruited from April 2016 to December 2019. Peripheral blood samples were obtained at radiological progression with first- or second-generation EGFR-TKI. Tissue samples were obtained for nine patients without EGFR-Thr790Met detectable in LB studies. Additionally, in two patients, six liquid biopsy samples obtained during first-line EGFR-TKI treatment were retrospectively analyzed for *EGFR*-Thr790Met detection.

All patients showed their agreement by signing the informed consent by the Health Department in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki and institutional regulations, and approved by the Hospital Ethics Committee.

4.2. Reference Materials

Wild type genomic DNA obtained from peripheral blood of healthy donors and genomic DNA from the H1975 cell line (*EGFR*-Thr790Met positive) were used for the standard curve preparation of the PNA Clamp TaqMan assay. For the validation procedure of both ultrasensitive assays in FFPE samples, we used wild type DNA from FFPE reference material obtained from Horizon Discovery (Waterbeach, UK) and FFPE samples previously characterized in-house. Multiplex I cfDNA Reference Standard Set (Horizon Discovery) containing wild type cfDNA and two aliquots of cfDNA harboring the *EGFR*-Thr790Met variant (0.1%, and 1%) were used in the validation procedure of ddPCR for LB assays.

4.3. DNA Extraction Plasma Isolation

All molecular analyses were carried out at the Molecular Biology Unit (UBM) of the Clinical Analysis Department, an ISO 15189-certified laboratory (*Entidad Nacional de Acreditación*, ENAC, Nº1302/LE2445). FFPE sections were macrodissected by a pathologist to select regions containing the highest proportion of tumor cells (≥30%). Genomic DNA (gDNA) was isolated from five 5 μm thick FFPE sections using Deparaffinization Solution and the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Peripheral blood was collected into two 8.5 mL Vacutainer PPT EDTA-K2 Gel separator tubes (BD Biosciences, Franklin Lakes, NJ, USA). Samples were centrifuged at 4 °C (1800 g) for 10 min. Supernatant was subsequently centrifuged at 4 °C (16,000 g) for 10 min to remove cell debris. cfDNA was isolated from 4 mL of cell-free plasma using a MagMAX Cell-Free DNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). gDNA from peripheral blood of healthy donors and from cell lines was isolated using an UltraClean™ Blood DNA Isolation Kit (MO-BIO, Carlsbad, CA, USA). In all samples, DNA concentration was assessed using a Qubit 3.0 fluorometer with the DNA HS (High Sensitivity) Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

4.4. EGFR Mutation Screening

EGFR mutation screening was routinely performed in FFPE and LB samples using Cobas® EGFR Mutation Test v2 (CE-IVD) (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions.

4.5. PNA Clamp TaqMan Assay

The peptide nucleic acid (PNA) Clamp TaqMan assay was implemented as described by Costa et al. [21]. PCR reactions, using previously described primers and probes, were carried out in a 7500 Fast Real-Time PCR System and results were analyzed with the provided software (Applied Biosystems, Foster City, CA, USA). For each sample, we analyzed the cycle of threshold (CT) of the wild type allele (in the absence of PNA) and the CT of the mutant *EGFR*-Thr790Met allele (in the presence of PNA). The difference (ΔCT) between the CT of the mutant allele and the CT of the wild type allele is an estimate of the percentage of the mutated allele since there is a logarithmic relationship between ΔCt and the proportion of the mutated allele in the total allele population (variant allele frequency, VAF).

To establish the logarithmic relationship between ΔCt and VAF, we prepared a standard curve as previously described. We diluted DNA from the H1975 cell line into increasing concentrations of wild type donor DNA. This dilution bank consisted of 9 dilutions of a theoretical VAF ranging from 7.84% to 0.03%, which were tested in quadruplicate to obtain the ΔCt values. Using this standard curve, we were able to interpolate the ΔCt values of tested samples to obtain the VAF. In order to reduce inter-assay variability, in every run, we processed three standard curve dilutions to obtain a mean conversion factor as follows: (ΔCt value of dilution in standard curve experiment/ΔCt value in experiment). We used this conversion factor to multiply the ΔCt value obtained for each sample before interpolating.

4.6. Digital Droplet PCR (ddPCR)

EGFR-Thr790Met mutation testing by digital droplet PCR (ddPCR) was carried out with a QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) by using the ddPCR Mutation Assay VAL EGFR T790M (dHsaMDV2010019). ddPCR was performed in duplicate on 20 ng of gDNA obtained from FFPE samples or cell lines. LB samples were tested in quadruplicate using 9 µL of cfDNA. Droplets were generated with a QX200 Droplet Generator and PCR reaction was carried out in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The cycling conditions for the PCR reaction included an initial incubation at 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 55 °C for 60 s and enzyme inactivation at 98 °C for 10 min. After thermal cycling, the plates were transferred to a QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA) for fluorescence reading. ddPCR data were analyzed with the QuantaSoft software (Bio-Rad, Hercules, CA, USA), detecting positive droplets for *EGFR*-Thr790Met mutant and wild type probes and then establishing the VAF of *EGFR*-Thr790Met mutation for FFPE sample testing and the *EGFR*-Thr790Met allele copies/mL of plasma for LB testing.

4.7. Limit of Blank (LOB) and Limit of Detection (LOD)

LOB and LOD were established for PNA Clamp TaqMan assay in FFPE samples and for ddPCR in both FFPE and LB samples [44]. The LOB was established as the highest mutant signal (VAF or mutant copies/mL of plasma) that could be detected in wild type samples: LOB= mean VAF + 1.645 × SD. Samples with low abundance of *EGFR*-Thr790Met were processed for LOD establishment as follows: $LOD = LOB + 1.645 \times SD_{(low\ concentration\ sample)}$.

To ensure that these limits were transferable to the tested samples, we used FFPE samples and cfDNA-like standards. For LOB establishment with FFPE samples, we analyzed seven FFPE *EGFR*-Thr790Met-negative samples previously characterized by next generation sequencing (NGS) (average read depth of 3500×) and 4 FFPE reference materials obtained from Horizon Discovery (Waterbeach, UK). Regarding LOD establishment, we studied five low *EGFR*-Thr790Met allele frequency samples (VAF= 0.100%, 0.150%, 0.200%, 0.220% and 0.230%). These samples were tested in triplicate and the mean of the SD obtained for each sample was used to calculate LOD. Regarding the LB validation

procedure, for LOB establishment 10 wild type standards were processed and five standards with a VAF of 0.1% and 1%, respectively, were used for LOD establishment.

