UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE BIOTECNOLOGÍA

ANÁLISIS MOLECULAR Y DE MARCADORES PRONÓSTICO EN SANGRE PERIFÉRICA EN PACIENTES CON CÁNCER NO MICROCÍTICO DE PULMÓN EN ESTADIO LOCALMENTE AVANZADO

TESIS DOCTORAL

Presentada por:

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Valencia, Mayo de 2013

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA Y DEL MEDIO NATURAL

DEPARTAMENTO DE BIOTECNOLOGÍA

ÁREA DE MICROBIOLOGÍA

D. Rafael Sirera Pérez, profesor doctor, perteneciente al Departamento de Biotecnología (Área de Microbiología) de la Universitat Politècnica de València,

CERTIFICA:

Que la tesis doctoral titulada **"Análisis molecular y de marcadores pronóstico en sangre periférica en pacientes con cáncer no microcítico de pulmón en estadio localmente avanzado",** que presenta Dña. Vega Iranzo González-Cruz para optar al grado de **Doctor por la Universitat Politècnica de València**, ha sido realizada bajo su dirección y reúne los requisitos adecuados para ser presentada como tesis doctoral ante el tribunal correspondiente para su lectura y defensa.

Y para que conste a los efectos oportunos, firma el presente certificado,

En Valencia, 30 de Mayo de 2013

Fdo.: D. Rafael Sirera Pérez

UNIVERSITAT DE VALÈNCIA

FACULTAT DE MEDICINA I ODONTOLOGIA

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D. Carlos Camps Herrero, profesor titular doctor, perteneciente al Departament de Medicina de la Universitat de València,

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“…fuerte y noble como la encina…”

Deseo expresar mi agradecimiento a todas aquellas personas que han hecho posible la realización de esta Tesis Doctoral.

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RESUMEN

El cáncer de pulmón es uno de los principales problemas de salud del mundo; es una enfermedad frecuente, grave y con incidencia en aumento. A estas características, se añade la complejidad de su tratamiento -cirugía, quimioterapia y radioterapia-, que es agresivo y habitualmente costoso siendo necesaria la colaboración organizada de numerosos profesionales distintos. En este sentido integral de la atención al paciente oncológico, la aportación de la biología molecular con nuevos conceptos y retos como la oncofarmacogénetica y oncofarmacogenómica ha permitido no sólo el desarrollo de fármacos usando biomarcadores para predecir la respuesta; sino también el análisis del comportamiento de las células tumorales; el estudio de mutaciones de genes, polimorfismos, metilaciones, etc.; estudios anatomopatológicos más precisos; y la individualización del tratamiento gracias al mejor conocimiento de los factores pronóstico y sobre todo, de los factores predictivos de respuesta.

En nuestro trabajo hemos tratado de identificar marcadores moleculares en sangre periférica de pacientes con cáncer no microcítico de pulmón en estadios localmente-avanzado y/o metastásicos no sólo para conocer y poder predecir la evolución y pronóstico de la enfermedad como el análisis del receptor del factor de crecimiento epidérmico (EGFR), el factor de crecimiento del endotelio vascular (VEGF) o la inactivación de p16; sino para adecuar la mejor opción de tratamiento de forma individualizada y conseguir altas tasas de eficacia con mínima toxicidad.

RESUM

El càncer de pulmó és un dels principals problemes de salut del món, és una malaltia freqüent, greu i amb una incidència en augment. A aquestes característiques s'hi afegeix la complexitat del seu tractament -cirurgia, quimioteràpia i radioteràpia-, que a mès d´agressiu i habitualment costós, necessita la col.laboració organitzada de nombrosos professionals. En aquest sentit de l'atenció integral al pacient oncològic, l'aportació de la biologia molecular amb nous conceptes i reptes com la oncofarmacogenètica i oncofarmacogenómica ha permès no només el desenvolupament de fàrmacs utilitzant biomarcadors per predir la resposta, sinó també l'anàlisi del comportament de les cèl.lules tumorals, l'estudi de mutacions de gens, polimorfismes, metilacions, etc., estudis anatomopatològics més precisos i la individualització del tractament gràcies al millor coneixement dels factors pronòstics i sobretot dels factors predictius de resposta.

En el nostre treball hem tractat d'identificar marcadors moleculars en sang perifèrica de pacients amb càncer no microcític de pulmó en estadis localment-avançat i/o metastàtics no només per conèixer i poder predir l'evolució i pronòstic de la malaltia com l'anàlisi d'EGFR, VEGF i inactivació de p16, sinó per a adequar la millor opció de tractament de forma individualitzada i aconseguir altes taxes d'eficàcia i mínima toxicitat.

ABSTRACT

Lung cancer is one of the major health problems in the world, is a common and severe disease, and its incidence is increasing. These features are added to the complexity of its treatment -surgery, chemotherapy and radiotherapy-, which are aggressive, often costly and also require the collaboration of many different organized professionals. In this sense, the contribution of molecular biology with new concepts and challenges such as farmacogenetics and farmacogenomics has allowed not only the development of new drugs using predictive biomarkers, but also the analysis of the behavior of tumor cells, the study of gene mutations, polymorphisms, etc. and so on pathological analysis and individualized treatments through a better knowledge of prognostic factors and especially of the predictors of response.

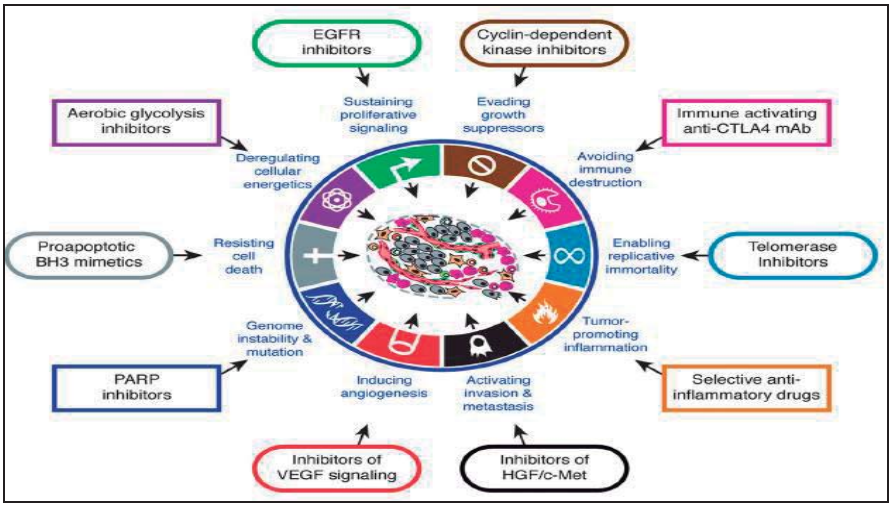
In our work we sought to identify, by pharmacogenomic analysis, molecular markers in peripheral blood of patients with non-small cell lung cancer in locally-advanced stages not only to know and be able to predict the evolution and prognosis of the disease as the analysis of EGFR, VEGF and p16 inactivation, but to bring the best treatment option individually and achieve high success rates and minimum toxicity.

I. INTRODUCCIÓN GENERAL

1. INTRODUCCIÓN

El cáncer es una enfermedad producida por cambios dinámicos en el genoma, basados en mutaciones que inducen un aumento de función de oncogenes o la pérdida de función de genes supresores. Todos los tumores presentan como característica común un proceso de diversas etapas, cada una de ellas gobernada por una alteración genética concreta, que conducen a la transformación de una célula normal en maligna. Dicho proceso se conoce como carcinogénesis, donde diversas alteraciones genéticas que condicionan una ventaja proliferativa a la célula se suceden hasta alcanzar el genotipo de célula maligna (Boveri,T. 2008). Las distintas etapas que debe atravesar una célula normal hasta su transformación en maligna se pueden dividir en: capacidad de proliferar en ausencia de señales mitogénicas; insensibilidad a señales de inhibición de crecimiento; evasión de apoptosis; replicación ilimitada; angiogénesis sostenida; y capacidad de invasión y metástatizar (Firvida,J.L. *et al.* 2009).

Figura 1: Características de la célula tumoral y nuevas opciones terapéuticas antidiana específicas. (Hanahan,D. and Weinberg,R.A. 2011)



2. BIOMARCADORES Y FACTORES PRONÓSTICO EN CANCER

2.1. IMPORTANCIA DE LOS BIOMARCADORES EN CÁNCER

Un biomarcador es cualquier indicador biológico que proviene de un organismo vivo. Los biomarcadores se utilizan principalmente como indicador de la evolución de una enfermedad: aumentan si la enfermedad avanza, se mantienen constantes si se estabiliza y disminuyen si remite (Simon,R. 2009). Estos biomarcadores suelen denominarse parámetros intermedios, farmacodinámicos o indirectos de valoración. En los estudios experimentales, los biomarcadores se utilizan con propósitos muy diversos: para determinar la eficacia de un tratamiento, evaluar la respuesta en función de la dosis e identificar a los candidatos más idóneos para su inclusión en ensayos. A fin de determinar si un criterio intermedio de valoración es un marcador válido del pronóstico clínico, hay que demostrar no sólo que los pacientes con tumores sensibles al tratamiento gozan de una mayor supervivencia, sino que el tratamiento que influye en el valor del biomarcador también lo hace en el pronóstico clínico. Para determinar la solidez de un marcador suele ser necesario analizar diversos ensayos clínicos aleatorizados que indiquen que las diferencias en la variabilidad del biomarcador en el grupo con tratamiento aleatorio coinciden con las diferencias en el pronóstico clínico (Korn,E.L. *et al.* 2005). Estos criterios son rigurosos por el papel primordial que tienen los resultados de los ensayos randomizados en la configuración de modelos de actuación.

Los biomarcadores también pueden utilizarse como indicadores previos al tratamiento con el fin de definir la enfermedad del paciente y determinar si éste necesita recibir un tratamiento específico. La genómica brinda una gran oportunidad para mejorar el tratamiento del cáncer, ya que permite seleccionar la opción terapéutica apropiada para el paciente adecuado. La práctica oncológica actual implica tratar a muchos pacientes que no obtienen ningún beneficio por cada uno que sí lo obtiene. Por ejemplo, los pacientes con un tumor sólido en fase inicial tienen un 80% de posibilidades o más de sobrevivir a largo plazo sin enfermedad recibiendo sólo tratamiento local. Existe un gran interés por encontrar una quimioterapia (QT) que

permita aumentar este porcentaje a un 85%. Esto representaría una reducción relativa del 25% del riesgo de recidivas. No obstante, el 80% de los pacientes no necesita el tratamiento, y esta mejoría absoluta del 5% se consigue en 1 de cada 20 pacientes tratados. Puesto que el cáncer es una enfermedad potencialmente mortal, tratar a 20 pacientes para que uno responda de forma satisfactoria es admisible, siempre y cuando, los beneficios sean considerables. Sin embargo, lo mejor para los pacientes sería que pudiésemos identificar con antelación a aquéllos cuyo pronóstico con sólo tratamiento local fuese lo suficientemente bueno como para prescindir del tratamiento complementario. En relación con los pacientes cuyo pronóstico con tratamiento local no es tan satisfactorio, también resultaría útil poder distinguir con precisión a aquellos que es probable que se beneficien de un régimen de QT específico de los que probablemente no se beneficiarán. Esta evaluación de un tratamiento específico suele denominarse biomarcador predictivo y se reserva para designar las pruebas orientadas a predecir la eficacia de un tratamiento específico. En ocasiones, el biomarcador predictivo puede ser la cantidad de una sustancia génica o proteica relacionada con la diana de un fármaco. En el caso de un anticuerpo monoclonal, la expresión del ligando en las células tumorales es una opción habitual. La expresión del ligando suele ser una condición necesaria, aunque insuficiente, para determinar la eficacia antineoplásica del anticuerpo. En algunos casos, la sobreexpresión del receptor puede estimular el crecimiento tumoral, mientras que en otros puede ser un efecto secundario. Si un cambio genómico da lugar a una alteración del metabolismo de la diana molecular, es más probable que la diana molecular tenga relevancia biológica para la invasión tumoral (Weinstein,I.B. and Joe,A.K. 2006). Las moléculas pequeñas, como los inhibidores de las tirosina quinasas (ITKs), a menudo se dirigen hacia distintas dianas, por lo que en el momento de diseñar un biomarcador predictivo útil será relevante saber cuáles son las dianas mutadas en el tumor de un paciente.

2.2. UTILIDAD DE LOS BIOMARCADORES EN CÁNCER

En oncología clínica se necesitan biomarcadores para realizar el diagnóstico precoz, predecir la respuesta al tratamiento y valorar el pronóstico del paciente. Estos biomarcadores, además, tienen que ser fáciles de utilizar para minimizar el uso de procedimientos cruentos y aumentar la conformidad de los pacientes. Para el diagnóstico precoz, los biomarcadores deben estar presentes en sangre, orina o heces, y tienen que poseer alta sensibilidad y especificidad para identificar a los pacientes con cáncer incipiente y realizar el diagnóstico diferencial de posibles enfermedades inflamatorias crónicas o de otro tipo, y así descartar posibles neoplasias. Una vez diagnosticado el tumor, los demás marcadores deberían permitirnos valorar el pronóstico del paciente para, de este modo, seleccionar la pauta terapéutica idónea, que habrá que individualizar en función de cada paciente y de cada tumor. En última instancia, la principal misión de los biomarcadores en oncología es la de ayudar a individualizar el diagnóstico y el tratamiento, y personalizar este último en función de cada paciente.

2.3. FACTORES PRONÓSTICOS EN CÁNCER

Por lo general, los pacientes oncológicos sufren una enfermedad sintomática que se diagnostica por la clínica y se confirma mediante histología. Para elegir el tratamiento y establecer el pronóstico del paciente se parte del estadio del tumor. En términos generales, el análisis histológico convencional y el estadiaje del tumor se realizan atendiendo a las categorías TNM de la Unión Internacional Contra el Cáncer (UICC) (UICC, 1988; Mountain,C.F. 1997; Mountain,C.F. 2002). Ahora bien, la valoración del pronóstico con estas categorías presenta limitaciones. En pacientes con tipos y estadios tumorales idénticos, la evolución de la neoplasia y la supervivencia a menudo varían de forma muy significativa. Pese a que se han estudiado una serie de marcadores pronósticos moleculares teóricamente mejores que los sistemas de estadificación histoanatómicos convencionales (Ahmed,F.E. 2005), apenas existen

datos sobre la integración de tales marcadores en la toma de decisiones clínicas todavía.

Clásicamente, se conocen como factores pronósticos (FP) a aquellos datos que nos proporcionan información a cerca de la evolución de la enfermedad. Son un conjunto de síntomas, signos, hechos o fechas que informan sobre el posible futuro de un enfermo, la supervivencia global (SG), la respuesta terapéutica, el intervalo libre de recaída o una complicación (González Barón,M. 2005). Las ventajas de conocer los FP son muchas, ya que no sólo somos capaces de predecir el futuro evolutivo de la enfermedad sino que, conociendo esa probabilidad, podremos: informar al paciente y familiares; comprender y vislumbrar hechos de la fisiopatología y patocronia de la enfermedad; comparar la eficacia de tratamientos al agrupar a los pacientes con pronósticos semejantes; diseñar ensayos clínicos definiendo criterios de inclusión que estratifiquen a los pacientes y los distribuyan equitativamente, etc.; e ir seleccionando subgrupos de pacientes para aplicar los tratamientos de forma ajustada a esos FP (George,S.L. 1998). Hay numerosos FP, algunos relacionados con el propio tumor (como localización anatómica, el estadio tumoral, nivel de diseminación, marcadores biológicos de diferenciación, receptores hormonales, número de mitosis, marcadores de proliferación, oncoproteínas, contenido de ácido desoxirribonucleico (DNA), cariotipo, protooncogenes, genes supresores, marcadores moleculares, receptores de membrana, fenotipos moleculares), otros pertenecen a características propias del paciente (la edad, el sexo, el estado general, el estado inmunitario, el estado psicológico y los relacionados con la sintomatología) y en otras ocasiones pueden depender del entorno, circunstancias sociales del paciente o terapéutica empleada (sensibilidad al tratamiento, tipo de cirugía, QT, dosificación, reducciones por toxicidad, tratamiento secuencial o concomitante, tiempos terapéuticos adecuados, o retrasos por neutropenia como analizaremos con detalle en uno de nuestros trabajos).

2.4. NUEVOS FACTORES PRONÓSTICOS MOLECULARES EN CÁNCER

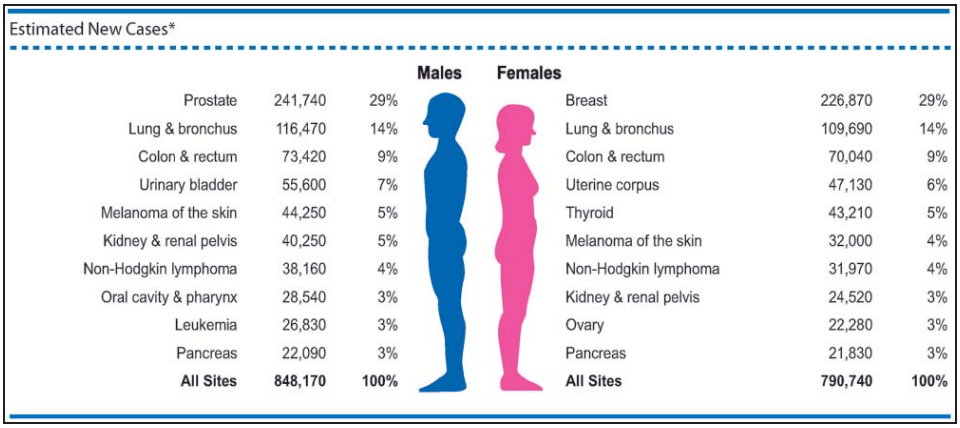
Existe gran expectación alrededor de la identificación de marcadores genéticos que puedan utilizarse como factores predictivos de respuesta a terapias o indicadores del curso clínico de la enfermedad, dentro de aquellos tumores con histologías homogéneas y con comportamientos muy diferentes. Gran parte de este interés está asociado al uso de nuevas terapias biológicas contra dianas moleculares, y se basa en la identificación de la población candidata a ser tratada y a identificar con exactitud aquellos pacientes que más se beneficien de la misma. De esta forma, amplificaciones de oncogenes o de genes implicados en el ciclo celular tienen valor pronóstico en algunos tipos de tumores, como HER-2/neu en las neoplasias de mama, la amplificación de N-myc en los neuroblastomas, deleciones de 13q o 17p13 en el mieloma múltiple, mutaciones puntuales de PI3K, del receptor def factor de crecimiento epidérmico (EGFR), KIT o BRAF, reordenamientos de BRC-ABL u otras ligadas a determinados tipos tumorales, como RET y PAX8 en tumores de tiroides (Maruvada,P. *et al.* 2005; Costa,J. 2006; Wu,M. and Merajver,S.D. 2005).

3. CÁNCER NO MICROCÍTICO DE PULMÓN

3.1. FACTORES EPIDEMIOLÓGICOS Y DE RIESGO

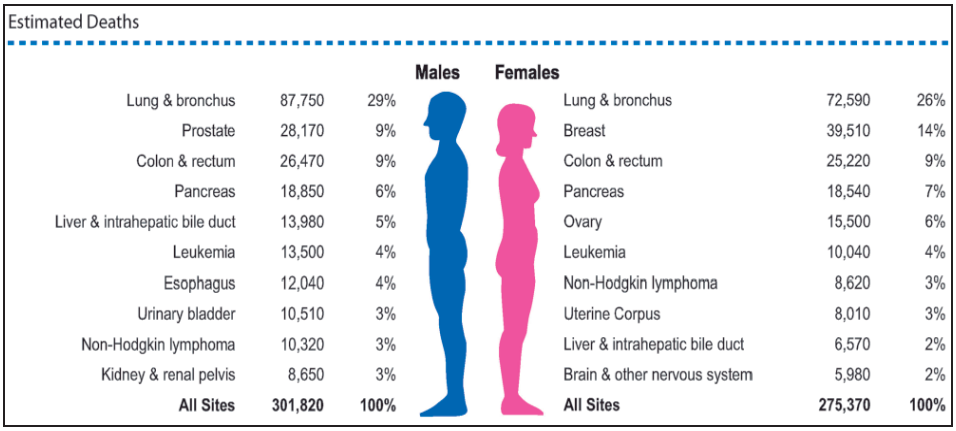
El cáncer de pulmón (CP) es uno de los cánceres con mayor incidencia siendo responsable de la primera causa de mortalidad por cáncer en el varón y la tercera después del colon y la mama en la mujer (Jemal,A. *et al.* 2007; Alberg,A.J. *et al.* 2005). En España se registran unos 18500 casos nuevos al año de CP. La razón varón:mujer es de 4,5 en Europa y de 11 en España, lo que refleja el retraso en la adquisición del hábito tabáquico y el menor riesgo laboral de las mujeres españolas. En términos de prevalencia parcial, la incidencia de los últimos años se ha traducido en 24000 casos prevalentes en España. La escasa diferencia entre el número de casos prevalentes e incidentes refleja la alta letalidad de este tumor (López-Albente,G. *et al.* 2003).

Figura 2. Incidencia estimada de Cáncer en EEUU para 2012.



Adaptado de Siegel,R. *et al* (2012) Cancer statistics, 2012. CA Cancer J Clin.62:10-29

Figura 3. Mortalidad estimada por Cáncer en EEUU para 2012.



Adaptado de Siegel,R. *et al* (2012) Cancer statistics, 2012. CA Cancer J Clin.62:10-29

El CP en la mujer tiene algunas características biológicas propias (Thomas,L. *et al.* 2005): el adenocarcinoma es el tipo histológico más frecuente, tanto en fumadoras como en no fumadoras; y la frecuencia de mutaciones del EGFR es mucho mayor lo que traduce un tipo de CP con un comportamiento biológico diferente y con

posibilidades de tratamiento con nuevos agentes dirigidos frente al EGFR con mejor supervivencia. Este fenómeno se ha intentado explicar por varias hipótesis: variaciones en polimorfismos de genes relacionados con el efecto carcinógeno del tabaco (Wei,Q. *et al.* 2000) y los estrógenos circulantes, y la expresión de receptores α y β pueden tener influencia en el desarrollo de adenocarcinomas.

La edad mediana de presentación del CP es de 69 años en varones y 67 en las mujeres. Más del 50% de los casos se diagnostican por encima de los 65 años y más del 30% por encima de los 70 (Wingo,P.A. *et al*. 2003).

En 1950 Doll y Hill demostraron la clara asociación epidemiológica entre fumar y la mortalidad por CP (Doll,R. and Hill,A.B. 1950). El riesgo relativo de CP de un fumador de larga evolución frente a un no fumador varía entre 10 y 30 veces, mientras que el riesgo acumulado entre grandes fumadores puede alcanzar el 30% frente al 1% de los no fumadores (Mattson,M.E. *et al.* 1987). Además el riesgo de carcinoma broncogénico es proporcional al tiempo total de consumo de tabaco y se incrementa tanto con el número de cigarrillos fumados al día como por la duración del hábito tabáquico (Ginsberg,R.J. *et al.* 1993). Como parece lógico, continuar fumando aumenta el riesgo frente a los que abandonan el tabaco y esta reducción del riesgo se hace evidente según estudios de cohortes en los exfumadores de más de 15 años entre un 80-90% respecto a los fumadores activos (United States Department of Health and Human Services (USDHHS) 1990). La exposición ambiental al tabaco, fumadores pasivos, también es causa de un aumento del riesgo de desarrollar CP, aunque parece que este riesgo va asociado a un factor de susceptibilidad individual (Hackshaw,A.K. *et al*. 1997). *Otros factores* implicados son los carcinógenos medioambientales como el gas radón, arsénico, éter bisclorometil, cromo hexavalente, gas mostaza, hidrocarburos policíclicos, etc. (Fraumeni,J.F. and Blot,W.T. 1982); la dieta y nutrición; factores familiares (Li,X. and Hemminki,K. 2005); la fibrosis pulmonar difusa (Hubbard ,R. et al. 2000) y la asbestosis (Hughes,J.M. and Weill,H. 1991).

