Int J Clin Exp Pathol 2015;8(3):2888-2898
www.ijcep.com /ISSN:1936-2625/IJCEP0004482

Original Article

Biomarkers of erlotinib response in non-small cell lung cancer tumors that do not harbor the more common epidermal growth factor receptor mutations

Blanca D López-Ayllón[1], Javier de Castro-Carpeño[2], Carlos Rodriguez[3], Olga Pernía[3], Inmaculada Ibañez de Cáceres[3], Cristobal Belda-Iniesta[4], Rosario Perona[1,5], Leandro Sastre[1,5]

[1]Instituto de Investigaciones Biomédicas CSIC/UAM; Biomarkers and Experimental Therapeutics in Cancer, IdiPaz, Madrid, Spain; [2]Department of Medical Oncology, La Paz University Hospital, IdiPaz, Madrid, Spain; [3]Cancer Epigenetics Laboratory, INGEMM, Biomarkers and Experimental Therapeutics in Cancer, IdiPaz, La Paz University Hospital, Madrid, Spain; [4]Department of Medical Oncology, Madrid Norte Sanchinarro University Hospital, Madrid, Spain; [5]CIBER de Enfermedades Raras, Valencia, Spain

Received December 5, 2014; Accepted February 3, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: Non-small cell lung cancer (NSCLC) represents approximately 85% of all lung cancers, which are the leading cause of cancer-related deaths in the world. Tyrosine kinase inhibitors such as erlotinib represent one therapeutic options presently recommended for tumors produced by activating mutations in the gene coding of epidermal growth factor receptor (EGFR). The aim of this study is the identification of possible biomarkers for tumor sensitivity to erlotinib in the absence of the main EGFR mutations. The erlotinib sensitivity of cells isolated from 41 untreated NSCLC patients was determined and compared with the presence of the more frequent EGFR mutations. Several patients had tumor cells highly sensitive to erlitinib in the absence of the EGFR mutations analyzed. The gene expression profile of 3 erlotinib-sensitive tumors was compared with that of 4 resistant tumors by DNA microarray hybridization. Sixteen genes were expressed at significantly higher levels in the resistant tumors than in the sensitive tumors. The possible correlation between erlotinib sensitivity and the expression of these genes was further analyzed using the data for the NSCLC, breast cancer and colon cancer cell lines of the NCI60 collection. The expression of these genes was correlated with the overall survival of 5 patients treated with erlotinib, according to The Cancer Genome Atlas (TCGA) database. Overlapping groups of 7, 5 and 3 genes, including UGT1A6, TRIB3, MET, MMP7, COL17A1, LCN2 and PTPRZ1, whose expression correlated with erlotinib activity was identified. In particular, low MET expression levels showed the strongest correlation.

Keywords: Biomarkers, EGFR, erlotinib sensitivity, MET, non-small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer-related mortality in the world [1], and non-small cell lung cancer (NSCLC) represents approximately 85% of the cases. Lung cancer treatment is hampered by a lack of specific treatments and by the frequent development of resistance to chemotherapy. There is an urgent need to determine the pathogenic and drug-resistance mechanisms involved in NSCLC. Activation of the epidermal growth factor receptor (EGFR) regulatory pathway is one of the mechanisms that result in NSCLC generation [2]. Inhibition of this pathway is considered a promising possibility for lung cancer treatment [3].

Several alterations result in EGFR pathway activation, including gene amplification, overexpression and mutation [4]. Constitutively activated proteins can derive from missense mutations or small deletions. Among the most frequent missense mutations is one changing leucine 585 into arginine; small in-frame deletions in exon 19 of the genes are also frequent [5]. Two general approaches have been used to inhibit the EGFR pathway [4]. First is the use of antibodies that recognize the extracellular region of the receptor and avoid ligand binding

and/or receptor activation. Among the presently used antibodies are cetuximab and necitumumab. Second is the use of molecules that inhibit of the receptor's tyrosine kinase activity, required for signal transduction. These molecules are known as tyrosine kinase inhibitors (TKIs), among which are erlotinib (Tarceva®), gefitinib, lapatinib and canertinib [4].