4.8. Statistical Analyses

Quantitative variables were summarized by their mean and standard deviation, and categorical variables by absolute and relative frequencies. Simple linear regression analysis and a Pearson correlation test were used to evaluate the relationship of theoretical VAF and that detected by ddPCR. Comparison among ddPCR results and PNA Clamp TaqMan assay results was made by determining overall agreement. Statistical association between the *EGFR*-Thr790Met genotype and qualitative variables was assessed by a chi-square test or the Fisher exact test. For PFS analyses, patients without radiological progression were censored. All time-to-event outcomes were estimated using the Kaplan–Meier method and compared across groups using log-rank testing (univariate analysis). A Cox proportional-hazards model was used to evaluate the association between the *EGFR*-Thr790Met genotype and the PFS of patients. Statistical analyses were carried out with the statistical package SPSS v.21 (IBM, Armonk, NY, USA) and GraphPad Prism Software version 7.0.2, (San Diego, CA, USA). p-values <0.05 were considered statistically significant.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Human Rights, the Conference of Helsinki, and approved by the Hospital Ethics Committee (2015/0713; 16 February 2016, 2017/0070; 29 March 2017 and 2016/0129; 11 October 2017).

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Article

Impact of Molecular Testing by Next Generation Sequencing in the Clinical Management of Non–Small Cell Lung Cancer Patients in a Public Healthcare Hospital

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Simple Summary: Precision medicine has revolutionized the treatment of advanced non-small cell lung cancer (NSCLC). Since the discovery of novel predictive biomarkers an exhaustive molecular characterization of the disease is required for an adequate clinical management. In this research we aim to evaluate the implementation to routine diagnostics of next generation sequencing (NGS) under a quality management system. In a cohort of 350 patients, NGS studies were able to reveal a distinct molecular profile of the disease according to sex and smoking status, as well as co-occurring and mutually exclusive relationships between molecular alterations. In stage IV patients, targeted therapies were associated with longer progression-free and overall survival. NGS has expanded the precision medicine in our center by increasing the percentage of patients with actionable molecular alterations. Our findings have consolidated the use of NGS as a molecular diagnostic tool in the clinical routine of a public healthcare hospital.

Abstract: Next Generation Sequencing (NGS) is a molecular approach able to provide a comprehensive molecular profiling of non-small cell lung cancer (NSCLC). The broad spectrum of biomarker-guided therapies has positioned molecular diagnostic laboratories as a central component of patient's clinical management. Here, we show the results of a UNE-EN ISO 15189:2013 NGS-accredited assay in a cohort of 350 patients. *TP53* (51.0%), *KRAS* (26.6%) and *EGFR* (12.9%) were the most frequently mutated genes. Furthermore, we detected co-occurring and mutually exclusive alterations, and also distinct molecular profiles according to sex and smoking habits. Actionable genetic alterations were significantly more frequent in female patients (80.5%, $p < 0.001$) and in never smokers (87.7%, $p < 0.001$). When NGS was established as the main molecular testing strategy, 36.4% of patients had received at least one line of targeted treatment. Among 200 stage-IV NSCLC patients, first-line treatment with targeted therapies was associated to a longer progression free survival (PFS) [13.4 months (95% CI, 10.2-16.6) ($p = 0.001$)]. Similarly, overall survival (OS) of patients receiving at least one targeted drug was significantly longer [26.2 months (95% CI, 11.8-40.5) ($p < 0.001$)]. Our results show that the implementation of NGS in the public healthcare system has provided a broader application of precision medicine.

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1. Introduction

Understanding of the genomic alterations that lead to cancer cells' proliferation opened the way for precision medicine as a new therapeutic paradigm in non-small cell lung cancer (NSCLC)[1]. As a consequence, molecular profiling of NSCLC has become central for the clinical management since of the discovery of predictive biomarkers and the clinical impact of targeted therapies.

In the recent years, the impressive growth of therapeutic strategies guided by molecular alterations has created the requirement of a rapid translational research in the field of molecular diagnosis in order to provide an up-to-date assessment of clinically relevant molecular alterations[2,3]. However, specimen adequacy is a significant issue in the molecular diagnosis of NSCLC. Reduced sample sizes of cytology and tissue biopsies as well as the quality of nucleic acids after fixation and paraffin embedding processes are important constraints that molecular diagnostic laboratories must deal with when implementing novel techniques[4].

Due to the actual requirements of precision medicine in NSCLC and the intrinsic limitations of the molecular diagnosis, high throughput approaches are needed. Next Generation Sequencing (NGS) has rapidly positioned as a central technique in molecular diagnostic laboratories because of its ability to simultaneously assess for different molecular alterations in a group of relevant genes.

Given the current implications of NGS studies for clinical management, translation to this molecular approach into routine care must be performed under strict quality standards. . In order to ensure technical requirements and the reliability of the results, the UNE-EN ISO 15189:2013 provides laboratories with the tools to manage the quality of the molecular studies[5,6].

Application of these techniques provides a comprehensive view of the disease from the molecular perspective, deciphering tumor heterogeneity and revealing co-occurring or mutually exclusive genetic alterations. The genetic profiling of NSCLC has revealed differences in the molecular profile of the disease according clinical-pathological characteristics such as ethnicity, histology, sex and smoking status[7,8].

The significant number of actionable molecular alterations that define the molecular spectrum of NSCLC has led current clinical guidelines to strongly recommend the use of gene panels for molecular diagnosis of NSCLC[9–12]. Apart from approved targeted therapies, NGS results may identify patients who are candidates for off-label drugs treatment or eligible in clinical trials.

In this work we report the integration of NGS studies into a reference public healthcare hospital under UNE-EN ISO 15189:2013 quality standard. We have evaluated the role of NGS studies in providing a comprehensive molecular profile of NSCLC cancer patients. Moreover, we have assessed the contribution of NGS in identifying patients eligible for targeted drug treatment and the influence of this therapeutic approach in the outcomes of our patients.

2. Materials and Methods

2.1. Patients and samples

The study included a cohort of 350 NSCLC patients diagnosed in the Medical Oncology Department at the Hospital Universitario y Politécnico La Fe in Valencia (Spain) from 2015 to May 2022. The epidemiological, clinical and pathological features of these patients are summarized in Table 1. All patients showed their agreement by signing the informed consent elaborated in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki and the study was approved by the Hos-

pital Ethics Committee. Tissue samples were examined in the Pathology Department and only those with at least 150 total cells and 10% of tumor content were considered valid for molecular analysis.

Table 1. Epidemiological and clinical-pathological characteristics of the patients recruited.

Variable	
Age, mean ± SD	63.2 ± 0.6
Sex, n(%)	
Male	217 (62.0)
Female	133 (38.0)
Smoking history, n (%)	
Never	65 (18.6)
Former smoker	127 (36.3)
Current smoker	158 (45.1)
Smoking load (Former and current smokers), median (IQR)	36 (23-50)
Years since quitting smoking (Former smokers), median (IQR)	12 (5-20)
Histology, n (%)	
Adenocarcinoma	288 (82.3)
Large cell carcinoma	9 (2.6)
Squamous	14 (4.0)
Sarcomatoid carcinoma	11 (3.1)
Adenosquamous carcinoma	3 (0.9)
Large cell neuroendocrine carcinoma	8 (2.3)
NOS	17 (4.9)
Stage, n (%)	
IA	38 (10.9)
IB	24 (6.9)
IIA	2 (0.6)
IIB	17 (4.9)
IIIA	28 (8.0)
IIIB	19 (5.4)
IIIC	9 (2.6)
IV	200 (57.1)
Unknown	13 (3.7)

SD: Standard deviation. IQR: Interquartile range.