3.2. PATOLOGÍA DEL CÁNCER NO MICROCÍTICO DE PULMÓN

Entre las diversas clasificaciones nosológicas del CP, la clasificación anatomopatológica más utilizada es la de la Organización Mundial de la Salud (OMS), actualizada en el año 2004 (Travis,W.D. *et al.* 2004). El carcinoma epidermoide de pulmón es el más frecuente, ya que supone el 44% de los carcinomas en el varón y el 25% en la mujer. El adenocarcinoma, el segundo más frecuente, corresponde al 28% de los casos en el varón y el 42% en la mujer; habiéndose observado en los últimos años un aumento en su incidencia. Los restantes tipos histológicos aparecen con una frecuencia muy baja (Parkin,D.M. *et al.* 2002).

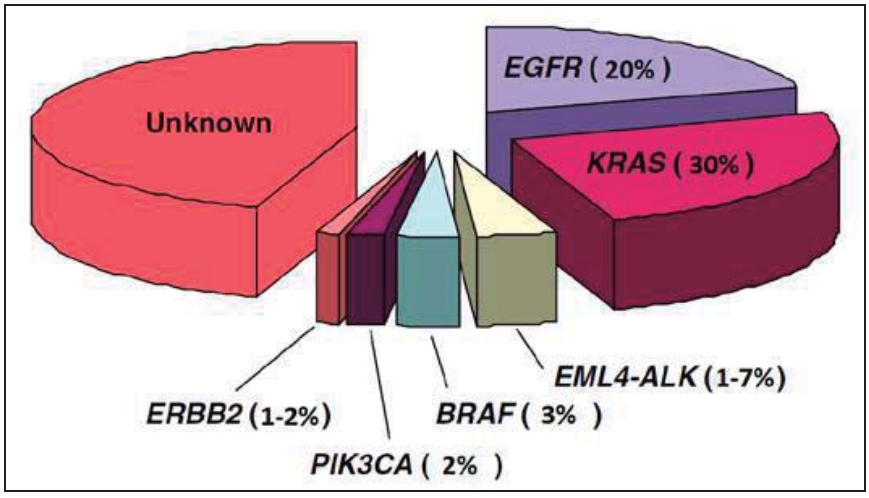
Los marcadores por inmunohistoquímia (IHQ) más utilizados son las citoqueratinas (CK), la apoproteína A del surfactante (SP-A) y el factor de transcripción de tiroides (TTF-1). Las CK están presentes en todos los carcinomas, aunque su expresión disminuye conforme se produce desdiferenciación del tumor. El TTF-1 se expresa normalmente en el tejido pulmonar y se está mostrando como uno de los marcadores más útiles para diagnosticar un CP y diferenciarlo de las metástasis. La SP-A se expresa en cerca de la mitad de los adenocarcinomas, y de forma más variable en los otros tipos de carcinoma. A pesar de ello, puede haber un gran solapamiento de los perfiles antigénicos entre los diferentes tipos histológicos que en ocasiones hace difícil el diagnóstico patológico.

3.3. NUEVA CLASIFICACION MOLECULAR DEL CÁNCER NO MICROCÍTICO DE PULMÓN

El estudio de las alteraciones moleculares en CP contribuye a conocer mejor el proceso de carcinogénesis, ya que la heterogeneidad histológica y biológica son fenómenos bien conocidos en Cáncer No Microcítico de Pulmón (CNMP). La generalización de técnicas de biología molecular aplicadas al conocimiento de las neoplasias permite identificar mutaciones relevantes clínicamente que mejoran la comprensión de la patogénesis del CP. La nueva clasificación molecular del CNMP conlleva una revolución hacia el tratamiento personalizado (Hirsch,F.R. *et al.,* 2010; Ladanyi,M. 2008; Pao,W. and Girard,N. 2011). Concretamente, la heterogeneidad

clínica, radiológica e histopatológica del adenocarcinoma de pulmón hace ya imprescindible obtener pruebas moleculares para clasificar y seleccionar a los pacientes candidatos a determinadas terapias según su perfil molecular. En la actualidad, el análisis mutacional del gen del EGFR debe ser la norma principal para la selección de pacientes que puedan ser candidatos a recibir tratamiento con ITKs de EGFR ya que representa la prueba disponible más exacta para la predicción de respuesta. Pero además, se han descrito nuevas mutaciones que tienen cierto impacto sobre la selección de tratamiento, por lo que la rutina del diagnóstico molecular en CP se ampliará más allá del perfil mutacional de EGFR (Figura 4) (Dacic,S. 2011;Ladanyi,M and Pao,W. 2008;Pao,W. and Girard,N. 2011). Por todo ello, en CP es ya una necesidad la integración del diagnóstico molecular en el flujo de trabajo de los laboratorios de patología; la disponibilidad de muestra biológica en cantidad y calidad suficiente; así como el desarrollo de técnicas estandarizadas, de alto rendimiento y alta sensibilidad, que permitan llevar a cabo las técnicas histológicas y moleculares requeridas (Dacic,S. 2011;Ladanyi,M. 2008).

Figura 4. Clasificación molecular del Adenocarcinoma de Pulmón.



4. FACTORES PRONÓSTICO EN CÁNCER DE PULMÓN

Es crucial definir factores capaces de predecir la evolución del CP o la eficacia de las diversas estrategias terapéuticas para conseguir un tratamiento individualizado para cada paciente (Redondo,A. *et al.* 2005). La detección de estos FP es muy difícil, dada la enorme heterogeneidad entre los distintos tipos tumorales que se encuadran dentro del término CP. No obstante, hay algunos FP de gran peso que se mantienen en los diversos trabajos y que han sido reconocidos por los principales grupos de estudio como la International Association for the Study of Lung Cancer (IASLC) (Feld,R. *et al.* 1997) y la American Society of Clinical Oncology (ASCO) (Pfister,D.G. *et al*. 2004) como son: el estadio, el estado general del paciente, la pérdida de peso, el sexo y la histología.

4.1. FACTORES PRONÓSTICO DEPENDIENTES DEL PACIENTE

Los factores clínicos más importantes asociados con el pronóstico en el CP son el estado general del paciente y la pérdida de peso, sin olvidar la edad, el sexo y las comorbilidades asociadas.

El *estado general* es quizás el FP más importante en la evolución de la enfermedad. Puede ser medido mediante las escalas de valoración de Karnofsky y del Eastern Cooperative Oncology Group (ECOG), que se correlacionan con la supervivencia. El estado general puede dar mucha información sobre la posible efectividad y tolerancia al tratamiento oncológico. En diferentes revisiones de los grupos cooperativos norteamericanos, la supervivencia ha sido inferior en pacientes con peor estado general. Por ello, en la práctica clínica y en muchos ensayos clínicos, los pacientes con mal estado general, definido como un ECOG igual o superior a 2, son excluidos de los tratamientos establecidos para pacientes con mejor situación general (Kelly,K. 2004).

La *pérdida de peso* en los 6 meses previos al diagnóstico es un FP independiente de supervivencia en muchas series, siendo fundamental a la hora de

decidir planteamientos terapéuticos, sobre todo cuando se van a aplicar tratamientos combinados de forma concomitante pues la pérdida de peso no sólo influye en la supervivencia sino también en el grado de toxicidad de los tratamientos (Pfister,D.G. *et al.* 2004).

La *edad* como FP en el CP es un tema controvertido y parece claro que la edad biológica no tiene peso como FP independiente, pero lo que es innegable es que la edad avanzada se asocia con mayor comorbilidad*,* lo que condiciona los resultados del tratamiento tanto en estadios precoces como avanzados (Gridelli,C. *et al.* 2005).

En *mujeres*, el CP tiene un comportamiento biológico intrínsecamente diferente y presenta algunas características especiales, como ya hemos visto, y sabemos que la histología más frecuente es la de adenocarcinoma, incluso en mujeres muy fumadoras; y que el riesgo de desarrollar CP en mujeres no fumadoras es 2.5 veces superior a los varones. Es más, en las mujeres la tasa de supervivencia a los 5 años es del 15,6% frente al 12,4% en varones y cuando se analiza por estadios, también la supervivencia es superior. Del mismo modo, la supervivencia de las mujeres es superior cuando se comparan series de pacientes sometidos a resección completa del tumor o a radioterapia (RT) o a QT (Visbal,A.L. *et al*. 2004). Para intentar explicar estas diferencias se han realizado múltiples estudios que han encontrado diferencias en los polimorfismos de genes relacionados con el efecto carcinógeno del tabaco y que demuestran que en las mujeres hay una mayor predisposición a los efectos carcinógenos incluso cuando el nivel de exposición es menor. Además, la frecuencia de mutaciones en el gen del EGFR es mucho mayor en mujeres, lo que tiene implicaciones terapéuticas importantes con los nuevos tratamientos con ITKs del EGFR (Paez,J.G. *et al.* 2004).

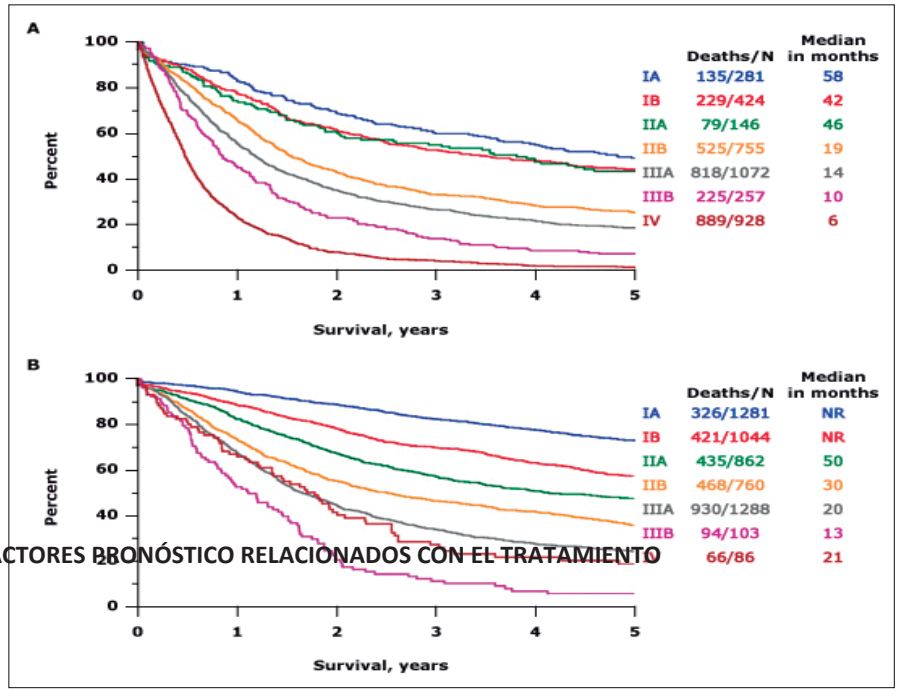
4.2. FACTORES PRONÓSTICO DEPENDIENTES DE LA NEOPLASIA

El estadio TNM es el FP más importante, de manera que la supervivencia a 5 años disminuye progresivamente desde 58 meses para el estadio IA a 4-6 meses para el estadio IV. La *figura 5A* muestra la supervivencia según el estadio clínico y la *figura*

5B según el estadio patológico de acuerdo a la 7ª edición del TNM (UICC, 1988; Mountain,C.F. 2002).

Se ha especulado que las *variantes histológicas* del CP pueden conferir una evolución diferente de la enfermedad y, en concreto, se ha asociado el carcinoma de célula grande con un peor pronóstico. No obstante, otras revisiones no han evidenciado una asociación con los tipos histológicos. Por otra parte, hay rasgos patológicos que se han relacionado con el pronóstico como la presencia de necrosis, el alto índice mitótico, un elevado pleomorfismo celular, una escasa diferenciación histológica o la infiltración de la pleura que confieren un peor pronóstico, mientras que el patrón ocupante de espacio alveolar parece tener una evolución más favorable. En los últimos años, se ha observado un crecimiento significativo de la incidencia de adenocarcinomas de pulmón en mujeres que nunca habían fumado. Este hecho ha adquirido especial relevancia cuando se ha demostrado la alta eficacia de los agentes inhibidores de EGFR en este subgrupo tumoral.

Figura 5. Supervivencia a 5 años en CNMP por estadios



4.3. FACTORES PRONÓSTICO RELACIONADOS CON EL TRATAMIENTO

Entre los FP relacionados con el tratamiento quirúrgico se considera que la resección mínima necesaria en el grupo de pacientes con estadios precoces es la lobectomía, excepto en pacientes que no puedan tolerar esta cirugía, ya que la aplicación de técnicas más conservadoras está ligada a una supervivencia menor (Ginsberg,R. *et al*. 1995). De forma inversa, en estadios avanzados, la obtención de una resección completa tras un tratamiento de inducción es uno de los principales factores para un pronóstico favorable (Eberhardt,W. *et al.* 1998). En cuanto a la RT, se conoce su efecto deletéreo para la supervivencia cuando se administra de forma postoperatoria en estadios I y II; no así, administrada en estadios avanzados (PORT Meta-analysis Trialists Group. 1998). Por último, es indudable el beneficio de la QT en supervivencia y calidad de vida (QoL) en los pacientes con enfermedad avanzada metastásica (NSCLC Collaborative Group. 1995).

4.4. FACTORES MOLECULARES CON VALOR PRONÓSTICO

Múltiples alteraciones genéticas han sido relacionadas con el desarrollo y evolución del CP, pero probablemente ninguna de ellas pueda ser considerada un FP determinante de esta neoplasia. Así se han descrito asociados al CNMP: aumento del número de copias de regiones cromosómicas como 1p, 1q, 3q,5p, 6p, 8q, 12, 17q, 19p, 19q, 20p, 20q y X, lo que sugiere que estas regiones son potenciales localizaciones de oncogenes; se han observado pérdidas de material genético en regiones como 2q, 3p, 4p, 8p, 9p, 10p, 11p, 11q, 13q y 17p, lo que a su vez sugiere que en ellas hay posiciones teóricas de genes supresores de tumores relevantes en la biología del CP; y relacionado con la progresión del tumor la sobreexpresión de genes como EGFR, ciclina D1, c-myc, Ras o bcl-2; y la inhibición de p53, p16, pRb, FHIT, PTEN, BAP1 o MKP (Kaminski,N. and Krupsky,M. 2004).A continuación, repasaremos la más relevantes.

4.4.1. MUTACIONES DE KRAS

Kras es un gen regulador de señales de transducción y de proliferación celular localizado en el cromosoma 12p12.1. Se activa mediante mutaciones puntuales en los codones 12, 13 y 61 sobretodo, siendo las más frecuentes transversiones G-T en el codón 12. El gen codifica para una proteína que se ancla en la cara interna de la membrana citoplasmática y que se activa en presencia de estímulos externos; su misión es transmitir la señal mitogéna del exterior al interior celular. Ras activado se une a la proteína raf-quinasa, que a su vez activa otra cadena de proteínas que se unen al DNA actuando como factores de transcripción que activan la división celular. Cuando se produce una mutación en el codón 12 de Ras se cambia la estructura de la proteína de forma que no responde a estímulos reguladores y permanece siempre activado, lo que genera proliferación celular constante.

Se observa en un 20-30% de todos los pacientes diagnosticados de CP, casi exclusiva de adenocarcinomas y se relaciona con el tabaco (Finberg,K.E. *et al.* 2007). Implicado en la señalización desde el EGFR hacia la vía MAPK, se traduce en un aumento en la proliferación celular y se correlaciona con peor supervivencia independientemente del tratamiento recibido (Rosell,R. *et al.* 1993). Además, es predictivo de ausencia de beneficio al tratamiento con cisplatino/vinorelbina o con ITKs antiEGFR.

4.4.2. MUTACIONES DEL RECEPTOR DEL FACTOR DE CRECIMIENTO EPIDÉRMICO

El EGFR ha sido reconocido como modulador clave de las funciones de las células tumorales, es una glicoproteína transmembrana ubicua compuesta por un dominio extracelular amino-terminal para la unión de ligando, una hélice transmembrana hidrófoba y un dominio citoplasmático que contiene el dominio de la tirosina quinasa y una región carboxiterminal que contiene residuos de tirosina y elementos reguladores del receptor. El EGFR puede ser activado por diversos ligandos como el factor de crecimiento epidérmico (EGF), el factor alfa transformante de

crecimiento (TGFa), la anfiregulina y la betacelulina. La unión de ligandos al dominio extracelular da lugar a la oligomerización del receptor que activa la tirosina quinasa del receptor y origina la autofosforilación de ambos dominios del receptor. Estas tirosinas fosforiladas sirven como sitios de unión para diferentes moléculas transductoras de señales citoplasmáticas. Se inicia así una cascada de acontecimientos intracelulares que conduce a la proliferación celular, protección frente a apoptosis, mayor supervivencia y transcripción génica. Una excesiva señalización de EGFR a nivel tumoral suele ser consecuencia de una sobreexpresión y/o una excesiva producción y disponibilidad de ligandos, lo que conlleva proliferación incontrolada, invasión y metástasis y aumento de la supervivencia celular.

Las mutaciones aparecen en el 10-15% de los pacientes caucásicos y en un 30-40% de los asiáticos. También son mayores en no fumadores, adenocarcinoma y mujeres. Aproximadamente, el 90% de las mutaciones de EGFR aparecen en los exones 19 y 21 en la región que codifica el dominio tirosina quinasa (Jänne,P.A. *et al.* 2005; Johnson,B.E. and Jänne,P.A. 2005). La más común es una deleción del exón 19, y en segundo lugar una mutación puntual del exón 21, que da como resultado un cambio de una leucina por una arginina en el codón 858 (L858R). Se han identificado varias líneas celulares con mutaciones de EGFR que son sensibles a erlotinib y gefitinib: PC-9, HCC-827, NCI-H1650, NCI-H1975, NCIH3255 y DFCILU-011. La fosforilación de EGFR en estas líneas celulares con mutación de EGFR se inhibe con bajas concentraciones de erlotinib y gefitinib. Las mutaciones de EGFR se traducen en cambios conformacionales que llevan a un incremento en la sensibilidad a ITKs.

El 80% de los pacientes con la mutación responden a ITK antiEGFR, mientras que un 10% de los pacientes sin mutación lo hacen (Rosell,R. *et al.* 2006). No existe correlación entre las mutaciones y el número de copias o la expresión por IHQ (Sequist,L.V. *et al.* 2007). En el estudio BR.21, pacientes con CP en segunda y tercera línea de tratamiento se aleatorizaron a recibir erlotinib frente a placebo, y tanto la respuesta, la supervivencia libre de progresión (SLP) como la SG fue superior para los pacientes que recibieron erlotinib comparado con placebo. Se realizó un análisis

multivariado en el que la histología de adenocarcinoma, no haber fumado nunca y la expresión de EGFR por IHQ se asoció con respuesta a erlotinib (Shepherd,F.A. *et al.* 2005). En el año 2004, dos estudios publicados simultáneamente analizaron mutaciones del gen EGFR en pacientes con respuestas relevantes a gefitinib (Paez,J.G. *et al* 2004; Lynch,T.J. *et al.* 2004). Trece de los 14 pacientes con respuesta tenían mutaciones somáticas en el dominio tirosina quinasa del EGFR, mientras que ninguno de los 11 pacientes que progresaron a gefitinib tenía mutaciones.

A pesar del éxito inicial del tratamiento con ITKs en pacientes con mutaciones de EGFR, la mayoría progresarán a dicho tratamiento. Los mecanismos de esta resistencia adquirida son de un gran interés clínico. Se ha encontrado una mutación secundaria, una sustitución de una metionina por treonina en la posición 790 (T790M) en pacientes en los que se ha realizado una nueva biopsia en el momento de la progresión (Kobayashi,S. *et al*. 2005). Estos hallazgos llevarán al desarrollo de nuevos fármacos activos contra este mecanismo de resistencia adquirido como HKI-272, que es un inhibidor irreversible de EGFR.

En España, desde abril de 2005, se ha analizado la mutación de EGFR en más de 1.600 pacientes a partir de los bloques de parafina, realizándose una microdisección láser del área tumoral, y luego determinándose la mutación por reacción en cadena de la polimerasa (PCR) y secuenciación posterior, siendo el porcentaje de enfermos portadores de mutaciones del 14,4%. La media de edad de los pacientes mutados es de 65 años, y un 67% son no fumadores. En el subgrupo de pacientes con mutaciones de EGFR que recibieron erlotinib como tratamiento de primera línea el porcentaje de respuestas ha sido del 85% (Paz-Ares ,L. *et al.* 2006).

4.4.3. EXPRESIÓN DEL ERCC1

La expresión del RNA mensajero (mRNA) de ERCC1 se ha asociado con un fenotipo platinorresistente. El cisplatino es uno de los fármacos fundamentales en los esquemas de QT utilizados en CP. Su actividad citotóxica se basa principalmente en la

formación de aductos mono/bifuncionales en el DNA. Aunque la resistencia a cisplatino es multifactorial, algunos mecanismos incluyen la disminución de la acumulación intracelular, el aumento de la eliminación y una capacidad de reparación del DNA altamente eficiente (Zeng-Rong,N. *et al.* 1995). Los sistemas de reparación de escision de nucleotidos (NER) y de bases nitrogenadas (BER) son procesos enzimáticos complejos relacionados con la reparación del daño no específico del DNA, incluyendo la radiación γ y ultravioleta y la formación de aductos. La enzima ERCC1 es un gen de reparación de 15 kb localizado en el cromosoma 19 y tiene un papel fundamental en el sistema NER; forma un heterodímero con XPF, y el complejo ERCC1/ XPF, como unidad, es el responsable de la incisión 5' en el sitio del daño del ADN. La hipótesis es que, en las células tumorales tratadas con cisplatino, la disminución en la función de NER/BER conlleva un superior daño celular y, por lo tanto, una superior muerte celular (Rosell,R. *et al.* 2003).

Se han realizado estudios clínicos correlacionando ERCC1y resistencia a compuestos de cisplatino, la mayoría por análisis de RNA o DNA. En un primer estudio en CP resecable, el estudio IALT Bio, la determinación de ERCC1 sugiere que aquellos pacientes sin expresión de ERCC1 tienen una supervivencia inferior si se tratan con cirugía exclusiva, pero se benefician de la QT adyuvante. Contrariamente, los pacientes con expresión de ERCC1 pueden tener una mayor supervivencia cuando se tratan con cirugía exclusiva, pero son refractarios a QT adyuvante basada en cisplatino. Sin embargo, los resultados del estudio IALT Bio no significan que ERCC1 sea un marcador de resistencia para todos los tipos de QT (Cecere,F. *et al.* 2006). En el primer estudio prospectivo aleatorizado testando el concepto del tratamiento individualizado en CP, se ha analizado el valor de ERCC1 en la predicción de la respuesta a la QT. 444 pacientes con CP estadio IV se incluyeron y se aleatorizaron al brazo control que recibieron cisplatino/docetaxel o al brazo genotípico, de manera que los pacientes con niveles bajos de ERCC1 recibieron cisplatino/docetaxel, y aquellos con niveles altos recibieron docetaxel/gemcitabina. De los 346 pacientes evaluables no se encontraron diferencias de supervivencia entre los brazos probablemente debido a que las

muestras obtenidas por una punción o por biopsia de broncoscopio son limitadas para establecer un tratamiento individualizado en función de un marcador genético (Cobo,M. *et al.* 2007).