Initial clinical trials of unclassified lung cancer patients indicated that the use of EGFR inhibitors did not lead to any significant improvement of overall survival or disease-free interval. However, treatment of patients who presented EGFR overexpression or activating mutations resulted in a significant objective response and improved overall survival [6, 7]. The presence of EGFR mutations is currently considered the most important predictor of clinical response to treatment with TKIs [8]. Erlotinib has been also approved by the US Food and Drug Administration and the European Medicines Agency for treatment of all patients with advanced NSCLC in second- and third-line. However, its efficacy for these treatments is still contentious and some clinical trials indicated that chemotherapy could be more effective for second-line treatment of previously treated NSCLC patients.

However, recent trials have shown that TKIs can also have activity against NSCLCs that do not present EGFR-activating mutations [9]. There is therefore a need to identify new relevant biomarkers that would allow the selection of TKI-sensitive patients independently of the presence of EGFR mutations. In this study, the sensitivity to the TKI erlotinib [10] was determined in NSCLC biopsies and compared with the presence of the more frequent EGFR mutations. A population of tumors was identified that were highly sensitive to erlotinib in the context of nonmutated EGFR. Comparative microarray analyses of gene expression allowed the identification of 16 genes that are underexpressed in sensitive tumors in comparison with erlotinib-resistant tumors. We propose that the expression of some of these genes could be a new biomarker of erlotinib sensitivity in NSCLC tumors.

Materials and methods

Processing of surgical samples

NSCLC samples were collected at La Paz University Hospital, Madrid (Spain), in DMEM/MixF12 Ham medium (Sigma, St. Louis, MO, USA). They were incubated with collagenase (0.3 mg/mL) and hyaluronidase (125 U/mL) (both from Sigma) at 37°C for 20 min, mechanically disaggregated and filtered [11].

Cell viability assays

Disaggregated cells were cultured in 96-well plates at a density of 50 cells/well. After 24 h of culture, various concentrations of erlotinib (Tarceva®, F. Hoffmann-La Roche Ltd, Basel, Switzerland) were added, and the culture continued for 72 h. Cell viability was estimated by MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) hydrolysis (Promega, Madison, WI, USA). The assays were performed in triplicate.

RNA and DNA isolation

RNA was extracted from surgical samples frozen in optimal cutting temperature (OCT) (Miles, Elkhart, IN, USA) using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). DNA was extracted from paraffin sections treated with xylene. The samples were incubated in 20 mM EDTA, 65 mM NaCl, 1% SDS and 0.5 μg/mL of proteinase K (Sigma, St. Louis, MO, USA) for 12-16 h at 37°C. DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol.

EGFR mutation detection

Detection of the EGFR exons 20 and 21 mutations was performed by allelic discrimination using quantitative PCR [12]. Oligonucleotides 5'-AGGCAGCCGAAGGGCA-3', 5'-CCTCACCTCCACCGTGCA-3', FAM-labeled 5'-TGAGCTGCATGATGA-3' and VIC-labeled 5'-TGAGCTGCGTGATGA-3' were used for discrimination of the exon 20 T790M mutation. Oligonucleotides 5'-AACACCGCAGCATGTCAAGA-3', 5'-TTCTCTTCCGCACCCAGC-3', FAM-labeled 5'-CAGATTTTGGGCGGGCCAAAC-3' and VIC-labeled 5'-TCACAGATTTTGGGCTGGCCAAAC-3' were used for detection of the exon 21 L858R mutation. Exon 21 was amplified by PCR before variant analysis using the oligonucleotides 5'-CTAACGTTCGCCAGCCATAAGTCC-3' and 5'-GCTGCGAGCTCACCCAGAATGTCTGG-3'. The PCR products were analyzed using the StepOnePlus Real-Time PCR System (Life Technologies, Applied