In January 2021, as a consequence of the accreditation of the NGS techniques under the UNE-EN ISO 15189:2013 standard [13], the Lung Cancer Committee of our hospital decided to establish NGS as the routine testing strategy for the molecular profiling of NSCLC. During this period, 128 samples had been analyzed through NGS. From sample receipt to report generation a median turnaround time of 10 days (Range: 5-25) was achieved.

2.2. Nucleic acid isolation

Genomic DNA was isolated from five 5 μm thick FFPE sections using Deparaffinization Solution and the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany). RNA was extracted from five 10 μm thick FFPE sections employing the RecoverAll™ Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). DNA and RNA concentration was assessed using Qubit 3.0 fluorometer with DNA HS or RNA HS Assay Kit (ThermoFisher Scientific). NGS analyses were performed at the Molecular Biology Unit (Clinical Analysis Department).

2.3. Next Generation Sequencing studies

NGS studies were conducted using Oncomine Solid Tumor (OST; ThermoFisher Scientific) in 104 samples and Oncomine Focus Assay (OFA; ThermoFisher Scientific) in the remaining 246 samples. Briefly, OST allows the detection of point mutations and small insertions/deletions in 22 genes and fusions transcripts involving 4 genes while OFA was designed to detect point mutations and small insertions/deletions in 35 genes, copy number variations of 19 genes and fusion transcripts of 23 driver genes. Library preparation and clonal amplification were performed by either manually or automated procedures with the Ion Chef™ Instrument (ThermoFisher Scientific) following manufacturer's protocols. Clonally amplified libraries were sequenced on the Ion PGM™ System or on the Ion GeneStudio™ S5 System (ThermoFisher Scientific). Raw data processing and alignment to the human reference genome (hg19) was performed with Torrent Server and variant calling/annotation was conducted with Ion Reporter Server by using commercial workflows. Intronic variants and synonymous changes were filtered out. Variants with low total read depth (<500 total) and/or low variant read depth (<20 reads) were excluded. Additionally, variants were visually examined using the Integrative Genomics Viewer (IGV) software.

2.4. Statistical Analyses

Quantitative variables were summarized by their mean and standard deviation or median and interquartile range, and categorical variables by absolute and relative frequencies. Statistical association between qualitative variables was assessed by chi-square test or the Fisher exact test. For progression free survival (PFS) and overall survival (OS) analyses, patients with incomplete clinical data, without radiological progression and/or being alive at the time of the analyses were censored. All time-to-event outcomes were estimated using Kaplan-Meier method and compared across groups using log-rank testing (univariate analysis). Cox proportional-hazards model was used to evaluate the association between predictor variables and survival. Statistical analyses were carried out with the Statistical Package SPSS v.21 (IBM, Armonk, NY, USA) and GraphPad Prism Software version 7.0.2, (San Diego, California USA). p-values < 0.05 were considered statistically significant. For the statistical analysis of the data, the clinical evidence of the identified variants (approved drugs and clinical trials) has been reviewed as of May 2022.

3. Results

3.1. Molecular alterations detected by next-generation sequencing

In our cohort, 54.3% of patients showed a unique molecular alteration, 22.3% showed at least two molecular alterations (Range: 2-4) and 23.4% of patients did not carry any molecular aberration (Figure 1). The most prevalently mutated gene was *TP53* (51.0%) followed by *KRAS* (26.6%), *EGFR* (12.9%), *BRAF* (6.9%) and *PIK3CA* (5.4%). Gene fusions involving *ALK* (4.0%), *MET* (3.6%) and *ROS1* (1.4%) were the most common fusion transcripts detected. Regarding copy number variations (CNV), gene amplifications of *EGFR* (2.4%) and *MYC* (2.0%) were the most frequently detected (Figure 1).

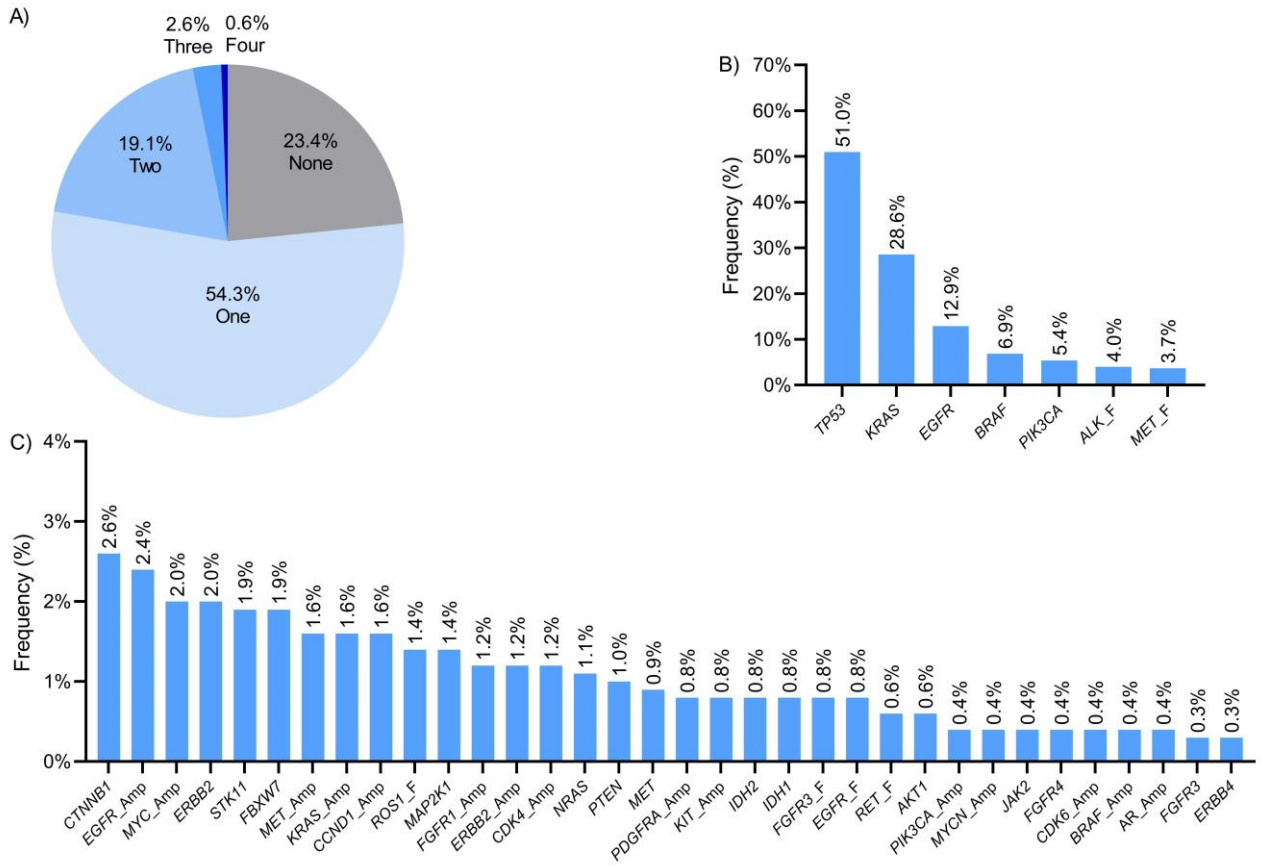

Figure 1. Molecular alterations detected in NGS studies. (A) Patient's distribution according to the number of genetic alterations detected. (B, C) Percentage of samples with molecular alterations in the genes included in the study. Variants detected at a frequency >3% (A) and <3% (B). Amp: Amplification. F: Fusion.