Los niveles altos de ERCC1, pues, son FP de supervivencia comparado con niveles bajos ya que la sobreexpresión de ERCC1 se ha correlacionado con alta capacidad de reparar el daño en el DNA independientemente del tratamiento recibido (Lord,R.V. *et al.* 2002). Es más los niveles de mRNA de ERCC1 pueden ser también pronósticos en ausencia de tratamiento quimioterápico. Simon y colaboradores evaluaron el efecto de los niveles de expresión de ERCC1 en la supervivencia analizando tejido congelado de 51 pacientes con CP operados observándose que mayores niveles de expresión de ERCC1 son factor predictivo independiente de mejoría de supervivencia probablemente debido a que ERCC1 repara también el daño del DNA de las células influyendo en la evolución del tumor (Simon,G.R. *et al.* 2005).

Finalmente ERCC1 puede ser un marcador útil para distinguir aquellos pacientes que se van a beneficiar del tratamiento de QT adyuvante de los que no. Olaussen y su equipo realizaron un análisis IHQ para determinar la expresión de la proteína ERCC1 en muestras de pacientes operados y demostraron un beneficio absoluto en la supervivencia a 5 años del 4,1% entre los pacientes aleatorizados a QT adyuvante basada en cisplatino. La QT adyuvante, al compararlo con observación, prolongó de forma significativa la supervivencia entre los pacientes con ERCC1 negativo, pero no entre los pacientes con tumores ERCC1 positivo (Olaussen,K.A. *et al*. 2006).

4.4.4. SOBREEXPRESIÓN DEL FACTOR DE CRECIMIENTO DEL ENDOTELIO VASCULAR

Numerosos estudios han investigado la relación entre los niveles de diversos marcadores angiogénicos en relación con características clínico-patológicas y/o supervivencia de los pacientes con cáncer. En 1996 se publicó el primer trabajo que estudió la expresión del factor de crecimiento del endotelio vascular (VEGF) en CP, en

el que se encontraba una asociación con la microdensidad de vasos (Mattern,J. *et al.*, 1996). Durante el año siguiente, se demostró que la mayor expresión de VEGF en CP se relacionaba con un peor pronóstico clínico (Mattern,J. *et al.*, 1997;Volm,M. *et al.*, 1997). Desde entonces, numerosos estudios en CNMP han analizado la expresión de VEGF asociados al pronóstico, mediante diferentes técnicas como IHQ, PCR cuantitativa a tiempo real (RT-PCR) o inmunoensayos (Bremnes,R.M. *et al.,* 2006; Salgia,R. 2011). La expresión o sobreexpresión de VEGF ha sido reportada en el 60% de los CNMP y la mayoría de estudios indican que hay una correlación positiva entre la presencia de VEGF y la vascularización del tumor (Zhu,C.Q. *et al.*, 2006). Además, en la gran mayoría de trabajos se ha encontrado una asociación con la progresión o la menor supervivencia de los pacientes (Bremnes,R.M, *et al.,* 2006; Salgia,R.2011), mientras que algunos estudios no han encontrado ninguna asociación (Baillie,R. *et al.,* 2001; Liao,M. *et al.,* 2001; Yano,T. *et al.,* 2000). Si bien, aunque otros miembros de la familia también han sido estudiados, su relevancia es más controvertida, ya que de todas las moléculas examinadas como biomarcaroders en CNMP, VEGF es el que se ha relacionado más consistentemente con la evolución de los pacientes (Salgia,R. 2011). Asimismo, según un metaanálisis de 2009, la sobreexpresión de VEGF tiene un impacto negativo en la supervivencia, con peor pronóstico para el subtipo adenocarcinoma (Zhan,P. *et al.*, 2009). No obstante, una isoforma de VEGF identificada recientemente ($VEGF_{165b}$) podría relacionarse con propiedades antiangiogénicas (Bates,D.O. *et al.*, 2002), lo cual necesita ser investigado más a fondo.

4.4.5. OTROS FACTORES MOLECULARES CON IMPLICACIÓN PRONÓSTICA

p53, gen supresor de tumores definido como el guardián del genoma, que actúa como temporizador del inicio de la apoptosis, aparecerá mutado en más del 50% de los casos de CP, la mayor parte en los exones 5 y 8, está relacionada con el tabaco y alcohol y suele asociarse con pacientes jóvenes en todas las histologías excepto en la variante bronquioloalveolar de adenocarcinoma. El 70-80% de las mutaciones son *missense* que prolongan la vida media de la proteína y pueden ser detectadas por IHQ;

el resto 20-30% son mutaciones *nonsense*, deleciones, inserciones o errores de *splicing* que no pueden detectarse por IHQ (Mitsudomi,T. et al. 2000).

El **nivel de expresión del gen RRM1** de la ribonucleótido reductasa se asocia en pacientes con CP resecado con mayor supervivencia, es un gen crucial para determinar el fenotipo tumoral, pues induce la expresión de PTEN e inhibe la migración celular, la invasión y la formación de metástasis. Sin embargo, en pacientes con enfermedad avanzada tratados con gemcitabina y cisplatino se ha visto peor supervivencia si la expresión de RRM1 es alta, probablemente por disminución de la eficacia del tratamiento quimioterápico (Rosell,R. *et al.* 2004).

Otros receptores de membrana activados en CP, aunque con menor frecuencia y preferentemente en adenocarcinomas, son CMET y ALK. **EML4-ALK** es un gen resultante de la fusión de *echinoderm microtubule-associated protein-like 4 gene (EML4)* con el receptor tirosina quinasa de *anaplastia lymphoma kinase gene (ALK)*. Este gen de fusión EML4-ALK se ha identificado en un 3-7% de los CP, siendo más frecuente en adenocarcinomas, pacientes no fumadores o que han fumado poco y jóvenes. Generalmente, es excluyente de mutaciones del EGFR y Ras. En un ensayo fase II con crizotinib presentado por Kwak y colaboradores en 2010 un 90% presentaron reducción tumoral, con respuestas mantenidas más de 15 meses y una SLP a los 6 meses del 72%. Por lo que se considera un factor predictivo de respuesta a los inhibidores de ALK, mientras que ninguno de los pacientes que lo presentan responden a los inhibidores de EGFR (Kwak,E.L. *et al.* 2010). Por otro lado, la amplificación de **CMET** está relacionada con la adquisición de resistencia secundaria a inhibidores de EGFR (Engelman, J.A. *et al*., 2007), de manera que éstos y otros hallazgos recientes de varios estudios nos van proporcionando nuevos conocimientos sobre las bases moleculares de la resistencia a ITKs del EGFR, incluyendo además de la activación de MET, y la expresión de EML4-ALK, la pérdida de PTEN, la mutación del gen KRAS, y la adquisición de la mutación de resistencia del EGFR (T790M). Por ello, las estrategias actuales se centran en lograr una inhibición más completa para superar

todas estas resistencias y optimizar así el uso de estas nuevas dianas terapéuticas (Vivanco,I. and Mellinghoff,I.K. 2010).

Por último, el gen **PIK3CA** se encuentra activado en CP preferentemente mediante amplificación génica y en menor medida por mutaciones puntuales, especialmente en carcinomas escamosos (Angulo,B. *et al.,* 2008; Samuels,Y. *et al.,* 2004). Este gen codifica la proteína p110 que es la subunidad catalítica de la fosfatidilinositol-3-quinasa (PI3K). La PI3K fosforila el fosfoinositido-3,4-difosfato (PIP2) a fosfoinositido-3,4,5-trifosfato (PIP3), ambas son moléculas mensajeras que regulan la localización y función de múltiples efectores.

II. OBJETIVO

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Las células tumorales desarrollan múltiples capacidades, como invasión tisular; replicación ilimitada; escape de apoptosis; producción de señales de crecimiento; etc. Estas propiedades se desarrollan a través de ganancias de función secundarias a alteraciones genéticas. La genésis, progresión y respuesta al tratamiento de una neoplasia es pues un subrogado del conjunto de las alteraciones genéticas de las células tumorales, dependiente a su vez del sistema inmune y del estroma, así como de variaciones polimórficas del genoma y del estado general del paciente. Las tecnologías en genómica y proteómica, capaces de analizar la diversidad y complejidad de cada tipo de cáncer, nos facilitan factores pronósticos moleculares que nos permiten dilucidar la evolución de la enfermedad y adecuar el tratamiento de forma individualizada. En este contexto y debido a la importancia del CP, el objetivo de este trabajo ha sido identificar marcadores pronóstico en sangre periférica en pacientes con CNMP en estadio localmente-avanzado y/o metastásico. La consecución de este objetivo general se llevó a cabo mediante el planteamiento de los siguientes objetivos específicos en los trabajos a continuación presentados:

1. Evaluar la influencia de VEGF, EGFR, niveles de DNA tumoral mediante análisis de telomerasa (hTERT), y mutaciones en codon 12 de Kras en la eficacia del tratamiento oncológico en pacientes con CNMP avanzado.
2. Determinar variantes genotípicas específicas en genes implicados en los mecanismos de reparación de DNA -ERCC1, XRCC3, XPD-23 y XPD-10- y su correlación con la eficacia y toxicidad hematológica del tratamiento.
3. Establecer el papel diagnóstico y pronóstico de la concentración de EGFR soluble (sEGFR) y su correspondencia con variables clínicas, patológicas y pronósticas en pacientes con CNMP avanzado en tratamiento
4. Analizar la frecuencia de inactivación de la proteína p16 en pacientes con CNMP avanzado y su relación con parámetros clínicos y de eficacia.

III. MATERIAL Y MÉTODO

PUBLICACIÓN 1

PUBLICACIÓN 1

Phase II clinical trial with gemcitabine and paclitaxel sequential monotherapy as first-line treatment of advanced non-small cell lung cancer (GECP 01-04)

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Abstract

Background In advanced-stage (IIIB or IV) non-small-cell lung cancer (NSCLC), combination chemotherapy has demonstrated response rates of 20% and a 1-year survival rate of 30%. We conducted a multicentre, open-label, nonrandomised phase II trial to determine the efficacy and tolerability of sequential monotherapy with gemcitabine followed by paclitaxel in chemotherapy-naïve patients with advanced NSCLC. ***Materials and methods*** Between December 2002 and July 2004, the Spanish Lung Cancer Group (SLCG) conducted a study in which 34 patients with advanced (stage IIIB or IV) NSCLC received 1200 mg/m^2 of i.v. gemcitabine on days 1, 8 and 15 of each 28-day cycle for a total of 3 cycles followed by 100 mg/m^2 of weekly i.v. paclitaxel for a maximum of 8 weeks. If objective response or stable disease was achieved, 70 mg/m^2 of weekly i.v. paclitaxel was maintained until disease progression was evident or toxic effects were intolerable. Lung Cancer Symptom Scale (LCSS) analysis was performed. Baseline levels of serum VEGF, EGFR, telomerase reverse transcriptase (hTERT) and K-ras mutations were analysed. The primary endpoint was the objective response rate. ***Results*** The median age of the 34 patients who were enrolled was 67 years (range 46–77), but later 8 patients were excluded; 78.8% were men, 81.8% had performance status 1 and also 81.8% had metastatic disease at diagnosis. The objective response rate was 28% (95% CI, 14.2–47.8); the median overall survival was 7.2 months (95% CI, 2.1–12.3) and the median time to progression (TTP) was 3.1 months (95% CI, 2.5–5.3). Grade 3 or 4 drug-related haematological toxicities were observed in 6 patients. Patients with lower baseline serum VEGF levels had significantly longer survival. ***Conclusions*** Sequential therapy with gemcitabine followed by paclitaxel was well tolerated with a low proportion of grade 3 or 4 adverse events, the absence of unexpected toxicity and with an improvement in quality of life. Unfortunately, the response rate did not meet the minimally required rate of 20% and the study was prematurely closed. VEGF was identified as a poor prognostic factor for TTP and survival.

Keywords

Sequential monotherapy · Pharmacogenomics · VEGF · EGFR · hTERT · Non-small-cell lung cancer

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of death by cancer worldwide, being responsible for up to one third of all cancer deaths. The median survival rate of patients suffering from advanced NSCLC who do not receive any therapy is approximately 5 months, with a 1-year survival rate of 10%. Currently, despite its modest efficacy and high toxicity, chemotherapy is the only therapeutic option that has been shown to favour longer survival rates, to reduce symptoms and to improve the quality of life (QoL) of patients with advanced NSCLC [1, 2]. ASCO and NCCN guidelines recommend that optimum first-line chemotherapy should include a cisplatin-based combination; when compared head-to-head in phase III studies, comparable efficacy has been observed (response rate around 20%, median survival about 8 months and a 1-year survival rate of 30%), with differences in toxicity profiles [3, 4]. At present, different drug combination strategies are under evaluation in order to improve these results either in efficacy and tolerability.

One of the advantages of sequential chemotherapy is that it allows each drug alone or in combination to be given at its maximum tolerated dose and maximises the dose density for the specific schedule. The selection and sequence of drug combination is critical in order to overcome possible resistance at molecular level [5, 6], to reduce toxicity [7] and to maximise drug activity. This therapeutic strategy has shown to be efficient and tolerable in selected subgroups, including elderly patients, and those with a poor performance status or previously treated [8–12].

The purpose of this phase II study was to evaluate the efficacy and tolerability of

sequential monotherapy with gemcitabine followed by paclitaxel in the treatment of chemotherapy-naïve patients with advanced (stage IIIB or IV) NSCLC.

Patients and methods

This multicentre, open-label, non-randomised phase II trial was designed by the Spanish Lung Cancer Group (SLCG). Inclusion criteria included histological or cytological diagnosis of stage IIIB (with pleural effusion) or stage IV NSCLC without prior chemotherapy and at least one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST, version 1.0), an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, age 18 years or older, estimated life expectancy of 12 weeks or more and adequate organ function. In case of previous radiotherapy, the measurable disease was not included in the radiation field. Exclusion criteria included a previous malignancy other than NSCLC, uncontrolled cardiac disease, clinically significant active infections, brain metastases, any other serious concomitant morbidities, and pregnancy or breastfeeding. Previous investigational treatment was completed at least 4 weeks before study enrolment. Patients were also excluded in case of persistent peripheral motor neuropathy or grade 2 or greater sensory neuropathy according to the National Cancer Institute Common Toxicity Criteria (version 2.0). The protocol was approved by the Institutional Review Board of each participating institution. We obtained written informed consent from all patients before study enrolment. The study was undertaken in accordance with the ethics principles of the Declaration of Helsinki and was consistent with good clinical practices and applicable laws and regulations.

Study design and treatment plan

All patients received gemcitabine 1200 mg/m^2 via a 30-min intravenous infusion on days 1, 8 and 15 of each 28-day cycle for a total of 3 cycles. Then they received weekly

paclitaxel 100 mg/m^2 via a 1-hour intravenous infusion for a maximum of 8 weeks; if the patients achieved objective response (complete or partial response) or stable disease (SD), weekly paclitaxel 70 mg/m^2 was administered until disease progression or unacceptable toxicity. According to local practice, all patients received prophylactic therapy before paclitaxel administration.

Before entering the study, patients underwent a medical history, physical examination, laboratory testing and tumour measurements assessed by imaging methods according to Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.0); assessment of toxic effects was obtained. Tumour response was re-assessed by imaging techniques after receiving 3 complete cycles of gemcitabine, 8 weeks of paclitaxel 100 mg/m^2, and in case of objective response or SD, every 2 cycles of paclitaxel 70 mg/m^2 (weeks 9–16, 17–24, 25–32, 33–40). Patients were assessed for toxicity according to the NCI-CTC 2.0. The study treatment was discontinued if documented disease progression or unacceptable toxicity occurred, or if the patient withdrew consent.

Quality of life

QoL was assessed with the Lung Cancer Symptom Scale (LCSS), and both investigators and patients filled in the questionnaires during the baseline visit, before each cycle (gemcitabine and paclitaxel) and during the follow-up period. For the purpose of data analysis, four time intervals were considered and were defined as follows: T1, within four weeks before study entry, and T2, T3 and T4 on day 1 of the corresponding cycle 1, 3 and the last one or follow-up cycle, respectively. The average, typical deviation, median and range for each patient were calculated, together with item and time interval and the scores were reverted by subtracting 100, thereby being able to interpret zero as death. The “Single Measure of QoL for each patient” was thus calculated, which reflected the four measures that summarise all items and the “Average Symptom Burden Index” was the average of the six symptom-specific

questions regarding anorexia, fatigue, cough, dyspnoea, haemoptysis and pain.

VEGF and EGFR determination

Peripheral blood was obtained at baseline and previously in cycle 3 and cycle 6 during the treatment phase. Blood samples were collected in sterile vacutainer tubes containing SST gel and clot activator (Becton Dickinson, UK). Serum was isolated after centrifugation at 2500 rpm for 10 min and stored at –80 ºC until further analysis. Serum samples were analysed and quantified for the extracellular fraction of VEGF-A and EGFR with two commercial ELISA kits (R&D systems, USA).

Extraction of DNA and quantification of hTERT

The extraction and purification of serum DNA was done with commercial kits based on affinity columns (QIAamp® Blood Mini Kit, QIAGEN) following the manu-facturer's recommendations. The quantification of hTERT was performed using a method based on real-time polymerase chain reaction (RT-PCR). The primers and the probe were designed to specifically amplify the gene of interest, hTERT, generating an amplicon of 98 base pairs as described previously [13].

Mutation analysis of K-ras at codon 12

K-ras was amplified by the PCRrestriction fragment length polymorphism (PCR-RFLP) enrichment method, where mismatch primers are designed to create two cleavage sites for the restriction enzyme Mva I (Roche Diagnostics, Germany) followed by direct sequencing as described previously [14].

Statistical analysis

The primary endpoint was objective response rate. Secondary endpoints included progression-free survival, overall survival (OS), QoL, safety and evaluation of the potential influence of several genetic alterations in therapy efficacy: determination in peripheral blood of vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and tumour DNA levels by quantification of human catalytic fraction of telomerase (hTERT), and K-ras oncogene mutations at codon 12. Biologic results were correlated with toxicity, progression-free survival, OS and QoL. We have worked with hTERT, VEGF and EGFR as a discrete variable using the methodology proposed by Schumacher [15] based on regression models. This methodology allows obtaining a cut-off point for the continuous variable that can identify two or more prognostic groups. With this methodology we may lose some information for the variable, but it improves the clinical usefulness for the variable. It allows us to identify groups of poor prognosis patients that are associated to one or more values of the variable.

Results

Patient characteristics

Between December 2002 and July 2004, a total of 34 patients were recruited from seven Spanish centres. One patient was ineligible because the baseline CT scan was not performed within 4 weeks of study entry. The remaining 33 patients were included in the safety and survival analysis. The baseline patient and disease characteristics are listed in Table 1.

First stage

A two-stage design was used to avoid unduly prolonging the study in case of not providing enough efficacy after the first stage. As per protocol requirement, if 4 or less patients out of the first 19 treated ones did respond, the treatment would not be considered efficient enough and the study would be stopped.

Table 1. Basal characteristics of the patients

Basal Characteristics	n=33 patients
Age	65.6 (45-77)
ECOG	
O	6 (18.2%)
1	27 (81,8%)
Histology	
Adenocarcinoma	13 (39,4%)
Epidermoid	13 (39,4%)
Large Cell	3 (9.1%)
Others	4 (12,1%)
Metastasis location	
Lung	16 (59,6%)
Liver	5 (18,5%)
CNS	1 (3,7%)
Others	5 (18,5%)
Primary location of the tumour	
Rigth	22 (66,7%)
Left	11 (33,3%)
Sex	
Male	26 (78.8%)
Fenale	7 (21,2%)
Stage	
IV	27 (81,8%)
IIIB	4 (12,1%)
Others	2 /6,1%)
Tumour size	10.3 (2.5 – 27)

Table 2 "Average symptom burden index" variable over time

Average Symptom Burden Index LCSS patients					
	T1	T2	T3	T4	P*
Typical deviation	73.6	71.4	44.2	32.5	0.003
Average	18.6	22.7	38.7	41.2	
Minimum-Maximum	77.7	78.7	65.9	0	
Median	25.7-94.5	0-94.5	0-96.2	0-97.2	
Average Symptom Burden Index LCSS observers					
	T1	T2	T3	T4	P*
Average	22.9	21.1	10.7	8.3	0.006
Typical deviation	14.2	14.1	11.7	13.2	
Median	16.7	16.7	8.3	0	
Minimum-Maximum	8.3-54.2	4.2-54.2	0-33.3	0-37.5	

P* = P value calculated with Kruskal-Wallis test

A total number of 26 patients were included at first stage, 19 of whom were evaluable (73%). Three partial responses (PR) were observed (15.8%), no complete responses (CR) (0%), 7 (36.8%) SD and 9 (47.4%) disease progression. The overall response rate (CR+PR) was 15.8%. As a consequence of these results, the study was closed in January 2005.

Efficacy

In patients with at least one measurable lesion, the tumour response was assessed according to RECIST (version 1.0). Eight out of 33 patients were not evaluable, 5 because of auto-exclusion by the patient, 1 for sudden death, 1 for cardiac problems and another for nausea grade III. Seven out of 25 evaluable patients achieved a PR; no

CR were observed; then, the overall response rate (CR+PR) was 28% [95% CI, 14.2–47.8] (Table 2). Five patients experienced SD (20%) and the other 13 patients, progressive disease (PD), despite study treatment (39.4%). The median survival rate was 7.2 months [95% CI, 2.1–12.3] and the median TTP was 3.1 months [95% CI, 2.5–5.3] in the 25 evaluable patients.

Safety

A total of 23 dose delays were needed: 10 (4.5%) for gemcitabine, 3 (1.2%) for 100 mg/m^2 paclitaxel and 10 (10%) for 70 mg/m^2 paclitaxel. The number of dose reductions was 8: 6 (2.7%) for gemcitabine in 6 different patients (18.2%), 1 (1.2%) reduced and delayed for 100 mg/m^2 paclitaxel and 1 (1.1%) for 70 mg/m^2 paclitaxel; the latter two dose reductions were in the same patient and caused by severe neuropathy. The median real dose intensity for gemcitabine was 1170.9 mg/m^2/week compared to the theoretical 1284.6 mg/m^2/week, with a median relative dose intensity of 1. For 100 mg/m^2 paclitaxel, the median real dose intensity was 167 mg/m^2/week compared to the theoretical 175 mg/m^2/week, the median relative dose intensity being 1; and in the same way, for 70 mg/m^2 paclitaxel, the median real dose intensity obtained was 110.8 mg/m^2/week compared to the theoretical 169.5 mg/m^2/week, with a median relative dose intensity of 0.7.

The most frequent haematological toxicity was anaemia, reported as grade 1 or 2 in up to 29.1% of administered cycles. Three (9.1%) patients had grade 3 or 4 neutropenia, and another 2 (6%) patients had febrile neutropenia related to respiratory tract infections. Six (18.2%) patients had mild thrombocytopenia and no grade 3 or 4 thrombocytopenia was reported. The most frequent non-haematological toxicities were fatigue (45.5%), anorexia (24.25), alopecia (15.2%), nausea and vomiting (33%) and peripheral sensory neuropathy with paraesthesia (9.1%). Five patients discontinued study treatment because of unacceptable toxicity (one patient had an allergic reaction related to paclitaxel administration in the third week, two

patients had grade 3 or 4 diarrhoea, one patient had grade 3 vomiting after day 8 of the first gemcitabine cycle and another patient had cardiac problems after the second gemcitabine cycle).

Quality of life analysis

Compliance in filling out the LCSS QoL questionnaires was around 48% among patients and 33% among investigators. The median scores obtained for all the gathered items descended in a statistically significant way over time when the patient filled out the questionnaire, except for cough (p=0.09); this was also the case for the "Single QoL Summative Item" variable and for the "Average Symptom Burden Index" variable. In contrast, regarding investigators' scores, only fatigue, cough and the "Average Symptom Burden Index" variable attained a statistical difference (Table 2). When the items for patients and investigators were assessed separately, there was no concordance in perceiving changes in certain items over time; thus, patients noticed a significant change over time in weight loss, fatigue, dyspnoea, haemoptysis and pain but not in cough, whereas investigators observed the significant change only in fatigue and cough. Patients and investigators only coincided with fatigue, as they both noticed a significant downward trend over time.

Pharmacogenomic study

At the time of diagnosis and according to clinical characteristics, there were no statistically signifi cant differences in any of the quantitatively analysed molecules, hTERT, EGFR and VEGF, depicted in Table 3. Additionally, we could determine baseline K-ras mutations in three patients and serum levels of these molecules did not signifi cantly differ in relation to the presence or absence of K-ras mutations at baseline.

The analysis of tumour response according to baseline serum hTERT, VEGF and EGFR concentrations and splitting the cohort in two groups (PR vs. SD and PD), as shown in

Table 3, revealed that patients with objective response (PR) had significantly lower VEGF levels than those patients without objective response (SD and PD). Furthermore, the presence of K-ras mutations was not associated with any trend with regards to treatment response.

Table 3 Pharmacogenomic study and response to therapy

	SD+PD	PR	P
hTERT basal			0.18
Below median 27.49	8(61.5)	5(83.3)	
Above Median 27.49	9(90)	1(10)	
EGFR basal			0.16
Below median 31601.46	10(90.9)	1(9.1)	
Above Median 31601.46	7(58.3)	5(41.7)	
VEGF basal			0.02
Below median 241.04	7(53.8)	6(46.2)	
Above Median 241.04	10(100)	0	
Kras			0.54
Mutated	3(100)	0	
No mutated	14(70)	6(30)	
Gender			0.99
Female	4(66.7)	2(33.3)	
Male	14(73.7)	5(26.3)	
ECOG			0.60
0	3(60)	2(40)	
1	15(75)	5(25)	
Histology			0.27
Adenocarcinoma	6(60)	4(40)	
Squamous cell	9(90)	1(10)	
Others	3(60)	2(40)	

As represented in Figure 1, we have found signifi cant differences in TTP according to baseline serum VEGF levels. The Kaplan–Meier analysis and the long-rank test have been done splitting our cohort in two groups attending to a cutoff placed at median baseline serum VEGF levels, 241.04 pg/ml. In the group of patients with baseline serum VEGF levels lower than 241.04 pg/ml, median TTP was 4.41 months [range from 0 to 14.99]. On the other hand, in the group of patients with baseline serum VEGF

levels higher than the cutoff, median TTP was 2.7 months [range from 1.97 to 3.42] (p=0.03).

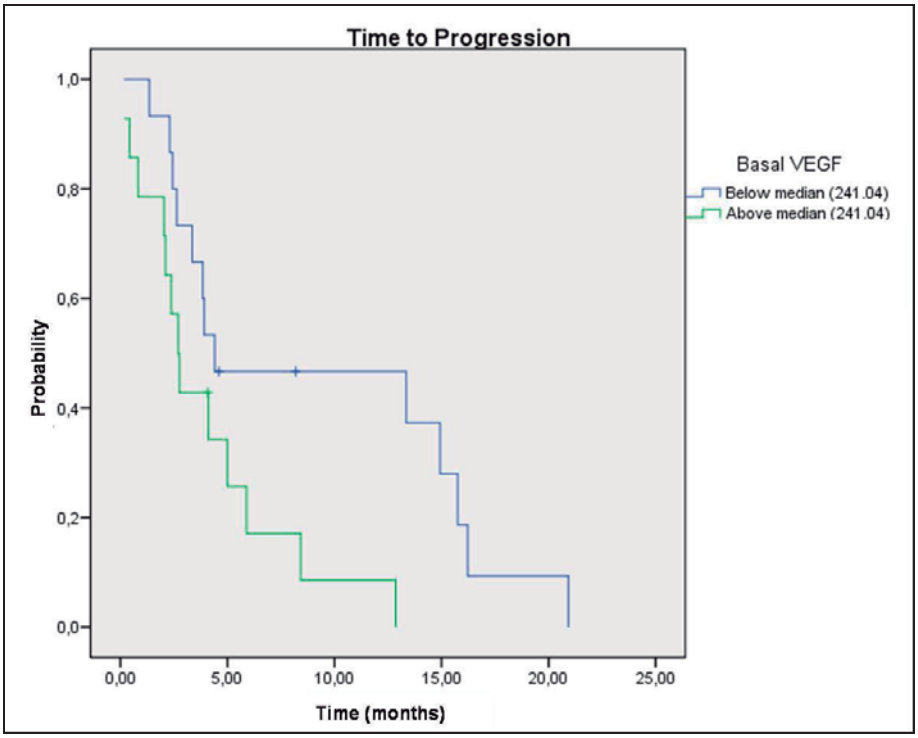
The OS analysis was done with the same criteria and cutoff at median baseline serum VEGF levels (represented in Fig. 2). Patients with baseline serum VEGF levels below the cutoff had a median survival of 9.74 months [range from 0.22 to 22.5], whereas those patients with baseline serum VEGF levels higher than 241.04 pg/ml showed a median survival of 5.72 months [range from 0.59 to 10.85]. This difference was also statistically significant (p=0.04).

Discussion

The standard first-line therapy in advanced NSCLC consists of the combination of platinum salts and a second or third-generation agent. Survival benefit and symptomatic control have been shown in patients undergoing this treatment, who experienced an improvement in their QoL. The responses obtained are within 10–25%, with a median survival of 9–11 months in phase II and III trials and a metaanalysis [1, 2, 4, 16, 17]. New strategies such as sequential drug administration, alternating regimens, maintenance chemotherapy, triplets, and addition of biological therapies and soon are being explored in various phase II trials with the purpose of improving these results.

Norton and Day decided on obtaining better results with chemotherapy by sequential administration [6]. In sequential regimens, switching to the second part of treatment (either a single agent or a drug combination) does not require documented disease progression; in contrast, the drugs are administered according to the planned schedule even if at the end of the first part of the sequence a PR has been achieved and there are no signs of treatment resistance [7].

Fig 1. Time to progression acording to basal VEGF levels

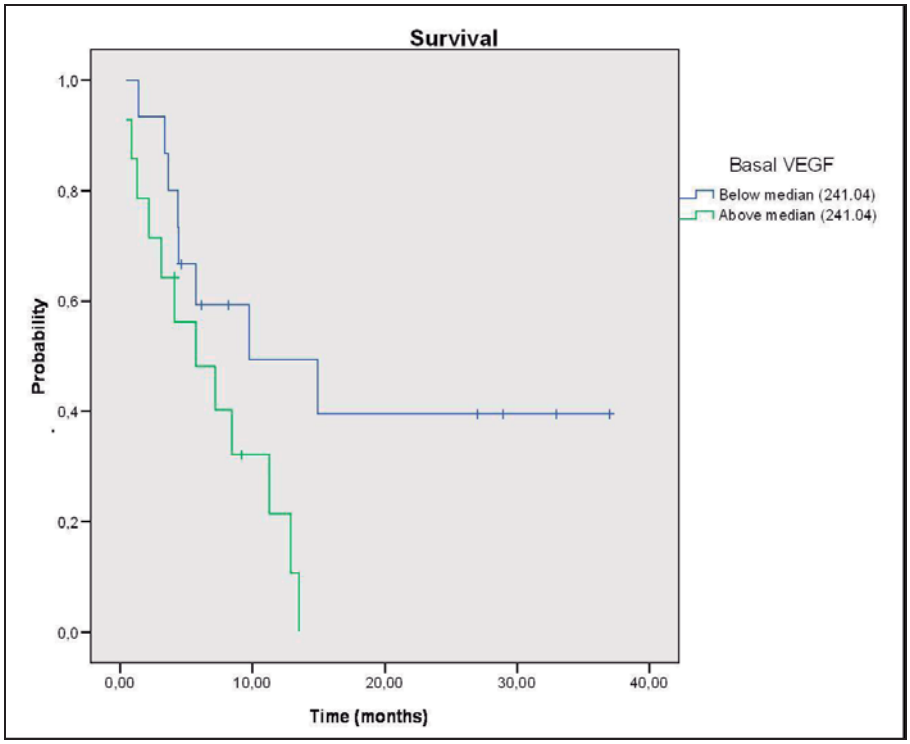


VEGF	Median (95% CI)	P
Below median 241.04	4.41 (0-14.99)	0.03
Above median 241.04	2.70 (1.97-3.42)	

Sequential chemotherapy allows administration of each single agent or drug combination at their highest tolerated dose and at the same time optimises the drug density for this regimen and limits its toxicity. The selection of drug sequence is fundamental in order to overcome any potential resistance at molecular level [5, 6] and thus is in agreement with the "worst drug rule" hypothesis. When dealing with two non-cross-resistance regimens, the least active regimen should be administered first, so after that the most active regimen would eliminate the resistant clones [18]. Various sequential chemotherapy trials have been recently conducted with different schedules of treatment in patients with high-risk advanced NSCLC (elderly, ECOG PS 2)

with objective response rates between 30% and 50% and TTP between 6 and 9 months [5, 8–12, 19–21]. In such a way, Manegold et al. proved the influence of drug sequence selection on QoL when treating locally advanced and/ or metastatic NSCLC with gemcitabine and docetaxel as monotherapy [21].

Fig 2. Overall Survival according to basal serum VEGF levels.



VEGF	Median (95% CI)	P
Below median 241.04	9.74 (0-22.55)	0.04
Above median 241.04	5.72 (0.59-10.85)	

In our trial, we aimed to determine the efficacy and tolerability of gemcitabine followed by paclitaxel as sequential monotherapy in the first-line treatment of locally advanced and/or metastatic NSCLC, with objective response rate assessment being the

primary endpoint. A two-stage design was used and if 4 or less objective responses were observed in the first 19 patients undergoing study therapy, the treatment would not be considered efficient enough and the study would be stopped early.

As objective responses, only a total of 3 PRs were observed (15.8%), which corresponded to the overall response rate (CR+PR) and did not meet the estimated efficacy objective leading to close the study down based on the predefined stopping boundaries. The response rate observed in our trial is worthy of attention; the response rate with gemcitabine as a single agent reported in several published studies is nearly 20%, with a 1-year survival rate up to 35% depending on the series [22, 23]. On the other hand, the response rate with paclitaxel as a single agent is between 23% and 38%, the estimated 1-year survival rate being 45% [24].

Sequential chemotherapy has been used for the treatment of other solid tumours prolonging survival [25–27], however, its role in lung cancer has yet to be defined. There are favourable data in selected subgroups such as elderly patients or those with a poor performance status who could predictably develop greater toxicity [12, 18]. In our trial, the median age was 67 (range 46–77) and the majority of patients had a good performance status (ECOG 1, 81.8%). Theoretically, they were potential candidates for therapy based on platinum combinations to obtain better response and survival rates as several trials have shown, such as both meta-analyses by Le Chevalier et al. and Delbaldo et al. [17, 28]. The good tolerance of the chosen treatment schedule provided and maintained our patients' QoL, which is a fundamental objective for chemotherapy with palliative intention in patients with advanced lung cancer, and this is one of the reasons why the sequential therapy could be one additional option in the therapeutic arsenal against advanced lung cancer.

On analysing QoL, and in agreement with the trial conducted by Gralla and Thatcher [29], the LCSS has been proved to be a great tool for determining the most significant changes over time and to treat the principle symptoms related with lung cancer as well as our patients' limitations and general QoL.

According to our previous results [14], we did not find a correlation between the

presence of mutant K-ras genotype in serum with any of the following parameters: disease stage, performance status, objective response rate, progression-free survival or OS. On the other hand, as high concentrations of total DNA is a negative prognostic factor and there is a linear relationship between free serum DNA and serum hTERT concentration, we could hypothesise that higher serum hTERT values could also be a marker of poor prognosis. We have not found any association between serum hTERT concentration and clinical and pathological characteristics of our cohort of patients, including age, histology and treatment response, or even with TTP or OS. This lack of predictive value was obtained when the patients were stratified in two categories according to serum hTERT concentration and these results do not confi rm our previous observations [13], probably due to the low number of patients in this study. Additionally, we could not find any relationship between baseline serum EGFR levels and disease stage, treatment response, TTP and OS. Our findings are consistent with those described by Spano et al. in colorectal cancer patients [30], while other studies in lung or colorectal cancer have shown that EGFR levels are related to stage of disease [31]. We could not find this association, mainly due to advanced stages in our patients (IIIB and IV), whereas in the other studies patients with early and late disease stages were included. Our study has not demonstrated that EGFR is an independent factor for treatment response, TTP or OS.

On the contrary, in our study patients with lower serum VEGF levels had a longer survival compared to those patients with higher levels. We have observed that VEGF was a poor prognostic factor for survival and disease progression, consistent with the data analysed in a recent review [32]. Furthermore, Laack et al. found a statistically significant difference when comparing survival of patients with resected stage I/II tumours [33].

In summary, our schedule of sequential therapy in advanced NSCLC patients was related to an acceptable tolerability but does not provide efficacy rates in the range of the common chemotherapy regimens. VEGF has been revealed as an important

prognostic factor. Learning more about overexpression, mutations, polymorphisms and gene methylation that contribute to resistance to a specific drug combination through pharmacogenomic studies will help us to offer our patients a more customised treatment.

Conflict of interest The authors declare no conflicts of interest or any financial disclosure.

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PUBLICACIÓN 2

PUBLICACIÓN 2

Chemotherapy-Induced Neutropenia Does Not Correlate With DNA Repair Gene Polymorphisms and Treatment Efficacy in Advanced Non–Small-Cell Lung Cancer Patients

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Abstract

Background: Platinum doublets are standard chemotherapy for advanced non–small cell lung cancer (NSCLC). The aim of this study was to assess whether neutropenia is: (1) an indicator for treatment efficacy, or (2) associated with specific polymorphisms. **Patients and Methods:** Four hundred ninety-four patients, treated with cisplatin-docetaxel were retrospectively analyzed. Relative dose intensity (RDI) was assessed for both drugs. Neutrophil counts were assessed only on Day 21 of each cycle. Genotyping was performed for 4 different polymorphisms in ERCC1, XRCC3, XPD-23, and XPD-10. **Results:** The median overall survival was 9 months. The mean RDI was 0.94 for cisplatin and 0.93 for docetaxel. Four hundred three patients received ≥ 3 cycles of chemotherapy, and 239 received ≥ 6 cycles. Thirty-one percent developed neutropenia, and 19% had Grade (G) 3-4 neutropenia. RDI was lower in patients with neutropenia (G1-4; 0.87-0.93) when compared with those without (G0; 0.94-0.95; *P* <.02). Male patients (*P* = .02) had inferior survival when compared with female patients, and ECOG (Eastern Cooperative Oncology Group) 1-2 patients (*P* < .001) had worse survival when compared with ECOG 0. There was no significant survival difference with respect to Grade of neutropenia (G0, 8.7 vs. G1-2, 11.6 vs. G3-4, 9.6months; *P* = .41). In ECOG 0 patients, survival was significantly better for neutropenic G1-4 (Hazard ratio [HR], 0.55; 95% confidence interval [CI], 0.31-0.96; *P* = .034) when compared with non-neutropenic (G0) patients. No association was observed between examined polymorphisms and neutropenia. **Conclusion:** RDI was significantly higher in patients who did not develop neutropenia during treatment, but as the nadir period was not explored in our study; the low occurrence of neutropenia in our cohort is considered underestimated. There was no significant survival difference with respect to grade of neutropenia. Finally, none of the examined single nucleotide polymorphisms (SNPs) were associated with the presence of neutropenia, disease characteristics, response rates, or survival.

Introduction

Non–small cell lung cancer (NSCLC) is responsible for up to a third of all cancer deaths. The average survival of untreated advanced NSCLC patients is approximately 4 to 5 months, with a 10% 1-year survival probability.[1] For decades chemotherapy has been the only treatment capable of prolonging the survival of these patients with rather limited benefits.[2,3] However, chemotherapy treatment has in different randomized trials demonstrated improvement of symptoms and health related quality of life (HRQOL).[4] Today, a 2-drug platinum-based combination regimen combined with supportive care is considered first-line therapy,[5] with response rates at around 20%, median overall survival 8 months, and a 30% 1-year survival.[6] Despite its modest efficacy and prevalent toxicity, chemotherapy is the most active treatment of advanced NSCLC. Some authors have proposed using chemotherapy-induced hematological toxicity as a measure of efficacy of such drugs.[7] Previous adjuvant chemotherapy studies in women with breast cancer have supported this theory. Longer intervals of disease free survival have been observed in breast cancer patients who developed neutropenia during treatment.[8-11] In a series of 1265 NSCLC patients treated with different chemotherapy schemes in various trials, Di Maio et al observed that neutropenia was associated with a survival improvement.[12] They hypothesized that the absence of neutropenia could be due to drug underdosing and hence a meager treatment efficacy.

Polymorphic variants, primarily single nucleotide polymorphisms (SNPs), can explain differences in both survival and adverse effects in cisplatin-treated NSCLC patients. SNPs in DNA repair genes can impair the removal of DNA adducts[13], which may modulate the response to cytotoxic agents and lead to neutropenia. Examining possible association between DNA repair genotypes and the appearance of neutropenia may help elucidate whether polymorphisms can adversely or favorably

influence chemotherapy outcomes[14].

The objectives of this study were to examine whether: (1) chemotherapy-induced neutropenia is associated with an increased survival; (2) lack of neutropenia is associated with undertreatment; and (3) specific genotype variants in DNA repair enzymes correlate with hematological toxicity in advanced NSCLC patients.

Materials and Methods

Patient Characterization

This was a retrospective study based on the Spanish Lung Cancer Group (SLCG) PLATAX trial, a multicenter single-arm pharmacogenomic study of cisplatin/docetaxel in 502 patients with NSCLC in stage IV and IIIB (malignant pleural effusion). The ethical committees of all participating hospitals approved the study and patients provided their informed consent.

Inclusion criteria included histological confirmation of NSCLC, age ≥18 years, stage IIIB or IV, ECOG (Eastern Cooperative Oncology Group) ≤2, and 1 measurable lesion according to the response evaluation criteria in solid tumours (RECIST) criteria[15], as well as adequate bone marrow (haemoglobin >10 g/dL, total neutrophils ≥ 2.0 x 10^9/L), hepatic (total bilirubinuria ≤ 1, aspartate aminotransferase [AST] and alanine aminotransferase [ALT] ≤ 1.5 times upper normal limits [UNL], alkaline phosphatase 5 times UNL), and renal function (creatinine ≤ 1.5 times UNL, creatinine clearance ≥ 60mL per minute) 1 week before inclusion. Patients treated with surgical interventions were included provided ≥30 days since surgery.

Those treated with radiotherapy were included if the irradiation included < 10% of the hematological bone marrow and if 21 days had passed since the last treatment session. Pregnant women or those in the lactation period were not included, norwere patients who had received previous chemotherapy or who presented with cerebral or leptomeningeal metastasis, peripherals neuropathy >2 according to the National

Cancer Institute common toxicity criteria (NCI-CTC) v.2.0[16] scale, malignant tumour history in the previous 5 years, serious comorbidity, or previous ascites and/or pericardial effusion.

Table 1. Patient characteristics

(n=493)	Global sample	≥3 cycles	≥6 cycles
Age, median (range)	59.8 (30-79)	59.2 (30.6-79)	59.8 (30-79)
Sex, n (%) Male Female	 412 (83.8%) 80 (16.2%)	 337 (83.6%) 66 (16.4%)	 193 (80.8%) 46 (19.2%)
ECOG, n (%) 0-1 2	 484 (98.4%) 8 (1.6%)	 396 (98.5%) 6 (1.5%)	 132 (97.5%) 6 (2.5%)
Histology, n (%) Adenocarcinoma Squamous cell Large-cell Others	 245 (49.7%) 153 (31%) 77 (15.6%) 18 (3.7%)	 196 (48.6%) 131 (32.5) 65 (16.1%) 11 (2.7%)	 118 (49.4%) 76 (31.8) 39 (16.3) 6 (2.5%)
Stage,n(%) IV / IIIB	418 (84.4%) 75 (15.2%)	339 (84.1%) 64 (15.9%)	207 (86.6) 32 (13.4%)

Abbreviation: ECOG = Eastern Cooperative Oncology Group.

Neutropenia was classified according to the patient's highest neutropenic grade (NCI-CTC) during the chemotherapy treatment.[16] The patients were categorized according to absence of neutropenia (G0), moderate neutropenia (G1-2) and severe neutropenia (G3-4). To avoid any bias on the basis of number of chemotherapy courses, performance status (PS), other side effects, etc, assessments were performed according to the following 3 subgroups of patients: the total sample group, patients who received at least 3 chemotherapy cycles, and those who received 6 or more cycles.

Table 2. Number of Patients According to Grade of Neutropenia

Neutropenia	**Patients n (%)**	**Neutropenia**	**Patients n (%)**
Grade 0	342 (69,4)		
Grade 1	34 (6.9)	**Grade 1-2**	60 (12.2)
Grade 2	26 (5.3)		
Grade 3	38 (7.7)	**Grade 3-4**	92 (18.6)
Grade 4	53 (10.8)		

Treatment and Follow-up

The chemotherapy regimen consisted of docetaxel 75mg/m^2 followed by cisplatin 75mg/m^2, both as 1-hour intravenous (I.V.) infusions on Day 1 of each cycle and repeated every 21 days up to maximum of 8 cycles. The chemotherapy doses were adjusted by 25% in cases of severe (G3-4) or no (G0) hematological toxicity. In cases of neutropenic fever, only the docetaxel dose was reduced by 25%. If neutrophils were <1.5 x 10^9 and/or platelets <100 x 10^9 on Day 21, treatment was delayed by 1 week. If no recovery by 2 weeks, the study treatment was terminated.