3.2. Clinical-pathological associations with molecular alterations

Regarding molecular alterations associations with clinical-pathological features, smoking status and sex had a strong impact in the mutation profile of the disease. *EGFR* mutations ($p < 0.001$), *ERBB2* mutations ($p = 0.013$) and *ALK* fusions ($p = 0.049$) were more frequently detected in female patients while *TP53* mutations were significantly associated with male patients ($p = 0.045$). Moreover, *KRAS* mutations were more frequent in former or current smokers ($p < 0.001$) while *EGFR* mutations ($p < 0.001$), *ERBB2* mutations ($p = 0.029$), *ALK* fusions ($p < 0.001$), *ROS1* fusions ($p = 0.030$), *RET* fusions ($p = 0.012$), and METEx14 ($p < 0.001$) were associated to never smoker patients (Figure 2). Moreover in former smokers patients, the index of pack/year was significantly lower in *EGFR* mutated patients (Median: 15) than in *EGFR*-WT patients (Median: 35), U = 302.5, $p = 0.03$) (Figure S1). Regarding age at diagnosis, patients harboring METEx14 were significantly older (Mean 75.1 ± 12.1) than those without this molecular alteration (Mean 62.6 ± 11.4), t(244) = -3.2, $p = 0.002$ (Figure S2).

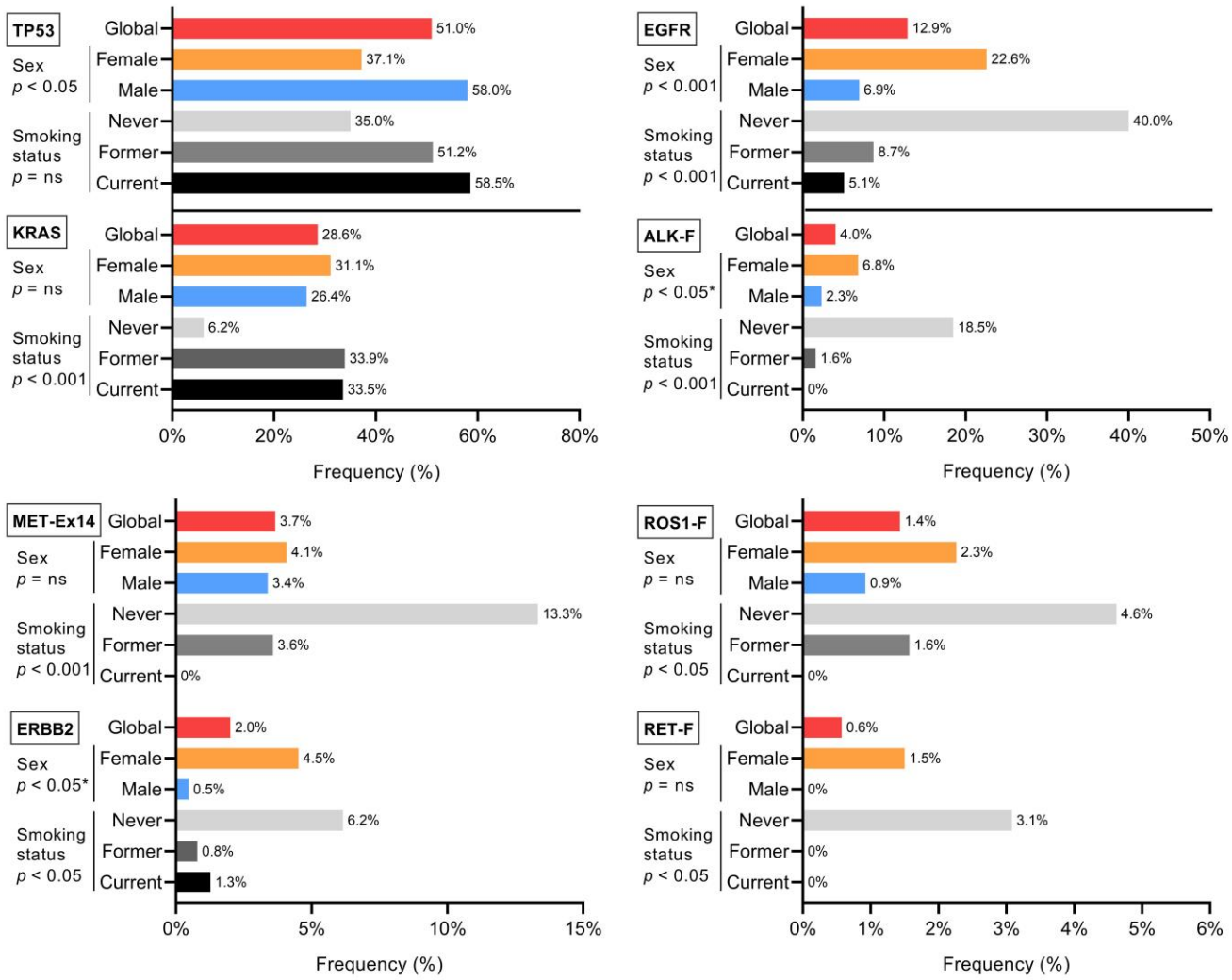

Figure 2. Association of sex and smoking status with specific molecular alterations. Frequency of gene alterations in the global cohort and in the evaluated subgroups is depicted. ns: not statistically significant. *Comparison using Fisher's Exact Test.

3.3. Co-occurring or mutually exclusive genetic alterations

NGS studies for the molecular profiling of NSCLC patients have revealed co-occurring and mutually exclusive genetic alterations in our cohort. *KRAS* mutations were highly exclusive with *EGFR* mutations ($p < 0.001$), *TP53* ($p = 0.01$) and *ALK* fusions ($p = 0.01$). *EGFR* amplifications were more frequent in *EGFR* mutated patients ($p < 0.01$). *MET* mutations were associated to METEx14 skipping ($p < 0.001$) and *KRAS* amplifications and *CCND1* amplifications frequently co-occurred in our patients ($p = 0.01$) (Figure 3). Our analysis suggested the existence of other associations between molecular alterations that did not reach statistical significance: Co-occurrence of *KRAS* mutations + *KRAS* amplifications, *NRAS* mutations + *MYC* amplifications, *CCND1* + *MET* amplifications and a mutually exclusion between *TP53* mutations and *ALK* fusions.