Table 3. Relative Dose Intensity

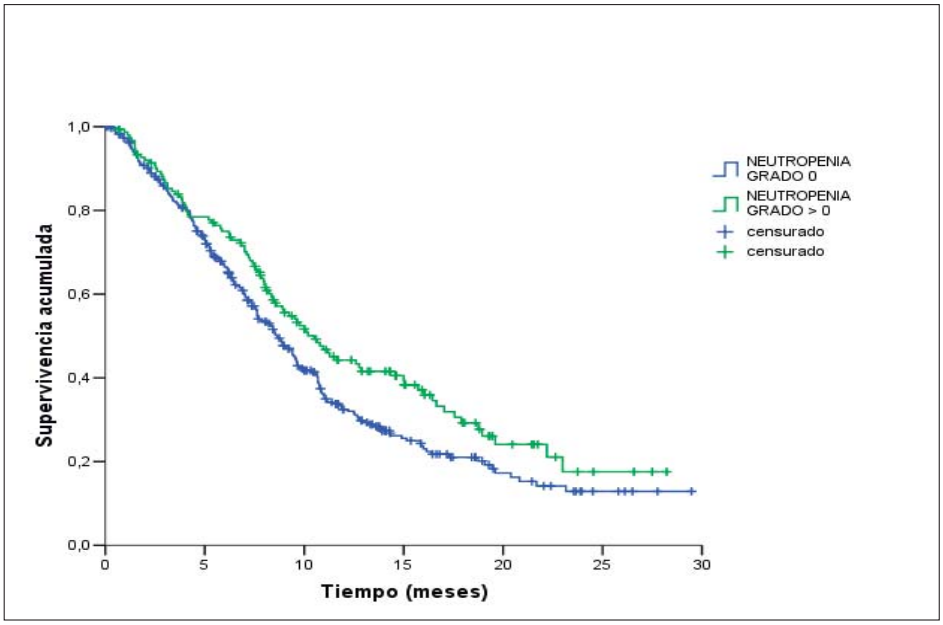
	DOCETAXEL			CISPLATIN		
Grade of neutropenia	Global (n=493)	³ 3 cycles (n=403)	³ 6 cycles (n=239)	Global (n=493)	³ 3 cycles (n=403)	³ 6 cycles (n=239)
G0	0.95	0.94	0.95	0.94	0.94	0.95
G1-2	0.92	o.91	0.93	0.92	0.91	0.92
G3-4	0.89	0.88	0.87	0.91	0.91	0.90
P	<.001	<.001	<.001	.011	NS	0.024

Abbreviation: NS = not significant.

The protocol did not allow primary prophylaxis with granulocyte colony-stimulating

factor (G-CSF). As secondary prophylaxis, lenogastrim 150g/m^2 per day could be administered subcutaneously from day 4 to 12 of every cycle to maintain dose intensity.

Figure 1. Overall Survival According to the Absense (G0) or Presence (G1-4) of Neutropenia



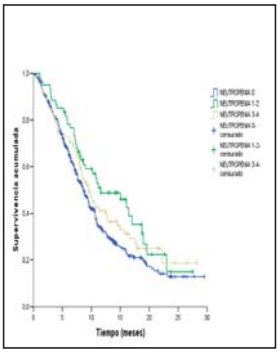
Disease assessment was carried out every 3 cycles according to the RECIST criteria.[15] Treatment was terminated in cases of progression, unacceptable toxicity, or according to the patient's preference.

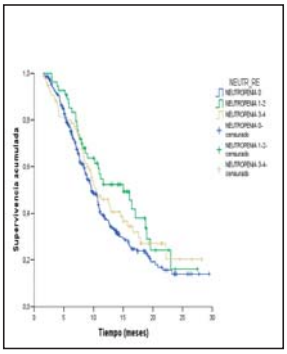
The dosage intensity of each drug was calculated by dividing the total number of administered doses by the total treatment time. The relative dosage intensity (RDI) was calculated using the coefficients of the received dosage intensity and the planned dosage intensity.

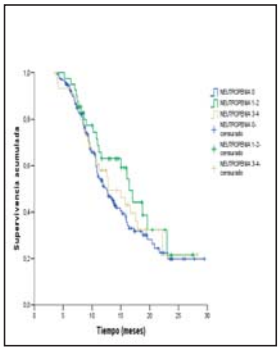
Sample Collection and Genotyping

Venous blood was collected at baseline from each subject in ethylenediaminetetraacetic acid (EDTA) tubes and sent to the reference lab. Leukocyte genomic DNA was later isolated using a commercial kit according to the manufacturer's instructions (QIAmpDNA blood Minikit, Qiagen). Polymorphisms were assessed using an allelic discrimination assay, as previously described[17] using an ABIPrism 7900 HT Sequence Detection System (Applied Biosystems). The following polymorphisms were analyzed: XRCC1-118, ERCC1-8902, XRCC3, XPD-23, and XPD-10

Figure 2. Overall Survival According to Grade of Neutropenia







	MUESTRA GLOBAL	AL MENOS 3 CICLOS	AL MENOS 6 CICLOS
NEUTROPENIA G0	8.7 m (7.7,9.6)	9.6 m (8.6,10.7)	12.5 m (10.5,14.6)
NEUTROPENIA G1 -2	11.6 m (6.3,16.9)	15 m (9.8,20.3)	16.4 m (13.9,18.9)
NEUTROPENIA G3 -4	9.6 m (7.7,11.5)	10.5 m (7.4,13.6)	12.9 m (8.8,16.9)
P	0.4	0.1	0.36

Statistical Methods

All statistical analyses were done using the statistical Packaged SPSS version 13. The^2 test and Fisher exact test were used to examine the association between SNP genotypes and the presence of neutropenia. Univariate analisys were done by using the Kaplan-Meier method, and statistical significance between survival curves was

assessed by the log-rank test. Overall survival was defined as the time from study inclusion until date of death. To assess the independent value of different variables on survival, in the presence of other variables, a multivariate analysis was carried out using the Cox proporcional hazards model. Probability for step wise entry and removal was set at .05 and .10, respectively. The significance level used was *P*>.05.

Results

Patients, Dose Intensity, and Neutropenia

Of 502 patients initially recruited, 9 were excluded because of no measurable disease (n=1), withdrawing consent (n=2), PS >2 (n=2), serious comorbidity (n=1), death prior to treatment start (n-=1), and previous chemotherapy (n=2). The remaining 493 patients were included in the analysis. Patient characteristics are shown in Table 1. Eighty-four percent of the patients were male, 98% had ECOG 0-1, 85% had distant metastatic disease, and 50% had adenocarcinoma.

The frequency and grade of neutropenia are presented in Table 2. Overall, 31% of the patients developed neutropenia, of which 12% had G1-2 and 19% G3-4.

The mean RDI was 0.94 (median, 0.97; range, 0.42-1.11) for cisplatin and 0.93 (median, 0.97; range, 0.48-1.08) for docetaxel. Table 3 shows the RDI of docetaxel and cisplatin according to grade of neutropenia and the Lumber of courses. For docetaxel, the mean RDI was significantly lower (<0.001) for G3-4 patients when compared with G0 patients in all treated patients as well as subgroups receiving ≥3 and ≥6 courses. For the subgroup administered ≥6 courses, the RDI was significantly lower in the G3-4 when compared with the G1-2 group (*P*=.033). For cisplatin, the mean RDI was also significantly lower for G3-4 patients when compared with G0 patients in all treated patients (*P*= .011) as well as the subgroups receiving ≥6 courses (*P*=.024). Also there were significantly reduced RDI for the G1-2 when compared with the G0 group (*P*=.048) among patients administered ≥3 courses.

Survival

In the total patient population the overall median survival was 9 months. For patients without neutropenia the median survival was 8.7 months, and for those with neutropenia 10.5 months; *P*=.18 (Figure 1). When analyzed according to grade of neutropenia, the median survival was 11.6 and 9.6 months in patients with G1-2 and G3-4, respectively (*P*=.40; Figure 2). There was no statistical significant difference between patients with G0 and G3-4 (*P*= .48).

Figure 2 presents the survival curves according to grade of neutropenia for all patients administered ≥3 and ≥6 courses. Among patients administered ≥3 courses there was no significant neutropenia-related differences in survival (G0, 9.6 vs. G1-2, 15.0 vs. G3-4, 10.5months; *P*= .10). Neither was this the case for patients administered ≥6 courses (G0, 12.5 vs. G1-2, 16.4 vs. G3-4, 12.9months; *P*=.36).

Multivariate Analysis

Table 4 summarizes the results of the multivariate analysis, which was stratified for the number of administered cycles. Patients with ECOG 1 to 2 had a higher risk of death when compared with patients with ECOG 0 (Hazard ratio [HR], 1.78; P=.001). The same effect was observed for male patients compared with female patients (HR, 1.5; P=.02). When this analysis was applied among patients who received ≥3 (ECOG 1-2 vs. ECOG 0: HR, 1.72; P=.001; male vs. female: HR, 1.8; P= .002) or ≥6 chemotherapy courses (ECOG 1-2 vs. ECOG 0: HR, 1.5; P=.04; male vs. female: HR, 2.1; P=.004), similar results were obtained.

In a subanalysis, selecting the ECOG 0 patients who developed neutropenia (G1-4) during treatment it was observed that this Group had significantly lower risk of death (HR, 0.55; 95%CI, 0.31-0.96; *P*= .034) when compared with ECOG 0 patients without neutropenia (G0). As seen in Figure 3, the mean survival of patients with ECOG 0 that did not develop neutropenia was 11 months (9.5-12.5), whereas mean survival of patients that developed neutropenia was 17 months (15.1-19.1), though these differences were not statistically significant (*P*=.15).

Table 4. Multivariate Analysis: Patients Stratified by the Number of QT Courses

	P	HR	IC 95%
Age	0.356	0.995	0.984-1.006
Sex (M vs F)	0.023	1.491	1.057-2.102
ECOG 1-2 vs 0	0.000	1.783	1.352-2.350
Histology	0.819		
SCC vs Adeno	0.746	1.044	0.805-1.354
SCC vs Large	0.791	1.048	0.741-1.483
SCC vs other	0.455	0.805	0.456-1.421
IV vs IIIB	0.404	1.144	0.834-1.569
Neutropenia	0.737		
1-2 vs 0	0.651	0.920	0.641-1.320
3-4 vs 0	0.476	0.897	0.666-1.209

Abbreviations: ECOG= Eastern Cooperative Oncology Group; HR= Hazard Ratio

Polymorphisms and Neutropenia

The allelic frequencies of DNA repair genes are presented in Table 5. No significant associations were observed between any of the investigated SNPs and the presence of neutropenia. Nor were there any significant correlations between the different genotypes and survival.

Discussion

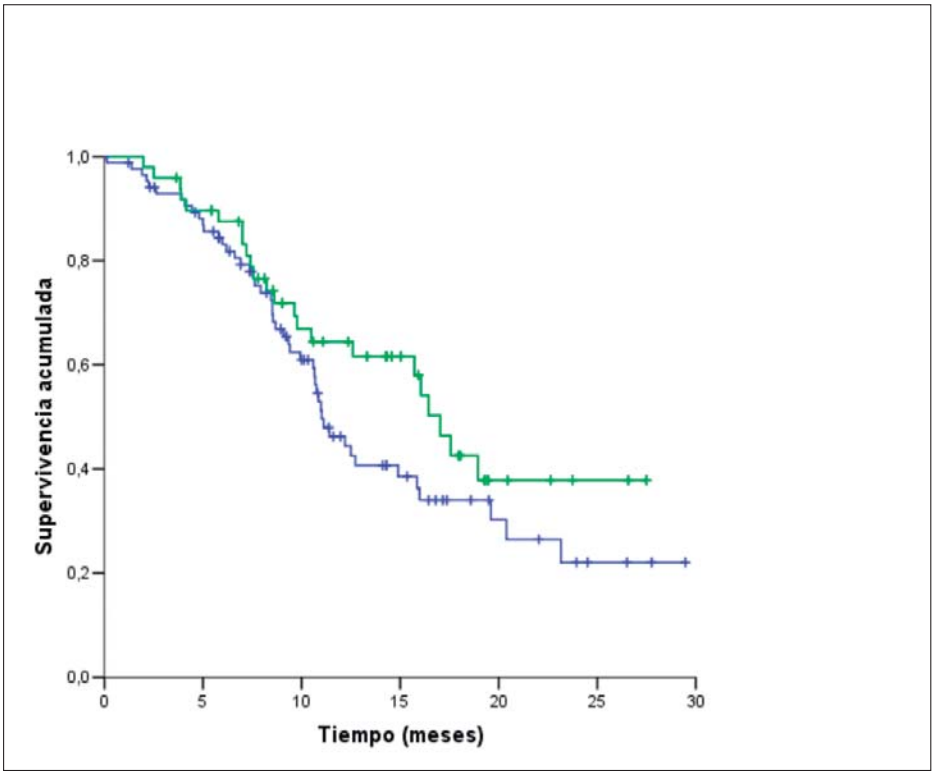
Herein, we have demonstrated that RDI was significantly higher in the NSCLC patients who did not develop neutropenia during chemotherapy treatment. There was no significant survival difference with respect to grade of neutropenia, but in ECOG 0 patients, the overall survival was significantly better for neutropenic (G1-4) than non-neutropenic (G0) patients. There was, however, no association between examined genotype variants and neutropenia or survival.

For more than a decade investigators have discussed the poor correlation between body surface area based chemotherapy dose calculation and the pharmacokinetic parameters for most cytotoxic agents.[18] Because pharmacokinetics and drug sensitivity largely reflect genetic predisposition, body surface area calculated doses will lead to high blood levels and clinical toxicity in some, while a higher proportion will receive doses that are too low, leading to a satisfying clinical course with feder side effects, but with an increased risk of treatment failure.[19,20] In the latter case, it has been stated that the treatment is not well-tolerated, but too well-tolerated.[21] This problem has led to searches for surrogate markers to optimize treatment efficacy, such as hematological toxicity.

In patients receiving cisplatin-based and non-cisplatin chemotherapy for advanced NSCLC, DiMaio et al[12] demonstrated that the occurrence of chemotherapy-induced neutropenia was associated with significantly longer survival. This was observed, however, only for ECOG 0 patients in our study. Their analyses were based on 1265 advanced NSCLC patients enrolled in 3 randomized trials of first-line chemotherapy.[12] In 436 patients who had completed 6 planned courses and were alive 180 days after randomization, median survival was 42.0 and 43.7 weeks for those experiencing G3-4 and G1-2 neutropenia, respectively, versus 31.4 weeks for those without neutropenia (G0; *P*=.012).

They also found that neutropenia was an independent prognostic marker for survival in multivariate analysis. In this Italian study,[12] in which only 20% received a cisplatin-based doublet while the rest were administered vinorelbine or gemcitabine single-drug or a vinorelbine and gemcitabine combination, the frequency of neutropenia was in fact greater than in our study employing the more toxic cisplatin-docetaxel combination (G1-4: 40% vs. 30%; G1-2: 21% vs.12%). While DiMaio et al[12] assessed neutrophil counts on Day 8 and 15 of each cycle, this was done only on Day 21 in our study. Although the median age was higher in the Italian study (71 vs. 59 years), the occurrence of neutropenia must be considered underestimated in our study, as the nadir period was not explored.

Figure 3. Overall Survival in ECOG 0 according to absence or presence of neutropenia.



The prognostic value of chemotherapy-induced neutropenia/leu-copenia has also been investigated in other cancer types. Most retrospective Studies have been done in breast cancer patients treated with adjuvant chemotherapy. In general, these studies have shown a significantly increased risk of death Hmong breast cancer patients who did not present neutropenia/leukopenia during adjuvant treatment, but the differences were not significant in the multivariate analysis[8-10,22] Leukopenia was proposed as a useful biological marker for chemotherapy efficacy in 1999.[9] In primary osteosarcoma, leukopenia during adjuvant chemotherapy was associated with a reduced risk of relapse.[23] In gastric cancer, chemotherapy-induced neutropenia was

found to be an independent prognostic factor for survival.[24]

Table 5. Allelic Frequencies of DNA Repair Enzymes and the presence and absence of neutropenia.

	n	Allelic Frequencies	Neutropenia				
			No (G0)		Yes (G1-4)		p
			n	Allelic frequencies	n	Allelic frequencies	
ERCC1 N118N (Exon 4)							.53
	574	0.623	478	0.627	96	0.600	
	348	0.377	284	0.373	64	0.400	
ERCC1 G8092T (3´UTR)							.92
	221	0.244	183	0.243	38	0.247	
	685	0.756	569	0.757	116	0.753	
XRCC3 T241M (Exon 7)							.37
	376	0.398	307	0.392	69	0.431	
	568	0.602	477	0.608	91	0.569	
XPD K751Q (Exon 23)							.72
	621	0.648	511	0.645	110	0.663	
	337	0.352	281	0.355	56	0.337	
XPD D312N (Exon 10)							.89
	136	0.335	111	0.332	25	0.347	
	270	0.665	223	0.668	47	0.653	

Abbreviation: UTR= untranslated region.

It has to be kept in mind that the retrospective analyses on the association between neutropenia and survival may be hampered by statistical bias, eg, patients with poor chemotherapy tolerance, because of metastases and poor performance status, may receive lower doses, and as such, might show a false positive dose-response relationship. This is not likely the case in the retrospective analyses of neutropenia as a surrogate marker for dose effect, because patients with neutropenia, for example, because of bone marrow metastasis, are expected to reduce the association between neutropenia and a favourable survival. DiMaio et al[12] argued that the absence of neutropenia may be a result of underdosing. Our analyses demonstrated that the RDI for both drugs was higher in patients without than those with neutropenia, but this does not exclude that they may have been “underdosed”

due to pharmacokinetic heterogeneity.

None of the examined SNPs evolved in DNA repair mechanisms were associated with the presence of neutropenia, disease characteristics, response rates, or survival. Hence, none of the polymorphisms has any prognostic relevance.

Conclusion

The results of the present study together with others suggest that neutropenia is a useful prognostic indicator for an effective chemotherapy dose in good performance status NSCLC patients. However, the demonstrated associations between neutropenia and improved survival have to be further tested in prospective trials.

Disclosure

The authors declare no conflicts of interest

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PUBLICACIÓN 3

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Analysis of the Prognostic Value of Soluble Epidermal Growth Factor Receptor (sEGFR) Plasma Concentration in Advanced Non–Small-Cell Lung Cancer Patients

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ABSTRACT

Background: Epidermal growth factor receptor (EGFR) is overexpressed in a variety of epithelial malignancies including lung cancer. A soluble fragment of the EGFR extracellular domain (sEGFR) can be detected in the blood of patients who have non–small-cell lung cancer (NSCLC), but its clinical/prognostic role must be further elucidated. **Methods:** sEGFR concentration was retrospectively determined by enzyme-linked immunosorbent assay in plasma samples from 308 advanced NSCLC patients (before treatment) and 109 healthy controls and correlated with clinico-pathological variables. **Results:** The concentration of sEGFR was lower in NSCLC patients than in controls (P <.0001). sEGFR behaves as a sensitive but not specific screening biomarker. No significant associations were observed between sEGFR concentration and demographic/clinical characteristics such as gender, Eastern Cooperative Oncology Group performance status, stage, and number or location of the metastatic sites. sEGFR was lower in patients with progressive disease or in squamous cell carcinoma compared with adenocarcinoma, but these differences were not significant. Patients with sEGFR≤34.56 ng/mL showed a shorter overall survival (median 9.1 versus 12.2 months, P=.019) than others. Moreover, in multivariate analysis, sEGFR remained a significant independent prognostic marker. **Conclusion:** Low baseline sEGFR is associated with reduced survival in advanced NSCLC. Therefore, our findings in this large cohort of patients suggest that the determination of sEGFR concentration provides valuable prognostic information.

Introduction

Most non–small-cell lung cancer (NSCLC) patients have advanced unresectable disease at diagnosis, which typically has a poor prognosis. (1) Development of therapies for these patients first requires a better assessment of prognostic determinants. To date, research on genetic markers in lung cancer has focused on the tumour tissue obtained by biopsy or surgery. Alternatively, the search for biomarkers in peripheral blood has an important potential advantage over the evaluation of tumour tissue, which is the possibility of evaluating all cancer patients without the need for surgical specimens or biopsy material. This advantage is an essential issue for advanced NSCLC patients because they do not always have readily available biopsy specimens. Multiple different biomarkers have been investigated in serum samples from patients with a variety of tumours. Among them, epidermal growth factor receptor (EGFR) has emerged as a potencial tumour-related biomarker because it is highly expressed in a variety of epithelial malignancies, including lung cancer. (2) EGFR is a member of the family of EGF-related tyrosine kinase (TK) receptors. Upon ligand binding, the receptor is activated by its intrinsic TK activity, which leads to the activation of several intracellular pathways, including the Ras-Raf-MAP-kinase pathway, the PI3K-Akt pathway, and the STAT pathway, ultimately affecting cell proliferation, differentiation, survival, angiogenesis, and migration. (3-5) In NSCLC tumour cells, EGFR are frequently overexpressed by 50% to 80%. (6-10) No definite conclusions have been reached about the prognostic value of the overexpression of EGFR by tumour cells. Briefly, some reports have indicated that NSCLC patients with poor prognoses have higher EGFR expression in tumour tissue than those with better prognoses. (11, 12) On the other hand, some studies have not found any association between EGFR tissue expression and survival in NSCLC. (8,10,13,14) The results of a meta-analysis show that EGFR expression is not a statistically significant prognostic factor for survival in NSCLC; that analysis concluded that EGFR expression might be a poor prognostic factor for survival in NSCLC, but the amplitude of its impact seems to

be small. (15) Therefore, the prognostic importance of EGFR expression in tumour tissues in patients with NSCLC remains controversial.

As mentioned above, the search for serum/plasma biomarkers is a challenge in advanced NSCLC. Several studies have detected the extracellular ligand domains of EGFR in the bloodstream of cancer patients, including NSCLC patients, (16) but the potential diagnostic or prognostic role of soluble EGFR (sEGFR) remains unclear because of the discordant results among the currently available data. (17-20)

This study investigated sEGFR concentration and correlated it with clinical, pathological, and prognostic variables to elucidate its potential utility as a biomarker in a large cohort of 308 advanced NSCLC patients.

Patients and Methods

Patients and Controls

We retrospectively analyzed 308 samples from patients enrolled in a multicenter study coordinated by the Spanish Lung Cancer Group carried out between February 2003 and January 2005. Eligible patients were those with histologically confirmed advanced NSCLC (stage IIIB disease with pleural effusion or stage IV). Other eligibility criteria included age ≥18 years, no prior chemotherapy, at least one disease measurable by the Response Evaluation Criteria in Solid Tumors (RECIST),(21) an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2, and adequate hematologic, renal, and hepatic function. Exclusion criteria included active metachronous cancer, severe heart disease, active infection, pregnancy, and other severe medical conditions. The control group consisted of 109 healthy volunteers without any acute or chronic inflammatory conditions. Control samples were collected during the same period as the patient samples. All individuals provided informed consent. The study was conducted in accordance with the Declaration of Helsinki and applicable local regulatory requirements and laws. The institutional ethical review board approved the study protocol.

Treatment and Evaluation

Patients were treated with cisplatin (75 mg/m2) and docetaxel (75 mg/m2) on day 1 every 3 weeks. Patients were restaged for objective response after the first three cycles of chemotherapy. Patients with complete or partial response or stabilization continued treatment until disease progression or a maximum of eight cycles in the absence of unacceptable toxicity. Patients progressing before or at first evaluationwere shifted to a second-line treatment. Responses were categorized according to RECIST and reported as best response achieved per patient. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria.

Samples

Peripheral blood samples were collected from controls and patients before chemotherapy. Ten milliliters of peripheral blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA). These tubes were sent to a reference laboratory within 24 hours of blood collection. Blood samples were subjected to two centrifugation steps to eliminate any possible cell fragments from the blood: an initial centrifugation for 10 minutes at 1,100 **x** g at room temperature and a second centrifugation of the supernatants for 10 minutes at 2,000 **x** g at room temperature. Plasma aliquots were immediately stored at -80°C until further analysis.

EGFR Quantification

Plasma EGFR concentration was determined at baseline using a commercial EGFR microtiter sandwich immunoassay (Duo Set, R&D Systems Minneapolis, MN, USA) following the manufacturer's instructions. The assay was performed using capture and probe antibodies specific to EGFR. A microplate reader (Victor3™- 1420 multilabel counter, Perkin Elmer Waltham, MA, USA) set to 450 nm and a reference at 620 nm

was used to determine the optical density of each well. All samples were assayed in duplicate.

Statistical Analysis

Because EGFR values were normally distributed, the comparisons and correlations with categorical variables were conducted using the Student's t-test. The association between categorical variables was evaluated using the X^2test. The performance of EGFR in the prediction of disease versus control status was evaluated using the receiver operating characteristic (ROC) curve, and the area under the curve (AUC) was measured. Overall survival (OS) was calculated from the date of diagnosis. Time-to-progression (TTP) was calculated from the date of treatment initiation. OS and TTP curves were plotted according to the Kaplan–Meier method, and differences between groups were assessed using the log-rank test. The univariate analysis (performed in parallel by Kaplan Meier and Cox methods) was performed for the following variables: age, stage, histology, PS, gender, metastatic locations, number of metastatic lesions and sEGFR. All the variables listed above were entered into the multivariate Cox analysis. A P value of less than .05 was considered statistically significant.

Results

Table 1 shows the most relevant demographic and clinico-pathological characteristics of the patient population. The median age was 59.8 years (range, 31 to 80 years), and 84% of the patients were male. The majority of patients were in ECOG PS = 1 (72%), 50% had adenocarcinoma, and 84% had stage IV NSCLC. In the control group, the median age at the time of sampling was 55.5 years (range, 34 to 85 years) and 77% were males. There were no significant differences in age or gender between patients and controls.

Plasma sEGFR Concentration in Patients and Controls

The plasma concentration of EGFR in cancer patients was statistically different from

that found in the control group (mean ± 2 standard error of mean [SEM]: 30.98 ng/mL ± 0.82 versus 34.91 ng/mL ± 1.10, respectively, P <0.0001). To evaluate the potential screening utility of sEGFR concentration for advanced NSCLC, we generated ROC curves comparing healthy controls to patients, yielding an AUC (underneath the no information line) of 0.341 (95% confidence interval [CI], 0.286-0.397). According to the Youden index, the best cutoff value for sEGFR to distinguish patients from controls was 31.62 ng/mL, resulting in 80% sensitivity and 49% specificity. We considered these values to represent an acceptable specificity but a poor sensitivity, therefore indicating that baseline sEGFR is not a strong biomarker to discriminate NSCLC patients from healthy controls

Correlations of sEGFR Concentration with Clinico-pathological Variables

To assess whether sEGFR concentration is associated with clinico-pathological and prognostic factors in NSCLC, we compared the values of sEGFR against gender, stage, ECOG-PS, different histological types, and the number and location of metastatic sites (classified as local or distant). No correlations were observed between baseline sEGFR concentration and gender (P = .112), stage (P = .174), ECOG PS (P = .323), number of metastatic sites (grouped as ≤2 or >2, P = .574) or locations of metastatic sites (P = .073). Comparing the different histological types showed a trend of higher sEGFR concentration in patients with adenocarcinoma (ADC) compared with squamous cell carcinoma (SCC), but the differences were not significant (mean ± 2 SEM; 31.74 ng/mL ± 1.14 versus 30.19 ng/mL ± 1.44, respectively, P = .081). When we evaluated the correlation between sEGFR and treatment response, we found that patients with progressive disease (PD) had lower sEGFR compared with the others (complete response [CR], partial response [PR], and stable disease [SD]), 30.23 ng/mL ± 1.38 versus 31.72 ng/mL ± 1.11, respectively, but again the differences were not significant (P= .094). From the whole cohort, 158 patients received a second-line treatment, and only 8 patients were treated with TK inhibitors (6 received gefitinib, and the other 2,

erlotinib). Considering the small number of TKI-treated patients, no statistical analyses could be performed.

Table 1. **Patient Characteristics**

Total	n=308	(%)
Age (y)		
Median	59.8 (range 31-80)	
Gender		
Male	258	83.8
Female	50	16.2
Histology		
ADC	153	49.7
SCC	95	30.8
LCC	48	15.6
Others	12	3.9
Stage		
IIIB	49	15.9
IV	259	84.1
ECOG-PS		
0	81	26.3
1	221	71.8
2	4	1.3
NA	2	0.6
RESPONSE		
CR	3	1.0
PR	70	22.7
SD	97	31.5
PD	107	34.7
NA	31	10.1

Abbreviations: ADC=adenocarcinomas; CR=complete response; ECOG=Eastern Cooperative Oncology Group;

LCC=large cell carcinomas; NA=not available; PD= progressive disease; PR=partial response; PS=performance status; SCC=squamous cell carcinomas; SD=stable disease. Tumor response was evaluated according to the Response Evaluations Criteria for Solid Tumors (RECIST).[21]

Correlation of sEGFR Concentration with Survival

Considering TTP as an endpoint, shorter intervals were observed in patients with PS≥1 (P= .005), in the group of patients with PD (P < .0001), in those patients with distal metastasis (P = .020), and in the group of patients with three or more metastatic sites (P < .0001). After the dichotomization of sEGFR using an arbitrary cutoff concentration of 34.56 ng/mL (3rd tercile), we found that patients with baseline sEGFR below this cutoff value displayed no significant difference in TTP compared with patients with sEGFR above this value (Fig 1). Survival analysis showed that patients affected by one of the following characteristics had worse outcomes in comparison with the opposite status of each variable: males (P = .018), PS ≥ 1 (P < .0001), presence of distant metastasis (P = .001), and presence of three or more metastatic sites (P < .0001). We found that patients with baseline sEGFR ≤34.56 ng/mL had a shorter OS compared with other patients; median survival: 9.1 months (95% CI 7.6-10.6) versus 12.2 months (95% CI 10.4-13.9), respectively (P = .019; Fig 2).

As shown inTable 2, the subgroup of patients 2, the subgroup of patients with sEGFR ≤34.56 ng/mL had a worse prognosis but was not significantly different from the other groups in the frequencies of histologic types, PS, gender, or treatment response. There were differences for stage (borderline, P=.071) and age (P=.003) between subgroups (Table 2). As the aim of this study was to determine the prognostic value of sEGFR, this variable was tested in the Cox model. In the univariate analysis, only gender, PS, localization of metastasis (local versus distant), number of metastatic sites, as well as sEGFR were significantly associated with survival (Table 3). By contrast, age, histology, and stage were not associated with survival in univariate Kaplan-Meier or Cox regression analyses (data not shown). In the multivariate analysis, sEGFR ≤34.56 ng/mL remained a significant independent prognosticator of reduced OS, after adjusting for

PS, gender, local versus distant metastasis, and the number of metastatic sites. Other significant variables for decreased survival included the following: male gender, PS ≥1, presence of distal metastasis, and the presence of three or more metastatic sites (Table 3). These data suggest that the differences observed in median OS between groups may be due, at least in part, to the different plasma EGFR concentrations.

Discussion

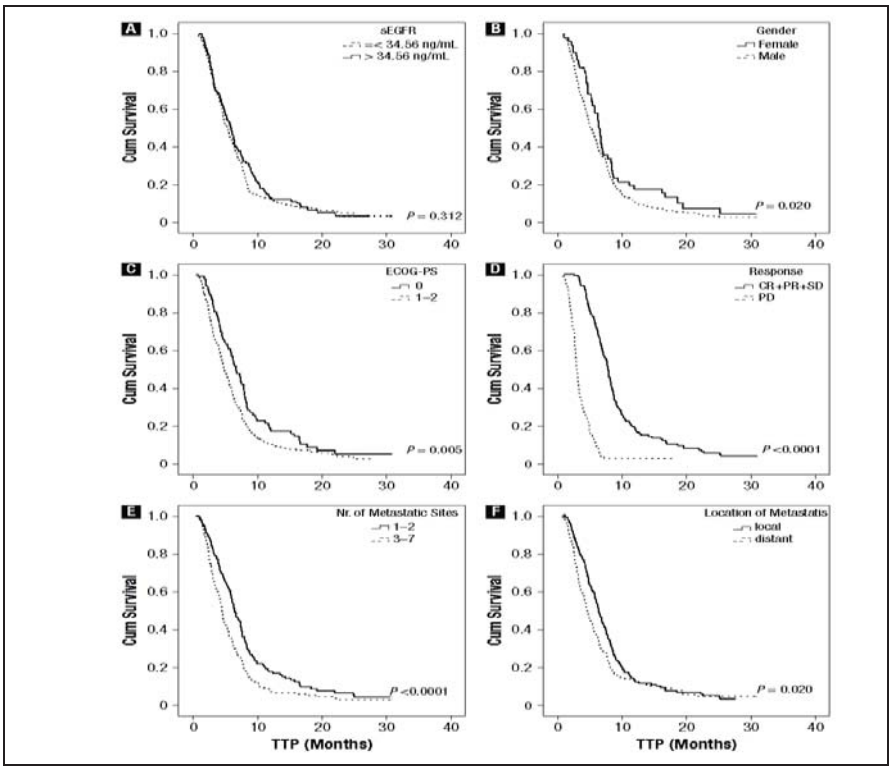
sEGFR consists of a set of differently sized fragments of the extracellular domains of the receptor generated mainly by alternate mRNA splicing (22,23) or by proteolytic cleavage. (24) Both, normal and malignant tissues may contribute to and regulate the pool of sEGFR in circulatory fluids.

Only a few studies have examined serum sEGFR as a prognostic biomarker of NSCLC, (18, 19, 25) and no studies have yet assessed the utility of plasma sEGFR as a prognosticator of NSCLC. Thus, this large-scale study analyzed baseline sEGFR plasma concentration in patients with advanced NSCLC and in healthy age matched controls to clarify the role of EGFR as a biomarker in lung cancer. We observed a significantly lower concentration of sEGFR in NSCLC patients than in controls, although sEGFR is a sensitive (80% sensitivity) but not specific (49% specificity) biomarker to distinguish NSCLC patients from controls. Our finding of lower sEGFR concentrations in samples from cancer patients compared with controls contradicts the results of other reports, (18,19,26) but it agrees with similar data from other tumors, such as metastatic breast (20,27) or ovarian cancer. (28)

Therefore, the field seems to contain mixed and unclear results, and one of the key points that should be addressed to improve the consistency between studies is the selection of the matrix used for the analysis of circulating biomarkers. (29) Many cytokines exhibit substantial differences in serum versus EDTA plasma concentrations (eg, EGF; Vascular Endothelial Growth Factor, VEGF; Interleukin-8, IL-8; monocyte chemoattractant protein-1, MCP-1) (30) that may modify the results, and thus, this difference should be considered to prevent bias and as a possible reason for the lack of

correlation between studies. Most of the above-mentioned studies used serum as the sample matrix, which may not be representative of in vivo conditions because proteases are activated during clotting and the contents of platelets and other blood cells are also released. (31) Consequently, we selected EDTA plasma as the most appropriate matrix for our analysis of the role of EGFR as a biomarker in NSCLC.

Figure 1. Kaplan-Meier Plots for Time-to-Pogression



The other key finding to address is the lower sEGFR concentration observed in NSCLC patients. Overexpression of EGFR is a common trait in NSCLC. (6-10) Thus far, the fact that NSCLC cells overexpress EGFR seems to be controversial, whereas sEGFR

concentration tends to be lower in cancer patients than in control individuals. One possible explanation for this discrepancy is that tumour cells and other non-tumour tissues release sEGFR, so that the sEGFR concentration measured in patients reflects the result of an adapted regulation resulting from tumour cell activity, rather than the simple result of the tumour burden. No correlations were observed between sEGFR concentration and other well-established pathological features of advanced NSCLC (eg, poor PS, stage, histology, gender, number of metastatic sites, and presence of distal metastasis). We found that patients with PD tended to have lower sEGFR concentrations, but this difference was not significant.

Table 2. Patient Characteristics according to sEGFR Concentrations

Patient demographic	sEGFR >34.56 ng/mL		sEGFR ≤34.56 ng/mL		P
	No.	%	No	%	
Total	105	34	203	66	
Age Mean +/- 2 sem	60.2 ± 1.36		56.2 ± 2.22		0.003
Gender					
Male	84	80	174	86	
Female	21	20	29	14	0.131
Histology					
ADC	52	49	101	50	
SCC	29	28	66	33	0.479
Others	24	23	36	17	
Stage					
IV	94	90	165	81	0-071
ECOG					
0	28	27	53	26	
1	76	72	145	71	
2	0	0	4	2	0.352
RESPONSE[a]					
CR	2	1.9	1	0.5	
PR	24	22.9	46	22.7	
EE	39	37.1	58	28.6	0.252
PD	33	31.4	74	36.5	
NA	7	6.7	24	11.8	

Abbreviations: ADC=adenocarcinomas; CR=complete response; ECOG=Eastern Cooperative Oncology Group; NA=not available; PD=progressive disease; PR=partial response; sEGFR=soluble fragmenre of the epidermal growth factor receptor. Tumour response was evaluated according to the Response Evaluation Criteria for Solid Tumors (RECIST).[21]

To date, the data regarding the potential of sEGFR as a prognostic biomarker for patients with various solid tumours are conflicting. In metastatic breast cancer, several reports show that patients with decreased concentrations of sEGFR have reduced survival, (20, 27, 32) whereas no association between sEGFR and prognosis was found in lung and cervical cancer. (18, 19, 26, 33, 34) Gregorc et al identified an increasing serum EGFR concentration at 28 days after gefitinib treatment in lung cancer patients as a significant indicator of disease progression and shorter progression-free survival. (35)

Table 3. Cox Regression Analysis for Overall Survival

Variable	Univariate				Multivariate			
	HR	95%IC		P	HR	95%IC		P
PS (1-2 vs. 0)	1.722	1.284	2.310	<0.001	1.658	1.231	2.232	.001
Gender (M vs. F)	1.536	1.074	2.197	.019	1.554	1.080	2.236	.017
Metastasic (distant vs local)	1.551	1.209	1.991	.001	1.369	1.055	1.776	.018
No. of Lesions (>2 vs. ≤2)	1.581	1.224	2.041	<.001	1.441	1.105	1.878	.007
sEGFR(ng/mL) (≤34.56 vs. >34.56)	1.367	1.051	1.780	.020	1.332	1.020	1.741	.036

Abbreviations: PS=performance status; sEGFR=soluble fragment of the epidermal growth factor receptor.

To our knowledge, these results are the first reported in a large cohort of NSCLC patients, demonstrating that patients with baseline sEGFR ≤ 34.56 ng/mL showed a poorer prognosis with regard to OS (P=.019). The poorer prognoses observed in this group of patients may have multiple hypothetical explanations: (1) the increased circulating concentrations of EGFR ligands observed in cancer patients could

easily form complexes with the circulating form of EGFR and consequently may be rapidly cleared from the bloodstream, and (2) the low concentration of sEGFR may prevent the formation of sEGFR/ EGFR dimers (36) which are capable of inhibiting the holoreceptor's intracellular kinase activity acting as negative regulators of the EGFR signaling cascade. Therefore, because of the trapping effect of sEGFR, in patients with low concentrations of sEGFR, there are more ligands (eg, EGF, TGF-alfa, amphiregulin) available to stimulate the EGFR pathway, finally leading to cancer cell survival as well as proliferation and consequently a poorer prognosis for this group of patients.

Conclusion

We studied the role of baseline plasma sEGFR concentration in advanced NSCLC, and our results indicate that sEGFR is a useful prognostic biomarker in these patients. In our cohort of 308 patients, lower sEGFR concentration was related to a poorer prognosis. Considering the fact that plasma samples are readily available in NSCLC patients, the low cost of this type of analytical test and the potential to provide valuable prognostic information, it could be interesting to further evaluate this marker in prospective large-scale studies as a predictor of response to chemotherapy-based treatments and also in trials designed to evaluate anti-angiogenic agents and TKIs.

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PUBLICACIÓN 4

PUBLICACION 4

Retrospective analysis of the prognostic role of p16 protein inactivation in plasma in patients with locally advanced non-small cell lung cancer

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SUMMARY

It has been analyzed the frequency of p16 inactivation in 67 blood samples of patients diagnosed with advanced non-small cell lung cancer (NSCLC), to establish the relationship between p16 inactivation and time to progression (TTP) and overall survival (OS), and its relationship with various clinical parameters. This is a retrospective study of 67 patients diagnosed with advanced NSCLC between August 2000 and July 2003 in the Hospital General de Valencia analysing p16 inactivation by assessing in plasma either loss of heterozygosity (LOH) or p16 promoter methylation. The study shows p16 inactivation in 28.3% (either by LOH or by p16 methylation). No significant differences were found between the group with p16 inactivation and the group without p16 inactivation, either in patients' TTP (31 weeks vs. 24 weeks; $p = 0.7$) or in OS (53 weeks vs. 43 weeks; $p = 0.48$). No relationship was found between the state of p16 and the clinical parameters analyzed (stage, ECOG, histology). Despite the fact that p16 is important in NSCLC carcinogenesis, the data obtained in our study do not allow the prognostic impact of this biological marker to be established.

KEYWORDS

Non-small cell lung cancer; NSCLC; Loss of heterozygosity; LOH; p16 methylation; Biological marker

1. Introduction

Lung cancer is the primary cause of cancer morbidity and dence of 30.9 for men and 12.6 for women per 100,000 inhabitants [1]. The average survival of advanced non-small cell lung cancer (NSCLC) is 8—10 months [2]. The genesis of NSCLC is the result of multiple genetic alterations including mutations in oncogenes (e.g. K-ras) and tumour suppressors genes (TSG) (e.g. p53, p16 and Rb) [3]. TSG codify proteins that take care of the negative regulation of cell growth as well as other functions related with invasivity and the metastastic potential of tumours. The loss of its protective function adds to the development of the tumour. According to Knudson's ''double impact'', the two alleles must be inactivated in order for the TSG to stop functioning [4]. In many cases, the first allele is inactivated by the loss of a region of DNA of the germ-line, the most frequent alterations being when inactivating the other allele: mutation, loss of heterozygosity (LOH) by deletion or hypermethylation of its promoters.

The LOH method is used to search for new TSGs in the human genome. This method is based on the use of microsatellites — short, repeated, non-coding and polymorphic nuclear DNA sequences that can be used as molecular markers — near a TSG. When an alteration is detected in one of these sequences there is a very high probability that an individual will be heterozygous for this gene. The loss of heterozygosity (LOH) occurs when the other allele is lost by deletion. The individual should be heterozygous for this region in order to analyze the presence of LOH. The chromosomes most frequently affected by LOH in NSCLC are 1p, 3p, 4q, 5q, 6q, 8p, 9p21, 10q, 11p, 13q14, 17p13, 18q, 19q and 22q [5,6].

Cytogenetic and molecular studies have shown the presence of TSG in chromosome 9p21, which encodes for a protein called p16 and has an important role in regulating the cell cycle in early stages, and its involvement in carcinogenesis of NSCLC [7—9].

The objectives of the study are to analyze the inactivation frequency of the p16 protein — either using LOH in the 9p21 chromosomic region or by methylating the

promoter — in 67 blood samples of patients diagnosed with advanced NSCLC, to establish the relationship between p16 inactivation, time to progression (TTP) and overall survival (OS), and its relationship with various clinical parameters as stage, ECOG and tumour histology.

2. Materials and methods

2.1. Patients and samples

A total of 67 plasma samples were analyzed. These samples were extracted between August 2000 and July 2003 in the Hospital General de Valencia from patients diagnosed with advanced NSCLC (stage IIIB and IV). Thirty-five patients were classified as stage IV and 32 as IIIB. Sixty-four patients were men (95%) and three women (5%). Median age of the patients was 62 (27—82). Sixty-three patients were smokers (95%) and four non-smokers (5%). Before beginning chemotherapy, 16 patients had ECOG 0, 44 ECOG 1 and 7 ECOG 2.

The histological subtype distribution was: 28 adenocarcinomas (42%), 20 squamous cell carcinoma (30%), 10 large cell carcinoma (15%) and nine non-differentiated carcinoma (13%). Forty-seven patients (70%) had no previous intervention, 66 (98%) had received no previous chemotherapy and 55 (82%) had no radiotherapy before beginning chemotherapy.

The 67 patients were treated with combined CDDP chemotherapy (70 mg/m^2 d1) + Gemcitabine (1200 mg/m^2 d1 and 8) every 21 days with a six-cycle treatment package. Fifty-seven patients (85%) completed three treatment cycles and 19 the six cycles (28%). The response was assessed via a thoracic and abdominal CT scan after the 3rd and 6th cycle.

The peripheral blood sample was taken from each patient before beginning chemotherapy by using a vacutainer tube with anticoagulant (EDTA). The lymphocytes were isolated and DNA was extracted from the lymphocytes and used as a control in order to study microsatellites.

2.2. LOH analysis

The DNA from the plasma and the lymphocytes was isolated by using the QiAamp Blood Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Four hundred microlitres of the initial plasma were used to isolate the DNA.

The amount of DNA was measured by means of the absorbance at 260/280/320 nm after each extraction by using the GeneQuant pro RNA/DNA spectrometer.

The D9S1747 marker was used to analyze the loss of heterozygosity (LOH) in the 9p21 chromosomic region. The PCR amplification was done with 0.12 mM of dNTPs (PCR Nucleotide Mix, Boehringer Mannheim), 2 mM of MgCl2, 0.1 µL of primers, 5 µLof10× buffer and 2.5 U of EcoTaq DNA Polymerase (Ecogen, España). One of the two primers used was marked with fluorescence (carbocyanine Cy5) with a total volume of 50 µL. The PCR program consisted of 35 cycles: 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s. 94 °C for 10 min was done before beginning the first cycle and 72 °C for 7 min before beginning the last. The Perkin-Elmer Gene Amp System 9600 thermocycle (Applied Biosystem, Norwalk, CT, USA) was used. The amount of sample added to the Master Mix was 5 µL for the plasmatic DNA and 1µL for the lymphocytic DNA. The primers used for the PCR were primer forward: 5-GGCTTTCTCTCTTTTTGTCTC-3and primer reverse: 5-GGAATAAATCAGGCTACCAGG-3

The PCR amplification products were placed in a 1% agarose gel embedded in a TBE buffer. A large fragment was amplified if a region of plasmatic DNA and lymphocytic DNA was detected in the same patient. If the chromosomic region was not found, a second PCR was carried out with the same primers and in the same conditions as the first, varying the number of centrifuge cycles from 35 to 25. The amount of DNA added to the Master Mix was then 1 µL of serological DNA and 0.5 µL lymphocytic DNA.

The PCR fragment length analysis was done with electrophoresis in an 8% polyacrylamide gel (Reprogel High Resolution, Amersham Biosciences, Sweden) and

was detected by fluorescence induced by helium—neon laser, using an automatic genomic sequencer (ALFWIN Express DNA Sequencer, Amersham Biosciences, Sweden).

The PCR from lymphocyte and plasma products were analyzed in the same gel. The size of the amplified allelic microsatellites was calculated mixing the PCR product with internal markers with a fluorescence of between 50 and 150 pb. External markers were used with a fluorescence of 50 and 500 pb (Amersham, Biosciences, Sweden). The data were analyzed with the Fragment Analyzer 1.02 program (Amersham, Biosciences, Sweden).