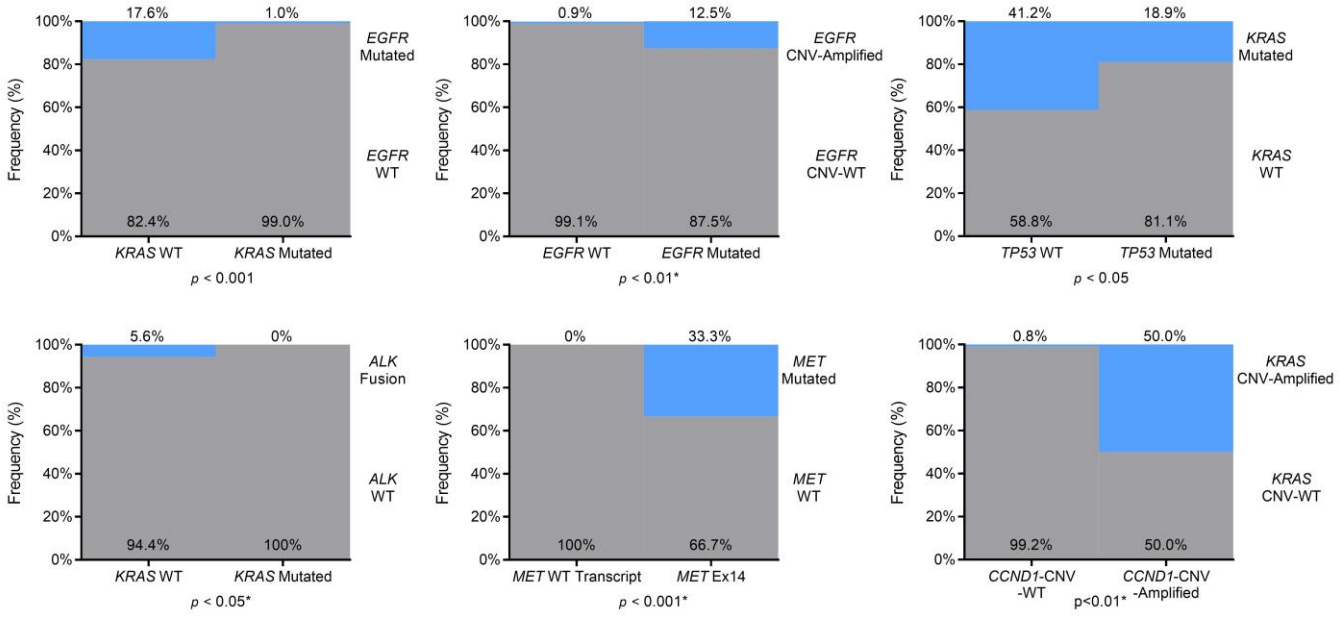

Figure 3. Mosaic plot showing relationships of co-occurrence and mutual exclusivity between the molecular alterations detected in our cohort. For each comparison the most frequently altered gene is depicted in the X-axis. Frequency of patients included in each of the four subgroups defined by the presence/absence of both genetic alterations is depicted in the graph. *Comparison using Fisher's Exact Test.

3.3. Clinically relevant genetic variants

In our cohort, actionable molecular alterations were detected in 65% of patients. Of these, 54.4% had molecular alterations for which there is an approved targeted therapy, while 45.6% could be enrolled in phase I or phase II clinical trials, based on their molecular profile. Actionable molecular alterations were significantly more frequent in female patients ($p < 0.001$). Moreover, clinical evidence associated to these variants also differed according to sex, with female patients harboring a higher percentage of molecular alterations targetable with approved drugs ($p < 0.001$) (Figure 4).

In this sense, smoking status also had an impact in the prevalence of actionable molecular alterations ($p < 0.001$). Eighty-seven percent of never smoker patients presented harbored molecular alterations, most of them (91%) being candidates for currently approved targeted therapies. Interestingly, no differences were observed in the percentage of patients with actionable molecular alterations and their associated clinical evidence between former and current smoker patients. When comparing the frequency of actionable molecular alterations regarding sex and smoking status, female patients presented more targeted therapy options than men regardless of smoking status. In both, female and male patients, no differences were observed between former and current smokers (Figure 4) (Figure S3).

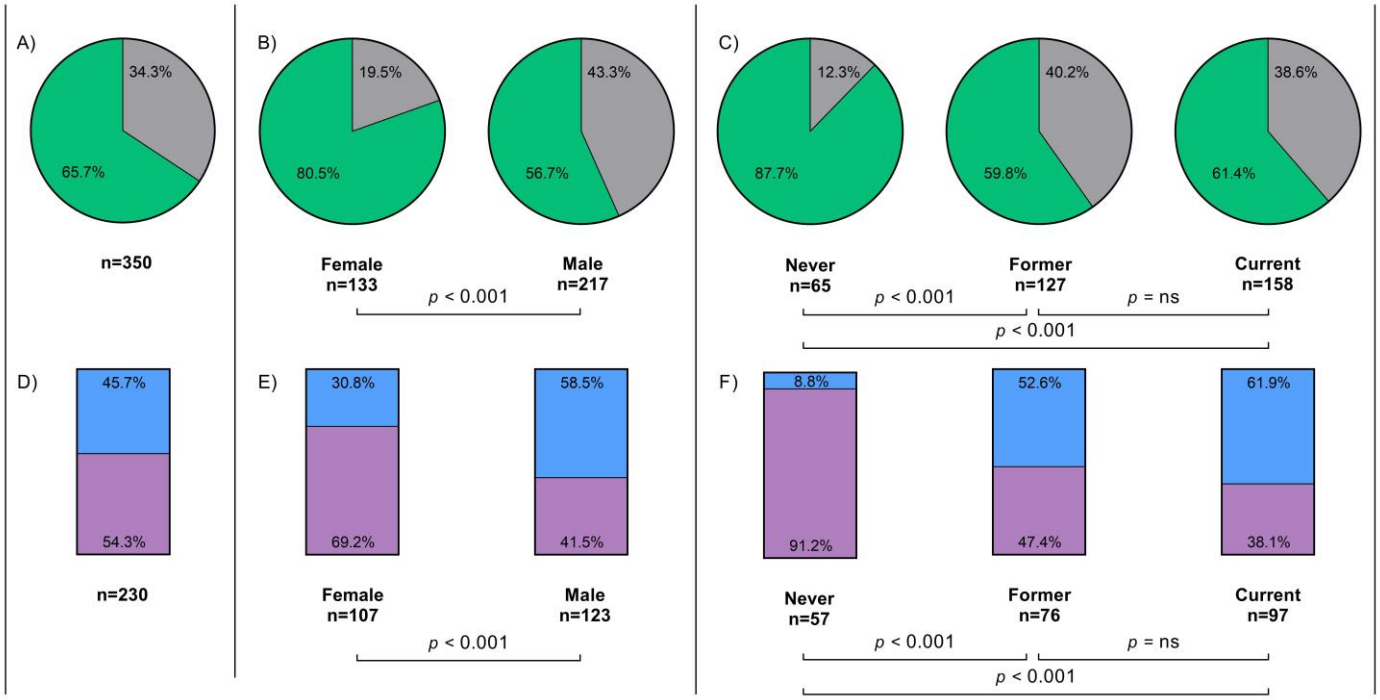

Figure 4. Actionable molecular alterations detected by NGS in our cohort. Percentage of patients with actionable genetic variants (Green) in the global cohort (A), according to sex (B) and according to smoking status (C). Patient distribution according to the level of evidence of the detected variants in the global cohort (D), according to sex (E) and according to smoking status (F). Purple: Patients who are candidates for treatment with approved drugs, Blue: patients eligible for Phase I-II clinical trials. ns: not statistically significant.