The peak corresponding to the DNA control sample was used to determine whether the sample was homozygotic (only one peak) or heterozygotic (two peaks). For each of the heterozygotic samples the size of the two alleles was calculated according to the two highest peaks. An automatic calculation was done of the peak areas and the ratio of the areas of the alleles (RA). The ratio of the alleles was calculated with the following formula. In cases where the RA was greater than 1, the ratio was inverted (1/RA) with the aim of normalizing the results. We consider that heterozygosity exists when the ratio between the PCR product of the serological sample and the normal tissue sample (lymphocytes) was greater than 50% of the cut-off point. We consider that there is a loss of heterozygosity when one of the two alleles completely disappears into the tumour as opposed to the normal tissue (lymphocytes), or when the relationship between the intensity of the two alleles of the tumoural sample differs at least by 40% with that observed in the normal tissue sample.

2.3. p16 promoter methylation analysis

p16 methylation-specific PCR was used to determine the methylation status of the CpG island of p16 in all plasma samples and in the matched lymphocytes as described [10]. Plasma DNA or DNA from lymphocytes was modified with sodium bisulfite. DNA samples were then purified with the Wizard DNA purification resin

(Promega, USA), again treated with sodium hydroxide, precipitated with ethanol, and resuspended in water. PCR 50 µl reaction volume contained DNA, each deoxynucleotide triphosphate at 300mM, 3mM MgCl2, 0,75mM PCR primers, and 1 unit of Hot Start DNA polymerase (Qiagen, USA). DNA was amplified by an initial cycle at 95°C for 15 min, followed by 40 cycles at 95°C for 30 s, annealing for 30 s (65°C for methylated and 63°C for unmethylated), and 72°C for 30 s, and ending with a 10-min extension at 72°C in a 2700 Thermocycler (Applied Biosystems, USA). Placental DNA treated in vitro with Sss I methyltransferase (New England Biolabs, USA) was used as a positive control for methylated alleles of p16, and DNA from patient's lymphocytes was used as negative control. Primers specific for methylated p16: primer forward: 5-TTATTAGAGGGTGGGGCGGATCGCGTCG-3and primer reverse: 5-ACCCGACCCCGAACCGCGACCGTAA-3¨; and primers specific for unmethylated p16: primer forward: 5-TTATTAGAGGGTGGGGTGGATTGT-3and primer reverse: 5-CAACCCCAAACCACAACCATAA-3as described previously [11, 12]. PCR products were loaded directly onto 1.5% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

2.4. Statistical analysis.

It was done using the SPSS package. The frequency distribution was analyzed using the chi-squared test. The survival curves were calculated with the Kaplan—Meier method and were compared by means of the log-rank test. The proportional risk of dying and the 95% confidence intervals for the multivariate analysis were estimated with Cox's regression model.

3. Results

Of the 67 samples analyzed, 9 (14%) were heterozygotes for marker D9S1747 and had a loss of heterozygosicity, 40 (60%) were homozygotes or heterozygotes for the marker with no loss of heterozygosicity and 18 (26%) were not-amplifiable samples.

Of the 67 samples analyzed, 12 (18%) had p16 promoter methylation, 50 (74%) had no p16 promoter methylation and five cases were unknown (8%).

Of the 67 samples analyzed, 19 (28%) had inactivation of the p16 protein (by loss of heterozygosicity and/or by p16 promoter methylation), 43 (64%) had no inactivation of the protein and 5 (8%) were unknown.

The distribution observed of the samples according to histology were: of the 19 samples with inactivation of the p16 protein, nine were adenocarcinomas (47%), three squamous carcinomas (15%), four large cell carcinomas (23%) and three anaplasic carcinomas (15%); of the 43 samples without inactivation of the p16 protein, 16 were adenocarcinomas (37%), 16 squamous carcinomas (37%), five large cell carcinomas (12%) and six anaplasic carcinomas (14%). No significant differences were found in the distribution of the samples according to the histological type between both groups with a $p = 0.52$.

The distribution observed of the samples according to the stage was: of the p16 protein inactivation group, nine were staged as EIIIB (47%) and 10 as EIV (53%); of the group without the p16 protein inactivation, 21 were staged as EIIIB (48%) and 22 as EIV (52%). No significant differences were found in the distribution of the samples according to the stage between both groups with a $p = 0.9$.

The distribution observed of the samples according to the ECOG was: of the p16 protein inactivation group, six had ECOG 0 (31%), 10 ECOG 1 (52%) and three ECOG 2 (17%); of the group without the p16 inactivation, nine had ECOG 0 (21%), 30 ECOG 1 (69%) and four ECOG 2 (10%) without any significant differences between both groups.

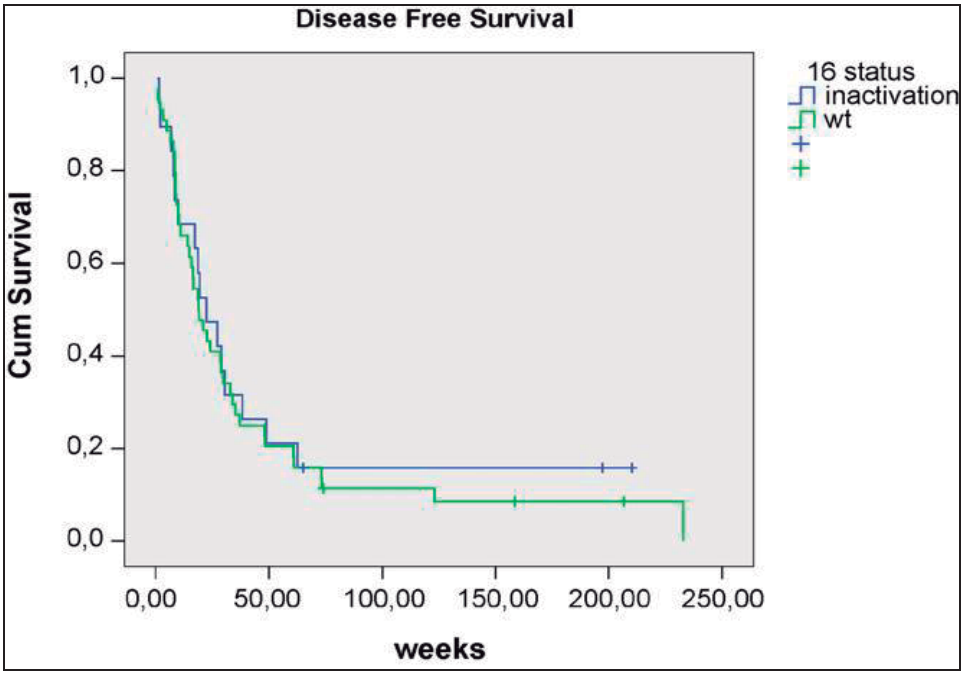
Neither was any relationship found among other clinical variables such as age, sex and smoking, and the state of the p16 protein (inactivated/non-inactivated).

No significant differences were found in time to progression (Fig. 1) and overall survival (Fig. 2) of patients between the p16 protein inactivation group and the group without the p16 protein inactivation (31 weeks vs. 24 weeks; $p = 0.7$ and 53 weeks vs. 43 weeks; $p = 0.48$, respectively).

4. Discussion

One of the lines of research of translational oncology over the last few years has been devoted to improving the prognostic prediction and response to treatment, combining clinical variables (tumour size, stage, histology, differentiation) with biochemical and genetic characteristics intrinsic to the tumour.

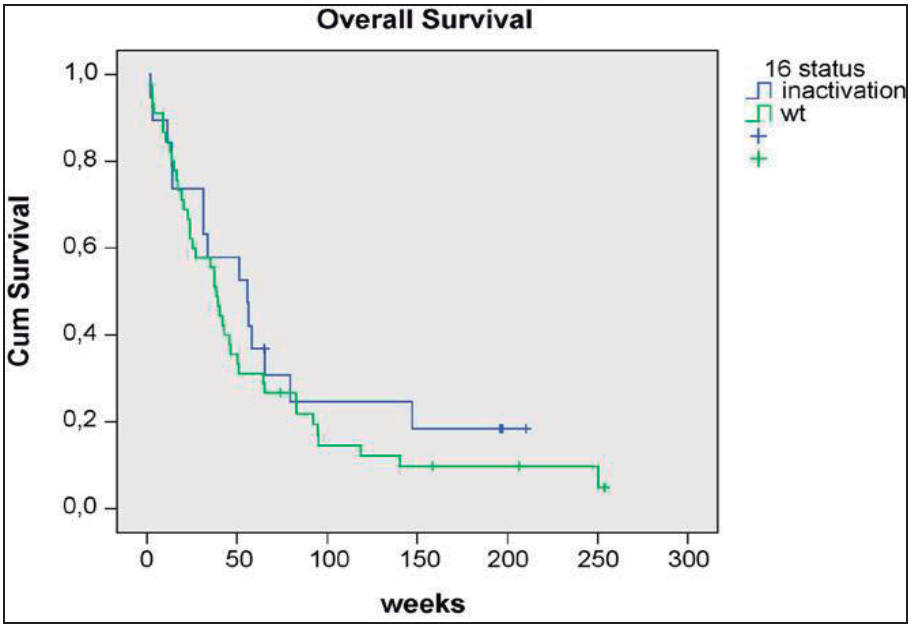
Fig. 1 Disease-free survival in advanced NSCLC patients according to p16 status in plasma. Inactivation means p16 promoter methylation or LOH in p16 chromosomic region.



Despite researching multiple molecular markers (involved in regulating the cell

cycle, apoptosis and angiogenesis), a clinically useful marker that contributes independent prognostic information has yet to be established [13]. It has been demonstrated that DNA concentrations in the serum are higher in cancer patients than in healthy individuals [14], this DNA present the same genetic alterations that the genetic material of the corresponding tumour [15] andthat DNA analysis could be related with the prognosis of the disease [16,17].

Fig. 2 Overall survival in advanced NSCLC patients according to p16 status in plasma. Inactivation means p16 promoter methylation or LOH in p16 chromosomic region.



Region 9p21 contains a suppressor gene called CDKN2A, which encodes for the p16 protein that belongs to the kinase family of inhibitory proteins dependent on cyclines, INK4, formed by: p16, p15, p18, p19. These proteins join and inhibit the formation of CDK4/6 complexes with cycline D, thereby impeding the progression of the cell cycle of phase G1 and phase S. When this mechanism is inhibited there is a high risk of uncontrolled cell growth [18, 19]. The metabolic mechanism of senescence $p16^{INK4a}$-cyclin D-CDK4-RB plays a major role in regulating the cell cycle in early stages (Table 1).

Table 1. Clinico-pathologic Characteristics of NSCLC Patients.

	p16 inactivation (n=19)	wt p16 (n=43)
Sex		
Male	19	40
Female	0	3
Stage		
IIIB	9	21
IV	10	22
Histology		
Adenocarcinoma	9	16
Squamous	3	5
Large Cell	4	6
Undifferenciated	3	6
ECOG		
0	6	9
1	10	30
2	3	4
Smoking history		
Yes	18	40
No	1	3

Cytogenetic and molecular studies have shown the presence of TSG in chromosome 9p involved in NSCLC carcinogenesis [8, 20, 21]. Mutations in the $p16^{INK4a}$ gene (including deletions, specific mutations and epigenetic alterations), which is present in a great variety of tumours, is observed in 30—70% of NSCLC cases [20]. In our analysis we observed p16 inactivation, either through loss of heterozygosity or through p16 promoter methylation in 28% of the samples analyzed, which is in accordance with data described in the literature.

Seventy serological samples from patients diagnosed with advanced NSCLC were analyzed and then classified into two groups: samples with p16 inactivation and those without. After analyzing the data we found no significant differences either in time to progression or in survival when comparing both groups. The lack of a prognostic impact by p16 inactivation found in serum can be related to the limited number of patients in the study. In fact Kaplan-Meyer analysis showed that there was a tendency in our series towards a better overall survival in patients with inactivated p16 in circulation and it seems that with a larger cohort the difference in survival could have become more evident.

Several studies on p16 show consistent data on the increased survival of those patients with a high expression of protein p16, although not all attain statistical significance [8]. Most of these studies have a greater sample size than ours (about 100 samples) and include initial stages of the illness which are important differences that could influence the negative results of our analysis.

As other have previously demonstrated we obtained no significant correlation among the clinical parameters (ECOG, histology and stage) and inactivation or not of p16 [22].In the study carried out by Esposito et al. with 105 patients diagnosed with stages I-III NSCLC a better prognosis is

The prognostic role of p16 protein inactivation in patients with advanced NSCLC observed with significant differences in those patients with p16 expression, cyclin-D and pRb2/p130, in the multivariate analysis. By putting the patients into three groups, depending if they have an adverse biological prognostic factor of 0 or 1, or 2 or 3, significant differences are observed in survival among the three groups, with a poor prognosis in those patients that express none of the three proteins. These data confirm the importance of the $p16^{INK4a}$-cyclin D-CDK4-RB regulating mechanism of the cell cycle in NSCLC pathogenesis and the interaction of several of its products.

Although the data available in the literature seem to clearly show the importance of p16 in NSCLC carcinogenesis, the results obtained in our study do not allow the prognostic impact of this biological marker to be established, and we thus

consider it necessary to carry out new studies in this direction and search for new therapeutic strategies directed at multiple targets related with cell cycle regulation.

Conflict of interest None.

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IV. DISCUSIÓN GENERAL

IV. DISCUSIÓN GENERAL

En la actualidad, se están estudiando diferentes marcadores predictivos moleculares (EGFR, EML4-ALK) y estrategias terapéuticas individualizadas (QT secuencial, mantenimientos, segndas líneas precoces, tripletes, antiangiogénicos, anticuerpos monoclonales, ITKs, etc.) que nos permitan seleccionar a los pacientes y mejorar estos resultados. En esta misma línea de investigación presentamos nuestros cuatro trabajos centrándonos en la determinación mediante análisis molecular de nuevos factores pronósticos en sangre periférica de pacientes con CNMP localmente avanzado o metastásico en diferentes estrategias de tratamiento.

El primer trabajo presentado propone la administración secuencial de fármacos como una opción terapéutica válida para el tratamiento del CNMP localmente-avanzado y/o metastásico. El tratamiento secuencial consiste en la alternancia en la administración de un número determinado de ciclos de un fármaco, seguido a continuación de un número determinado de ciclos del segundo fármaco, aunque existiese respuesta al primer régimen sin objetivarse resistencia. La elección de la combinación de fármacos y el orden de administración de los mismos es crítica para vencer las posibles resistencias que existen a nivel molecular (Pfister,D.G. *et al.* 2004; NSCLC Collaborative Group. 1995), reducir la toxicidad y maximizar la actividad de los fármacos. Diversos estudios de administración secuencial de QT se han llevado a cabo en los últimos años con diferentes regímenes de QT en pacientes con CNMP avanzado de elevado riesgo (ancianos, ECOG 2) con tasas de respuesta objetiva que oscilan entre el 30-50% y tiempo hasta la progresión entre 6-9 meses (Rixe,O. *et al.* 2005; Hirsh,V. *et al.* 2005; Hesketh,P.J. *et al.* 2006; Manegold,C. *et al.* 2006; Martoni,A.A. *et al*. 2006). El estudio fase II realizado por el grupo Oncopaz, que administró seis dosis de Paclitaxel semanal seguido del triplete Cisplatino/Gemcitabina/Vinorelbina, alcanzando una tasa de respuesta del 54% y un porcentaje de pacientes vivos al año del 56% (Feliu,J. *et al.* 2001); el estudio del SWOG que evalúo dos regimenes secuenciales con tres ciclos de Carboplatino/Gemcitabina seguidos de tres ciclos de Paclitaxel o tres ciclos de

Cisplatino/Vinorelbina seguido de Docetaxel, con resultados similares a los alcanzados con los dobletes, pero con mayor toxicidad hematológica (Edelman,M.J. *et al.* 2004); y finalmente el ensayo fase II/III de Manegold y colaboradores que trató de determinar si era mejor administrar el doblete Gemcitabina/Vinorelbina o tres ciclos de Gemcitabina seguidos de otros tres de Docetaxel, observando que la eficacia era similar pero que los costes con el esquema secuencial eran menores (Manegold,C. *et al.* 2005). Nuestro estudio trata de determinar la eficacia y tolerabilidad de la monoterapia secuencial con Gemcitabina y Paclitaxel en el tratamiento del CPNM localmente avanzado y/o metastásico siendo la tasa de respuestas objetivas, el objetivo principal del mismo. Entre los objetivos secundarios estaban: determinar los niveles en sangre periférica de VEGF, EGFR y hTERT así como analizar las posibles mutaciones del oncogén Kras en el DNA aislado, la toxicidad del esquema, la SLP, la SG y la QoL. Se empleó un diseño en dos fases para evitar prolongar el estudio excesivamente en caso de que la eficacia fuera demasiado baja tras la primera fase. En nuestro estudio, que resultó negativo, y a diferencia de otros estudios positivos en los que la estrategia de administración secuencial de fármacos se reservaba a pacientes ancianos y con mal estado funcional, la mediana de edad fue de 67 años (rango 46-77) y en general los pacientes incluidos presentaban buen estado funcional (ECOG 1 81,8%) en teoría posibles candidatos a recibir esquemas de combinación con platino y obtener mejores tasas de respuesta y supervivencia como han demostrado diversos estudios como el de Le Chevalier o el metaanálisis de Delbaldo (Le Chevalier,T. *et al.* 1994; Delbaldo,C. *et al*. 2004). Con nuestros resultados, podemos concluir que la monoterapia secuencial con Gemcitabina y Paclitaxel es bien tolerada con una proporción baja de efectos adversos grado 3-4 y sin toxicidad inesperada; por lo que podría ser considerada una alternativa más en el arsenal terapéutico del CNMP localmente avanzado y/o metastático. Con respecto al análisis farmacogenómico encontramos que la baja expresión de VEGF se relacionaba con un menor riesgo de muerte en nuestra población, siendo esta relación estadísticamente significativa para progresión y supervivencia (p=0.03 y 0.04, respectivamente) y consistente con los

datos de la literatura (Bremnes,R.M. *et al.* 2006; Laack,E. *et al.* 2002). La angiogénesis es el proceso por el cual las células tumorales reclutan células endoteliales para crear un sistema de suministro de oxígeno y nutrientes suficientes más allá del limite de difusión de éstos. La secreción del VEGF, entre otros, es el estímulo más potente para la inducción de este proceso. Se ha demostrado que las células de CNMP son capaces de secretar esta proteína y de translocar su receptor de membrana, generando así un bucle autocrino incontrolado (Fontanini,J.R. *et al*. 2002). Además la presencia de VEGF intratumoral en pacientes intervenidos de CNMP se asocia a peor pronóstico (Han,H. *et al.* 2001). Es más se puede correlacionar la determinación sérica de este VEGF con la presencia de expresión intratumoral, por lo que su evaluación en sangre periférica tiene un gran valor potencial en la estratificación pronóstica de estos pacientes (Brattström,D. *et al.* 2002).

Otro de los principales objetivos en investigación clínica en los pacientes con CNMP es la búsqueda de factores genéticos relacionados con la respuesta al tratamiento con la intencionalidad de personalizarlos al máximo y conseguir la mayor eficacia con la menor toxicidad. En el segundo trabajo presentado, tratamos de averiguar si la neutropenia secundaria al tratamiento quimioterápico en pacientes con CNMP avanzado se asociaba con un aumento en la SG. La hipótesis de que la neutropenia se comporta como un marcador de la actividad del fármaco, se sustenta en la idea de que la célula tumoral es una diana para la QT, de la misma forma que lo son las células sanas del organismo y particularmente las células hematopoyéticas (Kvinnsland,S. 1999). Los factores farmacocinéticos como el metabolismo, la distribución y el catabolismo que condicionan la biodisponibilidad de la droga, son los mismos para ambos tipos de células, como también puede serlo la predisposición genética para la quimiosensibilidad. Como objetivos secundarios de nuestro trabajo estaban: establecer si aquellos pacientes que no desarrollan neutropenia habían sido infratratados, y analizar polimorfismos (SNPs) específicos de genes implicados en los mecanismos de reparación del DNA como XRCC1-118, ERCC1-8902, XRCC3, XPD-23,

and XPD-10 y valorar su correlación con las tasas de neutropenia, pues la capacidad de reparación del DNA y por tanto de la eliminación de los aductos de DNA formados por el cisplatino es determinante en los mecanismos de resistencia al fármaco, de manera que variaciones individuales en polimorfismos de genes implicados en los sistemas de reparación podrían condicionar la respuesta a los tratamientos y por tanto su eficacia.

Los resultados de nuestro análisis muestran, que aunque existe una tendencia favorable en términos de SG en los pacientes que desarrollan neutropenia, las diferencias no alcanzan la significación estadística. Dichos resultados contrastan con los observados en estudios similares al nuestro como el publicado por Di Maio (Di Maio,M. *et al.* 2005), que analizaba un total de 1265 pacientes incluidos en 3 ensayos clínicos diferentes (The Elderly Lung Cancer Vinorelbine Italian Study Group. 1999; Gridelli,C. *et al.* 2003; Gridelli,C., Perrone,F. *et al.* 2003) encontrando que la SG de los pacientes que desarrollaban neutropenia era superior a la de aquellos que no lo hacían: 42 vs. 31,4 semanas (p=0.0118). Además de tener una muestra de pacientes mayor a la nuestra, en el análisis de Di Maio los pacientes recibían un tratamiento diferente al nuestro, con tan sólo un 20% de pacientes tratados con un esquema basado en cisplatino; y que, a pesar del uso de esquemas con menor toxicidad hematopoyética que el nuestro, la tasa de neutropenia en el análisis de Di Maio fue siempre superior, probablemente porque a los pacientes se les realizaba un control analítico los días 1 y 8 de cada ciclo, siendo mayor la probabilidad de detectar la neutropenia en comparación con nuestro control analítico que sólo era realizado el día 1 de cada ciclo; tal vez si en nuestro análisis se hubiese realizado un nadir el día 8 o incluso el día 15 de cada ciclo, la tasa de neutropenia hubiese sido mayor, y nuestros resultados diferentes. Por otra parte, los tres ensayos sobre los que realiza el análisis Di Maio estaban destinados a pacientes de edad avanzada y consecuentemente las diferentes características clínicas y concretamente de la reserva hematopoyética en el paciente anciano también influyen en la interpretación de los resultados (Repetto,L.. 2003).

En nuestro estudio el subgrupo de pacientes con buen estado general (ECOG 0), la aparición de neutropenia sí fue un factor pronóstico importante, presentando un riesgo de muerte inferior a aquellos que no la desarrollaron. De confirmarse que la neutropenia es un marcador de actividad antitumoral de la QT, se podrían plantear esquemas de tratamiento con dosis de fármacos variables en función de la presencia de toxicidad hematológica. Para ello se deberían plantear estudios prospectivos que confirmen estos hallazgos mediante la comparación de la eficacia de los fármacos a dosis fijas, con otras dosis ajustadas en función del desarrollo de toxicidad hematológica.

Por otro lado, no hemos encontrado en nuestro estudio correlación alguna entre los SNPs analizados -ERCC1 N118N (exon 4), ERCC1 G8092T (3´UTR), XRCC3 T241M (exon 7), XPD K571Q (exon 23) y XPD D312N (exon 10)- y la presencia de neutropenia.