Since NGS was established as the main molecular testing strategy, 128 samples had been prospectively analyzed through NGS. Of all these patients, 83 were diagnosed with de novo stage IV NSCLC and 55 started systemic treatment for advanced disease. Based on the NGS results, 20 patients (36.4%) have received at least one line of targeted treatment (Table 2). Moreover, among the remaining 35 patients, eight (22.9%) could initiate targeted treatment after progression to their current treatment lines (Six with novel KRAS p.(Gly12Cys) inhibitors and two with novel EGFR Exon 20 targeted agents).

Table 2. Stage IV NSCLC patients treated with targeted therapies based on the NGS results.

Molecular alteration	n	Drug
EGFR: p.(Leu858Arg)	4	Osimertinib
EGFR: p.(Glu746_Ala750del)	3	Osimertinib
EGFR: p.(Leu858Arg) + *EGFR* Amplification	1	Osimertinib
EGFR: p.(Gly719Ala) + p.(Ser768Ile)	1	Osimertinib
EGFR: p.(Leu861Gln)	1	Osimertinib
EGFR: p.(Glu709_Thr710delinsAsp)	1	Osimertinib
EGFR: p.(Ala767_Val769dup)	1	Amivantamab
EML4(13)-ALK(20)	1	Alectinib
KIF5B(17)-ALK(20)	1	Brigatinib
ALK Fusion (Unknown Partner)	1	Alectinib
MET(13) - MET(15)	1	Capmatinib
KRAS: p.(Gly12Cys)	1	Sotorasib

BRAF: p.(Val600Glu)	1	Dabrafenib + trametinib
SLC34A2(13)-ROS1(32)	1	Crizotinib
KIF5B(15)-RET(12) + *IDH1*: p.(Arg132His) + *MYC* Amplification	1	Selpercatinib

3.4. First-line treatment analyses

The main clinical and pathological features of the 200 de novo stage IV NSCLC patients included in the study are shown in Table 3. Among them, 146 started a first-line treatment for advanced disease: 69 (47.3%) were treated with chemotherapy, 33 (22.6%) with targeted therapies and 44 (30.1%) started an immunotherapy-based treatment regimen. Analysis of progression-free-survival (PFS) showed statistically significant differences in the outcome of patients according to each treatment strategy ($p = 0.01$). Patients treated with targeted therapy achieved a significantly longer PFS than patients in the chemotherapy group: 13.4 months (95% CI, 10.2-16.6) vs. 5.2 months (95% CI, 4.2-6.2) ($p = 0.001$). In this comparison, chemotherapy treatment had a significantly higher risk of progression: HR: 2.3 (95% CI: 1.4-3.8). Similarly, patients treated with immunotherapy achieved a longer median PFS [7.8 months (95% CI, 3.5-12.1)] ($p = 0.011$) and had a significant higher risk of progression than those treated with immunotherapy [HR 1.8, (95% CI: 1.1-3.0)]. Although the median PFS of patients treated with targeted therapies was higher than the achieved by patients treated with immunotherapies these differences did not reach statistical significance. Outcome of immunotherapy treated patients was diverse, while a 31.6% of patients have progressed first 5 months after treatment start, a 20.5% or patients achieved prolonged responses (> 60 months) (Figure 5).

Table 3. Epidemiological and clinical-pathological characteristics of stage IV NSCLC patients.

Variable	
Age, mean ± SD	**63.1 ± 12.1**
Sex, n(%)	
Male	78 (39.0)
Female	122 (61.0)
Smoking history, n (%)	
Never	46 (23.0)
Former smoker	63 (31.5)
Current smoker	91 (45.5)
Sex and smoking history, n (%)	
Never smoker female	31 (15.5)
Former smoker female	16 (8.0)
Current smoker female	31 (15.5)
Never smoker male	15 (7.5)
Former smoker male	47 (23.5)
Current smoker male	60 (30.0)
Histology, n (%)	
Adenocarcinoma	162 (81.0)
Large cell carcinoma	5 (2.5)
Squamous	7 (3.5)
Sarcomatoid carcinoma	5 (2.5)
Adenosquamous carcinoma	2 (1.0)

Large cell neuroendocrine carcinoma	5 (2.5)
NOS	14 (7.0)
Systemic treatment, n (%)	
No	37 (19.0)
Yes	158 (81.0)

SD: Standard deviation.

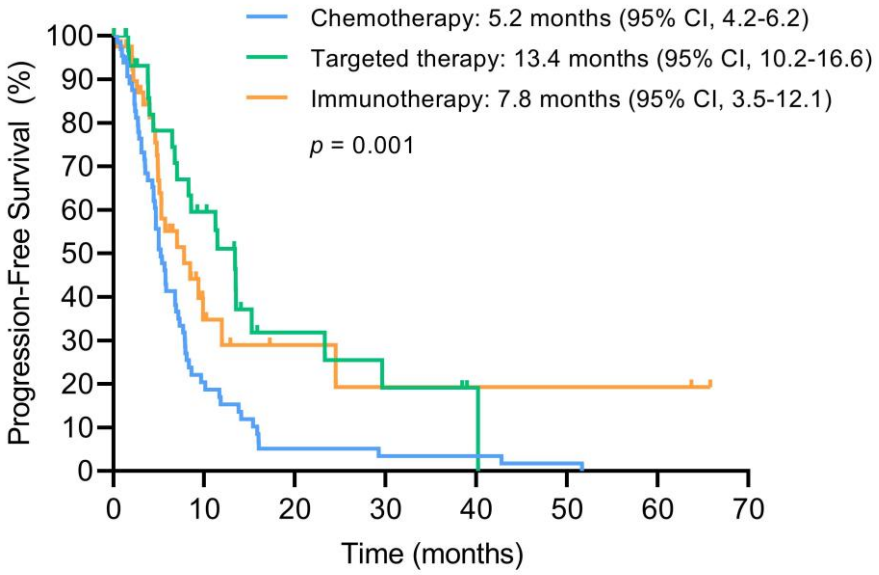

Figure 5. First-line progression-free survival for stage IV NSCLC patients stratified by therapeutic approach.