El cisplatino actúa preferentemente sobre las bases de DNA y en particular con los residuos de la posición N7 de la guanina y de la adenina para formar aductos mono o bifuncionales que pueden reaccionar para formar enlaces opuestos cruzados entre las dos cadenas de DNA, lo que se traduce en modificaciones en la estructura y función del mismo, y consecuentemente, en una fuerte inhibición de la síntesis de DNA (Chaney,S.G. *et al.* 1996). En 27 pacientes con CNMP localmente avanzado tratados con quimiorradioterapia concomitante con cisplatino se observó como factor pronostico independiente en el análisis multivariante que los pacientes con low cisplatin-DNA-adduct staining presentaban menor tasa de supervivencia (van der Vaart ,P.J. *et al.* 2000). La capacidad de reparación del DNA, y por tanto, de la eliminación de los aductos de DNA formados por el cisplatino, es determinante en los mecanismos de resistencia al fármaco, de manera que variaciones individuales en SNPs de genes implicados en los sistemas de reparación condicionarán la respuesta a los tratamientos (García-Campelo,R. *et al.* 2006). Así se ha visto que pacientes con CNMP avanzado tratados con cisplatino y con menor capacidad de reparacion de DNA presentan mayor

tasa de superviencia que aquellos con mayor capacidad de reparación (8.9 m versus 15.8 m (P=0.04) siendo el riesgo relativo de muerte el doble para los pacientes con mayor capacidad de reparación del DNA (RR=2.72; 95%IC=1.24-5.95; p=0.01) (Bosken,C.H. *et al.* 2002). También se han analizado los SNPs en XPD y XRCC1 en 103 pacientes con CNMP avanzado tratados con QT basada en doblete de sales de platino, observando que las variantes alélicas XPD Asp312Asn y XRCC1 Arg399Gln presentan tasas menores de supervivencia independientemente de estadio, performance status y régimen de tratamiento, por lo que los autores concluyen que los SNPs en XPD y XRCC1 son importantes factores pronósticos y de respuesta a los tratamientos en el CNMP (Gurubhagavatula,S. *et al*. 2004). Así mismo, en el estudio realizado por Camps en 39 pacientes con CNMP avanzado tratados con cisplatino/gemcitabina, el subgrupo de pacientes con enfermedad metastásica portadores de los SNPs XPD Asp312Asn y Asn312Asn presentaban una tendencia hacia una mejor respuesta al tratamiento que el subgrupo Asp312Asp (40% vs. 16.7%; p = 0.7) (Camps,C. *et al.* 2003).

Determinados SNPs del ERCC1, implicado en la vía NER, se han relacionado con mejores supervivencias en pacientes con CNMP tratados con dobletes de platino, así de acuerdo con los datos de Zhou en 128 pacientes se alcanzaban medias de supervivencia de 22.3 meses en pacientes con ERCC1 C8092C frente a 13.4 meses en los grupos ERCC1 C8092A y A8092A (Zhou,W. *et al.* 2004; Rosell,R *et al* 2003). Existe una fuerte correlación aunque no estadísticamente significativa entre los niveles bajos de expresión de ERCC1 en las biopsias de los pacientes antes de iniciar el tratamiento y las medianas de supervivencia alcanzadas por los pacientes tratados con gemcitabine plus cisplatin (13.7 months for patients with low levels and 9.5 m for those with high levels, p = 0.19) (Rosell,R. *et al.* 2004).

El XRCC3 presenta un papel fundamental entre los mecanismos de reparación de DNA y concretamente en el mecanismo de reparación de la doble cadena de DNA mediante recombinación homologa. El SNP en el codon 241 (Thr a Met) del XRCC3 se ha asociado con los niveles de aductos de DNA en leucocitos de sujetos sanos. Los sujetos portadores de XRCC3 Met241Met presentan niveles mayores de aductos de

DNA independientemente del hábito tabáquico (Matullo,G. *et al.* 2000), lo que implicaría una reparación ineficiente del DNA y podría traducirse en una mayor sensibilidad a los tratamientos.

El tercer trabajo analiza en profundidad el papel del EGFR como factor pronóstico en CNMP. El EGFR es un miembro de la familia de receptores de la proteína quinasa transmembrana conocida como familia del receptor erbB o HER: EGFR (HER1 o erbB1), erbB2 (HER2), erbB3 (HER3) y erbB4 (HER4) (Mendelsohn,J. and Baselga,J 2003). El EGFR está codificado por un gen localizado en el locus 7p11.2 del cromosoma 7. Este gen está formado por 28 exones de alrededor de 200 kilobases. El EGFR se sintetiza a partir de un precursor polipéptido de 1210 residuos; tras la escisión de la secuencia N-terminal, se inserta una proteína de 1186 residuos en la membrana celular (Ullrich,A. *et al*. 1984). El receptor del EGFR es pues una glicoproteína transmembrana formada por la unión de un ligando extracelular, una región hidrófoba transmembrana y un dominio intracelular con actividad TK para la transducción de la señal. La parte extracelular del EGFR (o ectodominio) está formada por cuatro dominios: dos (los llamados pliegues β-solenoide o β-helicoidal) donde se une el ligando y otros dos con un asa grande que contacta con el mismo dominio del otro receptor en la dimerización (Ogiso,H. *et al.* 2002). La región transmembrana es un dominio α-helicoidal que continúa en el dominio yuxtamembrana. La región intracelular está formada por tres dominios a su vez: 1) la región yuxtamembrana, de aproximadamente 50 aminoácidos y numerosas funciones reguladoras, como hiporregulación y acontecimientos de internalización dependientes del ligando, clasificación basolateral del EGFR en células polarizadas, y asociación con proteínas como eps8, calmodulina, proteína quinasa C (PKC) y proteínas quinasas activadas por mitógenos (MAPK) y ERK (He,C. *et al*. 2002; Castagnino,P. *et al.* 1995) 2) el dominio catalítico, de aproximadamente 250 aminoácidos; y 3) el dominio carboxi-terminal, que contiene residuos de tirosina donde la fosforilación modula la señal de transducción mediada por EGFR. También hay numerosos residuos serina/treonina (y otros residuos tirosina) en los cuales se

considera importante la fosforilación para los procesos de hiporregulación y endocitosis del receptor (Wells,A. 1999). El EGFR se activa cuando sus ligandos específicos, como el EGF, el TGF-α, la anfirregulina, la betacelulina, el EGF unido a heparina o la epirregulina, se unen a su dominio extracelular presentando la conformación abierta. Esta unión provoca un cambio conformacional que expone el brazo de dimerización y determina la dimerización del receptor con otro EGFR (homodimerización) u otro miembro de la familia de EGFR (heterodimerización). Tras la dimerización del receptor se produce la activación de la proteína TK intrínseca y la autofosforilación de la tirosina, iniciando una cascada de señalización mitógena intracelular y otras actividades celulares. La activación del EGFR también activa la internalización del receptor en un proceso que implica la endocitosis en surcos recubiertos de clatrina. Los receptores internalizados son degradados dentro de los compartimentos endosómicos, o reciclados y devueltos a la superficie celular. La clasificación se basa principalmente en la composición del dímero. Los homodímeros o heterodímeros que contienen ErbB-1 son generalmente los objetivos para la degradación, los que contienen ErbB3 son reciclados, y los que contienen ErbB2 sufren tasas más lentas de endocitosis y aumento del reciclado a la superficie celular. Parece que una vía de señalización importante de la familia erbB es la de la proteína quinasa activada por el mitógeno Ras-Raf. La activación de Ras induce una cascada de fosforilación de múltiples pasos que determina la activación de MAPK, ERK1, y ERK2, que regula la transcripción de moléculas relacionadas con la proliferación celular y la supervivencia in vitro (Lewis,T.S. *et al.* 1998). Otra vía importante en la señalización del EGFR es la fosfatidilinositol 3-quinasa (PI3K) y la proteína subsiguiente serina/treonina cinasa Akt (Vivanco,I and Sawyers,C.L. 2002). La tercera vía importante es la activación de la vía de la proteína quinasa activada por el estrés, que implica a PKC y Jak/Stat. Otros objetivos de señalización del EGFR importantes incluyen los transductores de la señal del factor de transcripción y los activadores de la transcripción 3 y fosfolipasa C-n1. Tras estos y otros efectores de señalización mitógena se regulan diversos procesos biológicos, incluyendo la apoptosis, la diferenciación, la proliferación celular, la

motilidad, la invasión, la adhesión, la reparación del DNA y la supervivencia (Alroy,I. and Yarden,Y. 1997; Burgering,B.M. and Coffer,P.J. 1995).

La señalización del EGFR tiene efectos sobre numerosos aspectos de la biología del tumor. La activación del EGFR se ha demostrado que favorece procesos implicados en el crecimiento y la progresión del tumor, como la proliferación, la angiogénesis, la invasión y la metástasis, y en la inhibición de la apoptosis (Baselga,J. 2000). La expresión del EGFR varía ampliamente en muchos tumores, incluyendo los de cabeza y cuello (80-100%), renales (50-90%), de pulmón (40-80%), de mama (14-90%), colorrectales (25-77%), ováricos (25-70%), de próstata (39-47%), gliomas (40-63%), de páncreas (30-50%) y de vejiga (31-48%) (Herbst,R.S. and Shin,D.M. 2002). Un alto grado de expresión de la proteína EGFR en los tumores se ha correlacionado con enfermedad más agresiva, mal pronóstico y disminución de la supervivencia, mala respuesta al tratamiento y desarrollo de resistencia a agentes citotóxicos en algunos tipos de tumores (Brabender,J. *et al.* 2001). Por ello, no es de extrañar que sea una de las principales dianas para su bloqueo farmacológico. Este bloqueo dependerá del modelo de activación del receptor, ya sea por exceso de ligando, por exceso del propio receptor o por activación constitutiva debido a mutaciones del dominio intracelular que permiten la señalización en ausencia de ligando o de pareja de dimerización. En ese sentido, la sobreexpresión y las mutaciones del receptor son los modelos de activación que mayor interés diagnóstico han despertado en los pacientes con CNMP. Este interés se ha suscitado tanto por la disponibilidad de fármacos frente a esta diana como por la controversia generada para garantizar la máxima fiabilidad en su identificación. Así, numerosos estudios han evaluado el valor de diferentes técnicas, como la IHQ, la PCR o la hibridación fluorescente in situ (FISH) para la identificación de EGFR. La validez y aplicabilidad de estos procedimientos ha sido controvertida, ya que muchos estudios han sido retrospectivos y sin un grupo control para comparar. La estimación del valor predictivo positivo de la IHQ, PCR y FISH ha sido del 6,5% al 82%, del 7% al 100% y del 11% al 89%, respectivamente. A pesar de este inmenso rango, se

ha publicado un metaanálisis que incluye a más de 5.000 pacientes, en el que se considera que los tres métodos pueden predecir una respuesta a gefitinib, ITK frente a EGFR (Cappuzzo,F. *et al.* 2005). Por el contrario, tan sólo se dispone de un estudio que compare entre sí todos estos métodos para predecir una respuesta, y se fundamenta en el tratamiento con erlotinib, otro ITK frente a EGFR. En cualquier caso, la principal limitación de estas tecnologías es que la presencia de EGFR no supone, al menos a priori, una dependencia proliferativa de la célula que lo expresa.

Sólo unos pocos estudios han examinado EGFR en suero (sEGFR) como biomarcador en CNMP, sin estar clara todavía su utilidad como un factor pronóstico. (Ciledag,A. *et al.* 2008; Jacot,W. *et al.* 2004; Sasaki,H. *et al*. 2003) Nuestros resultados indican que sEGFR puede llegar a ser un buen biomarcador pronóstico en pacientes con CNMP avanzado. En nuestra cohorte de 308 pacientes con CNMP avanzado y 109 controles sanos, se observó una concentración significativamente menor de sEGFR en los pacientes con CPNM que en los controles sanos, con una sensibilidad del 80% y una especificidad del 49%, lo que indica que sEGFR no es un biomarcador válido para discriminar a los pacientes con CPNM de los controles sanos. Nuestro hallazgo de una menor concentración de sEGFR en las muestras de pacientes con cáncer en comparación con los controles es similar a algunos datos de la literatura (Sandri,M.T. *et al.* 2007; Muller,V. et al. 2006; Baron,A.T. *et al.* 2005) y contradice otros estudios, (Jacot,W. *et al.* 2004; Ciledag,A. *et al.* 2008; Abdel Salam,I. *et al* 2009) probablemente debido a las diferentes matrices empleadas para el análisis, nosotros empleamos EDTA pues parece que refleja más las condiciones reales del tumor en la expresión de diferentes CK como EGF, VEGF, IL8, IL1, MCP1, etc. (Kavsak,P.A. *et al.* 2008; Kavsak,P.A. *et al.* 2010; Jelkmann,W. 2001) y al hecho de que probablemente el tumor y otros tejidos circundantes liberan sEGFR, de modo que la concentración medida en los pacientes refleja el resultado de una adaptada regulación a la actividad de las células tumorales y no solo la carga tumoral.

No se observaron correlaciones entre la concentración de sEGFR y las características clinico-patológicas habituales. sEGFR fue menor en pacientes con

enfermedad progresiva y en el carcinoma de células escamosas, aunque estas diferencias no fueron significativas.

Con respecto a la superviviencia y el tiempo a la progresión, los pacientes con sEGFR baja mostraron una menor SG (mediana de 9,1 en comparación con 12,2 meses, p= 0.019) frente a los pacientes con mayores concentraciones, siendo pues un buen marcador pronóstico independiente.

Considerando el hecho de que las muestras de plasma son fácilmente disponibles en pacientes NSCLC, el bajo coste de este tipo de análisis y el potencial valor de sEGFR para proporcionar valiosa información pronóstica, podría ser interesante evaluar más a fondo este marcador en ensayos prospectivos como predictor de respuesta al tratamiento quimioterápico y concretamente al tratamiento con ITKs.

El último trabajo presentado trata de determinar el significado pronóstico de la presencia de inactivación del gen supresor de tumor p16 en pacientes con CNMP avanzado. En la actualidad se considera el cáncer como una enfermedad genética, resultante de la acumulación de alteraciones genéticas que participan en el control del crecimiento celular (Hanahan,D. and Weinberg,R.A. 2011). Dentro de estas alteraciones genéticas, la región cromosómica 9p21 está involucrada en inversiones, traslocaciones y deleciones hetero y homocigotas en una gran variedad de líneas celulares malignas incluyendo las procedentes de CNMP (De Vos,S. *et al.* 1995). Se ha demostrado que la región contiene un gen llamado MTS1 (supresor múltiple de tumores) que codifica un inhibidor previamente identificado (p16) de la kinasa 4 ciclina-dependiente. La proteína p16 se une al CDK4 e inhibe la capacidad de CDK4 de interaccionar con la ciclina D y estimular el paso a través de la fase G1 del ciclo celular. Las deleciones o mutaciones el gen p16 pueden afectar al balance relativo entre el p16 funcional y la ciclina D, dando lugar a un crecimiento celular anormal. Se ha obsevado una alta frecuencia de deleciones y mutaciones del p16 en muchas líneas celulares tumorales lo que apoya la hipótesis del papel principal del gen p16 en la inhibición del

desarrollo de tumores malignos (Kamb,A. *et al.* 1994). Es importante señalar que la capacidad del p16 para inducir la parada del ciclo celular se pierde en células carentes de proteína funcional del gen Rb. Así, la pérdida de p16, la sobreexpresión de ciclinas, y la pérdida del gen Rb tienen efectos semejantes en la progresión de G1, y podrían representar un sendero común en la tumorigénesis. La inactivación de genes supresores de tumores por deleciones grandes, mutaciones intragénicas, mutaciones que alteran el splicing y mutaciones que afectan al promotor, pero además se ha demostrado un mecanismo de inactivación que no implica pérdida del material genético. Merlo y colaboradores demostraron que aunque la pérdida de heterocigosidad (LOH) en 9p21 es una de las alteraciones genéticas más frecuentes identificadas en cáncer humano, las mutaciones puntuales de p16 en el otro cromosoma son relativamente raras (Merlo,A. *et al.* 1994). En líneas celulares monosómicas con p16 sin anomalías estructurales, este gen se encontraba metilado en su isla CpG situada en 5'. Este patrón de metilación está asociado con un bloqueo transcripcional completo, que es reversible tras tratamiento con 5-deoxyazacitidina. Además, la metilación de novo de la isla CpG de 5' de p16 se halló en aproximadamente el 20% de diferentes neoplasias primarias, pero no en células normales, representando potencialmente una vía común de inactivación de genes supresores de tumores en cánceres humanos. Se ha sugerido la existencia de una actividad metilante sobre p16 dependiente de un gen regulador, capaz de frenar la inhibición de la proliferación celular inducida por p16 (Herman JG et al. 1995). El conocimiento de estos mecanismos podría contribuir al desarrollo de nuevos tratamientos.

En nuestro analisis se observó inactivación de p16, bien a través de la pérdida de heterocigosidad o por medio de la metilación del promotor, en el 28% de las muestras analizadas, lo que está de acuerdo con los datos descritos en la literatura ya que las mutaciones en el gen p16INK4a (incluyendo deleciones, mutaciones específicas y alteraciones epigenéticas), presentes en una gran variedad de tumores, se observan hasta en 30-70% de los casos CNMP (Sekido,Y. *et al.* 1998).

Tras analizar nuestros datos no encontramos diferencias significativas ni en el tiempo hasta la progresión ni en la supervivencia entre ambos grupos probablemente debido a que nuestro tamaño muestral es inferior al de los ensayos disponibles en la literatura y a que nuestros pacientes se encontraban en estadio localmente avanzado y/o metastásico. Los datos disponibles en la literatura parecen poner de manifiesto claramente la importancia de p16 en la carcinogénesis del CNMP, y un aumento en la supervivencia en aquellos pacientes con una alta expresión de la proteína p16 en la fase temprana de la enfermedad. (Weist,J.S. *et al.* 1997).

Los resultados obtenidos en nuestro estudio no nos permiten establecer la inactivación de p16 – mediante LOH o por hipermetilación del promotor- como factor pronóstico y por lo tanto consideramos necesario llevar a cabo nuevos estudios en este sentido y la búsqueda de nuevas estrategias terapéuticas relacionadas con la regulación del ciclo celular.

V. CONCLUSIONES

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En los pacientes con CNMP localmente avanzado y/o metastásico:

1. La baja expresión de VEGF está asociada con un menor riesgo de mortalidad cuando éstos son tratados con monoterapia secuencial con Gemcitabina y Paclitaxel.
2. La monoterapia secuencial con Gemcitabina y Paclitaxel es bien tolerada y puede ser considerada una alternativa más en su arsenal terapéutico.
3. El subgrupo que mantiene un buen estado general (PS 0-1) y desarrolla neutropenia durante el tratamiento con Cisplatino y Docetaxel presenta un riesgo de muerte inferior frente a aquellos que no la desarrollan.
4. No se ha encontrado correlación alguna en el análisis de variaciones individuales en polimorfismos de genes implicados en los sistemas de reparación del DNA y la presencia de neutropenia durante el tratamiento con Cisplatino y Docetaxel.
5. No se ha hallado correlación entre polimorfismos de genes implicados en los sistemas de reparación del DNA y supervivencia en pacientes tratados con Cisplatino y Docetaxel.
6. La baja expresión de sEGFR se correlaciona con menor supervivencia global y tiempo hasta la progresión.
7. No se ha establecido que la inactivación de p16 sea un factor pronóstico, siendo probablemente mayor su implicación en la carcinogénesis y en las fases tempranas de la enfermedad.

VI. BIBLIOGRAFÍA

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VII. ABREVIATURAS

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ALT: Alanina Aminotransferasa

ALK: *Anaplastic lymphoma receptor tyrosine kinase.* Receptor tirosina quinasa de linfoma anaplásico.

ASCO: *American Society of Clinical Oncology*

AST: Aspartato Aminotransferasa

AUC: *Area under curve*. Área bajo la curva.

BER: reparación por escision de bases

CK: Citoqueratinas

CNMP: Cáncer No Microcítico de Pulmón

CP: Cáncer de Pulmón

CR: *Complete response*.Respuesta completa.

DNA: Ácido Desoxirribonucleico

EDTA: *Ethylenediaminetetraacetic acid* Ácido etilendiaminotetraacético.

ECOG: *Eastern Cooperative Oncology Group*

EGF: *Epidermal growth factor.* Factor de Crecimiento Epidérmico

EGFR: *Epidermal growth factor receptor*. Receptor del Factor de Crecimiento Epidermico

EML4: EML4: *Echinoderm microtubule-associated protein-like 4.* Proteína equinoderma asociada a microtubulos 4.

FISH: Hibridación Fluorescente In Situ

FP: Factor Pronóstico

G-CSF: *Granulocyte colony-stimulating factor* Factor estimulante de colonias.

GECP: Grupo Español de Cáncer de Pulmón

hTERT: Telomerasa reversa transcriptasa

IASLT: *International Association for the Study of Lung Cancer*

IHQ: Inmunohistoquimia

ILE: Intervalo Libre de Enfermedad

ITKs: Inhibidores de las Tirosina Quinasas

KRAS: *v-Ki-ras2 Kristen rat sarcoma viral oncogene homolog.*

LCSS: *Lung Cancer Symptom Scale*

LOH: *Loss of heterozygosity* Pérdida de heterocigosidad.

MAPK: *Mitogen-activated protein kinases.* Proteína quinasa activada por mitógenos.

mRNA: RNA Mensajero

MTS1: Supresor Múltiple de Tumores

MYC: *v-myc myelocytomatosis viral oncogene homolog.* Oncogén homologo de la mielocitomatosis viral.

NER: reparación por escision de nucleótidos

NCI-CTC: *National Cancer Institute Common Toxicity Criteria*

NCCN: *National Comprehensive Cancer Network*

NER: Escisión de Nucleótidos

NSCLC: *Non-Small Cell Lung Cancer*

OMS: Organización Mundial de la Salud

OS: *Overall Survival.*Supervivencia global.

PCR: *Polymerase Chain Reaction.* Reacción en cadena de la polimerasa.

PI3K: *Phosphatidylinositol 3-kinase*. Fosfatidil-inositol 3-quinasa.

PD: *Progression disease.* Progresion de enfermedad.

PR: *Partial response.*Respuesta parcial.

PS: *Performance status.* Estado funcional.

PTEN: *Phosphatase and tensin homolog.* Homólogo de fosfatasa y tensina.

QoL: *Quality Of Life.* Calidad de vida.

QT: Quimioterapia

RDI: *Relative Dose Intensity.* Intensidad relativa de dosis.

RECIST: *Response evaluation criteria in solid tumors.* Criterios de evaluación de respuesta en tumores sólidos.

RNA: Ácido Ribonucleico

ROC: *Receiver operating curve.* Curva de característica operativa relativa.

RR: Ribonucleótido Reductasa

RT: Radioterapia

RTPCR: Real time quantitative polymerase chain reaction.PCR cuantitativa a tiempo real.

SD: .*Stable disease.* Enfermedad estable.

sEGFR: *Soluble Epidermal Growth Factor Receptor*

SG: Supervivencia Global.

SLCG: *Spanish Lung Cancer Group.*

SLP: Superviviencia Libre de Progresión

SNPs: *Single nucleotide polymorphisms.* Polimorfismos

SP-A: Apoproteína A del Surfactante

TGFa: Factor de Crecimiento Tumoral Alfa

TK: *Tirosine-kinase*. Tirosina-quinasa.

TSG: *Tumour suppressors genes* Genes supresores de tumor.

TF-1: Factor de Transcripción de Tiroides

TTP: *Time To Progression* Tiempo hasta la progresion.

UICC: Unión Internacional Contra El Cáncer

UNL: *Upper normal limits* Dentro de limites normales.

USDHHS: *United States Department of Health and Human Services*

VEGF: *Vascular endotelial growth factor* Factor de Crecimiento del Endotelio Vascular.

VEGFR: *Vascular endotelial growth factor receptor* Receptor del Factor de Crecimiento del Endotelio Vascular.