3.5. Overall survival

To evaluate the impact of the distinct treatment strategies in the overall survival (OS) of our stage IV NSCLC patients (n=200) we have grouped them into three categories according to the treatment received: Those who have been treated only with chemotherapy (41, 28.1%), those who received at least one targeted therapy agent (40, 27.4%), those who initiated at least one immunotherapy-based regimen (55, 37.7%) and a subgroup of patients treated with both targeted therapies and immunotherapy (10, 6.9%). Overall survival was significantly different among groups ($p < 0.001$), with patients in the chemotherapy group showing the worst OS [8.8 months (95% CI, 4.5-13.1)] (Figure 6). Compared to the patients in the chemotherapy group, patients treated with targeted therapy [HR: 0.3 (95% CI, 0.2-0.6)], immunotherapy [HR: 0.2 (95% CI, 0.1-0.4)], or both strategies [HR: 0.2 (95% CI, 0.1-0.6)] had a significant decreased risk of death.

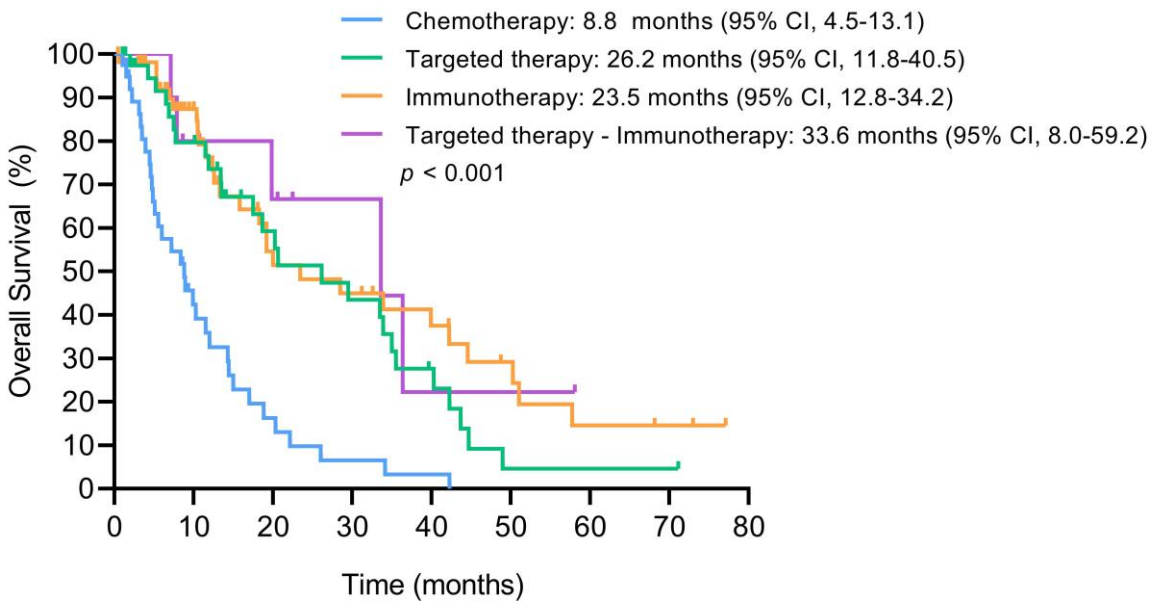

Figure 6. Overall survival of stage IV NSCLC patients stratified by the treatment strategies administered during the course of the disease.

4. Discussion

NSCLC has become the tumor type with the most available targeted therapies as a result of research to better understand the molecular basis of the disease. The evolving landscape of this therapeutic approach requires molecular biology laboratories to implement the most up-to-date molecular techniques. In this scenario, NGS has rapidly positioned as the main molecular testing strategy for advanced NSCLC due to its ability to assess for different molecular alterations with minimum sample requirements. In this paper we show the role UNE-EN ISO 15189:2013 certified NGS in understanding the molecular basis of NSCLC as well as its impact in the clinical management of patients in our institution.

Clinical-pathological characteristics of the recruited patients show that our work is representative of the clinical reality of non-small cell lung cancer in our community setting[14]. Molecular alteration's frequency in our cohort is concordant with previously published studies[15–17]. We have also found significant differences in the molecular profile according to sex and smoking status. Sex has been considered a risk factor for lung cancer development and, in women, has been considered a separate disease with individual characteristics[18–20]. Moreover, distinct clinical outcomes have been reported between men and women and its use as a prognostic biomarker has also been discussed[21,22]. As previously reported, *EGFR* mutations and *ALK* fusion transcripts were found to be more frequent in female patients[23,24] while TP53 mutations were significantly associate to male patients[25]. Regarding *ERBB2* mutations, our results are consistent with several articles suggesting their association with male sex[26,27].

The impact of smoking on the process of carcinogenesis determines that NSCLC in non-smokers is considered as a separate disease with specific clinical features[28,29]. Our results support this observation as the molecular profile of never-smoker patients was enriched in *EGFR* and *ERBB2* mutations as well as in *ALK*, *ROS1*, *RET* and METEx14 fusion transcripts[30–34]. Interestingly, the heterogeneous group of former smoker patients may reflect the distinct evolution of NSCLC according to smoking history. In our cohort, a significant lower smoking load was observed in former smokers with *EGFR* mutations and consequently its disease may be classified as "never smoker-like"[30].

Application of NGS to diagnostics routine provided an overview of tumor heterogeneity revealing co-occurring and mutual exclusion relationships among molecular alterations. In patients treated with targeted therapies, this information is particularly relevant since the strong selective pressure of treatment may enhance the proliferation of resistant clones[35,36]. *EGFR* activating mutations and *EGFR* gene amplification frequently co-occurred in our patients. This relationship has been previously reported and in the majority of cases, amplification occurs in the mutated *EGFR* allele. Due to the higher amount of mutated *EGFR* in these tumors, these patients could constitute a unique subgroup in terms of responses to EGFR-TKI treatment[37,38]. As previously reported, in our cohort, *KRAS* mutations showed a mutual exclusion pattern with other main oncogenic drivers such as *EGFR* mutations and *ALK* fusions[39,40]. In contrast, *KRAS* mutations were associated to *KRAS* amplifications suggesting a synergistic role in driving cancer proliferation[41]. Interestingly, *KRAS* amplifications and *KRAS* amplifications were found to be concomitant in our cohort, which may reflect the cooperation of both alterations in NSCLC proliferation[42]. Finally, in our patients, an association relationship has been identified between *MET* exon 14 skipping and *MET* gene amplification, which could be particularly relevant in terms of the response to *MET* inhibition treatment[43,44].

Sex- and smoking-related genetic differences in our cohort led to a distinct relevance of targeted therapies. Patients with actionable genetic alterations were most frequently female and never smokers[45]. Moreover, in these subgroups, actionable genetic alterations were majority related to currently approved treatments. In this sense, in our cohort, never smoker women constituted a unique subgroup of patients in which up to 91% could benefit from targeted treatment. Based on this percentage, maximum efforts should be made to offer a NGS study in this subgroup of patients[16,46]. In contrast, although former smokers constitute a heterogeneous group in which smoking load and smoking abstinence should be taken into consideration. In our cohort, former and current smokers had a similar molecular profile. Our results show a significant lower prevalence of actionable alteration in tobacco associated lung cancer[47].

In advanced NSCLC patients, PFS for first line treatment was significantly different among therapeutic strategies. Chemotherapy-treated patients showed the worst outcome while targeted therapy-treated patients exhibited the longest PFS. Interestingly, outcome of immunotherapy treated patients was diverse; almost one-third of patients with progressive disease in the first 5 months of treatment while a 20% of patients achieved prolonged responses. This result, reflect the lack of robust biomarkers to identify patients who will benefit most of this therapeutic approach[48,49].

OS analysis of the patients included in this study clearly reveals the impact of targeted therapies and immunotherapies in the survival of NSCLC. Patients who received at least one line of treatment based on this therapeutic approach experienced a significantly longer survival than those who only received chemotherapy-based regimens. These results justify the application of NGS to increase the number of patients harboring molecular alterations related to approved, or ongoing clinical trials for targeted therapy[50–52].

Following current recommendations, we have integrated the NGS molecular profiling of advanced NSCLC to routine molecular diagnosis[9–12]. Accreditation of this testing strategy under UNE-EN ISO 15189:2013 scope guarantees compliance with technical requirements, ensuring the reliability of the results and the consequent therapeutic decision making in a clinically practical turnaround time. Implementation of NGS has been a milestone for biomarker-selected treatments in our institution since 36.4% of patients who started a first-line treatment, have received targeted therapies based on the provided NGS results.

5. Conclusions

Our work shows the establishment of an UNE-EN ISO 15189:2013 certified NGS assay to routine molecular diagnostic of NSCLC in a public reference healthcare hospital.

Our results have revealed distinct molecular profiles according to clinical-pathological features and have provided insights into tumor heterogeneity. Moreover, targeted therapies have been associated to better outcomes in our patients. Implementation of NGS has expanded the application of precision medicine to a greater number of patients.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Smoking burden (pack/year) in former smokers according to the presence of activating mutations in the *EGFR* gene; Figure S2: Age at diagnosis according to the presence METEx14 molecular alteration; Figure S3: Percentage of female and male patients harboring actionable genetic alterations according to the smoking status.

Author Contributions: Conceptualization, J.S., Ó.J. and S.P.; methodology, J.S., G.P.-S. and S.P.; investigation, J.S., Ó.J. and S.P.; resources, N.M., E.A., C.F.M.-N. and J.G.-C.; writing—original draft preparation, J.S.; writing—review and editing, S.P.; visualization, J.S.; supervision, S.P.; funding acquisition, S.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Human Rights, the Conference of Helsinki, and approved by the Hospital Ethics Committee (2015/0713; 16 February 2016 and 2017/0070; 29 March 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary Materials: Impact of Molecular Testing by Next Generation Sequencing in the Clinical Management of Non–Small Cell Lung Cancer Patients in a Public Healthcare Hospital

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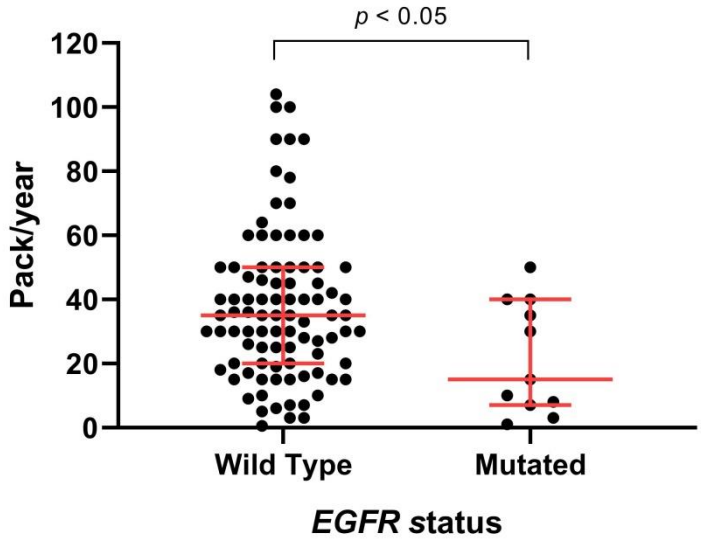

Figure S1. Smoking burden (pack/year) in former smokers according to the presence of activating mutations in the *EGFR* gene

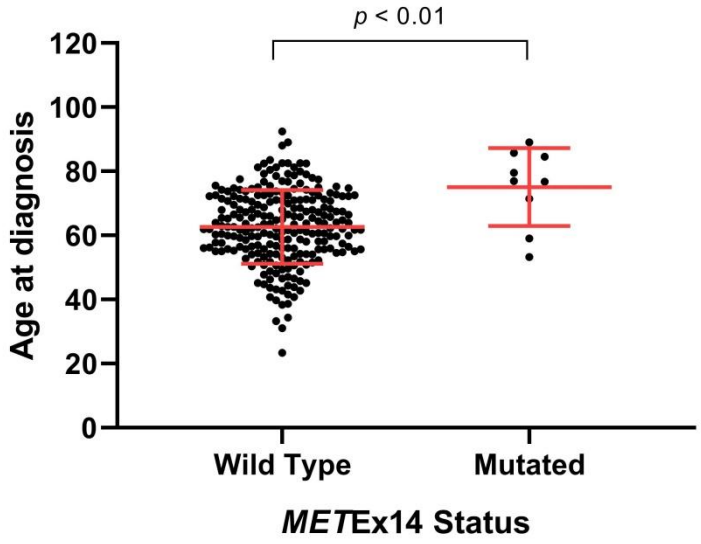

Figure S2. Age at diagnosis according to the presence METEx14 molecular alteration.

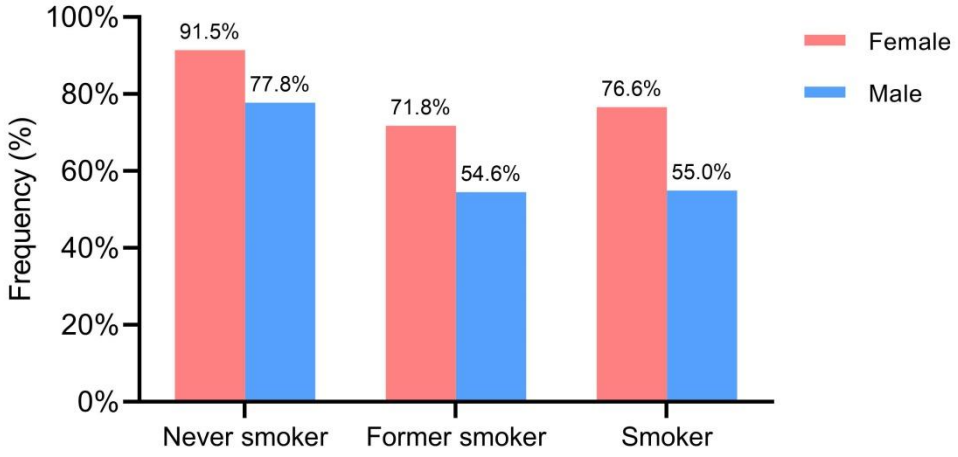

Figure S3. Percentage of female and male patients harboring actionable genetic alterations according to the smoking status.