Table 1. General characteristics of the patients analyzed in the study

Patient	Age	Sex	Smoker	Histology[a]	Stage	Genotype	Sensitivity[b]
1	74	M	No	AdCa	III	WT	90
2	57	F	-	EpCa	III	WT	90
3	70	F	No	AdCa	III	WT	100
4	65	F	-	Other	-	WT	100
5	53	F	-	EpCa	-	WT	100
6	72	F	-	EpCa	III	WT	90
7	44	M	Yes	Other	I	WT	70
8	86	F	-	EpCa	I	WT	90
9	52	F	Ex	AdCa	III	WT	100
10	61	F	Yes	AdCa	I	WT	100
11	77	F	Yes	Other	I	WT	100
12	75	F	-	LCCa	I	WT	100
13	66	F	Ex	EpCa	III	WT	100
14	61	F	-	Other	I	WT	40
15	72	F	-	LCCa	I	WT	100
16	77	F	No	AdCa	I	WT	100
17	81	F	-	AdCa	I	WT	90
18	62	F	-	EpCa	I	WT	70
19	46	F	Ex	EpCa	II	WT	100
20	52	F	Ex	AdCa	I	WT	80
21	48	M	Ex	AdCa	III	WT	60
22	68	F	Ex	EpCa	III	WT	100
23	72	F	Yes	EpCa	I	WT	100
24	67	F	Yes	AdCa	II	WT	60
25	80	F	No	EpCa	I	WT	100
26	62	F	No	EpCa	III	WT	70
27	84	F	No	EpCa	II	WT	50
28	45	F	Ex	AdCa	III	WT	70
29	67	M	Ex	Other	I	Mut_Ex19	60
30	75	F	-	EpCa	-	WT	30
31	60	F	Yes	EpCa	III	Mut_Ex19	70
32	61	F	Yes	EpCa	III	WT	30
33	47	M	Yes	EpCa	I	WT	60
34	49	F	Ex	AdCa	II	WT	100
35	64	M	Yes	AdCa	I	WT	40
36	60	F	Yes	EpCa	III	WT	60
37	59	M	No	AdCa	I	WT	30
38	66	F	-	Other	I	WT	50
39	67	F	No	AdCa	II	WT	100
40	63	F	Yes	EpCa	II	WT	60
41	60	F	Yes	EpCa	I	WT	0

[a]AdCa: adenocarcinoma; EpCa: epidermoid carcinoma; LCCa: large-cell carcinoma. [b]Percentage of cell viability after 24 hours of treatment with 10 μM erlotinib.

Biosystems, Carlsbad, CA, USA). Exon 19 mutations were detected by DNA sequencing after amplification of the exon using the oligonucleotides 5'-GTGATCGCTGGTAACATCC-3' and 5'-CATAGAAAGTGAACATTTAGGATGTG-3'. Oligonucleotides were obtained from Applied Biosystems and Sigma.

DNA microarray hybridization and analysis of the results

Total RNA (200 ng) was amplified using the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) and purified using the RNeasy Mini Kit (Qiagen). The labeled probes were hybridized to Whole Human Genome Microarrays 4x44K G4112F (Agilent). Hybridization, data extraction and analysis of the data were performed at the CNB (National Biotechnology Center, Madrid, Spain). Differential expression was determined using the rank product method [13]. Data were filtered and visualized as previously described [14]. Genes with an FDR lower than 0.1 were considered as differentially expressed. The GOTree Machine (GOTM) [15] was used to analyze functional enrichment in the selected genes.

Reverse transcription and quantitative PCR

One microgram of RNA was converted into cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative PCR amplification was performed using TaqMan probes and the HT7900 platform (Applied Biosystems). The TaqMan probes used were: Hs03929097_g1 (GAPDH), Hs00899658_m1 (MMP1), Hs00950669_m1 (AREG) and Hs01565584_m1 (MET). Relative gene expression was calculated by the threshold cycle method [16] using GAPDH as endogenous control.

Table 2. Summary of the genes identified in the DNA Microarray analyses

Gene	Accession number	Description	Exp. Difference
LCN2	NM_005564	Lipocalin 2	-9.12
IGF2	NM_00100713	Insulin-like growth factor 2	-7.88
UGT1A6	NM_001072	UDP glucuronosyltransferase 1 family	-7.87
MMP1	NM_002421	Matrix metallopeptidase 1	-7.36
COL17A1	NM_000494	Collagen 17	-6.94
PIGR	NM_002644	Polymeric immunoglobulin receptor	-6.77
AREG	NM_001657	Amphiregulin	-6.58
IGHG4	ENST00000379913	IgA1-A2 lambda hybrid	-6.57
PTPRZ1	NM_002851	Protein tyrosine phosphatase; pleiotrophin receptor	-6.5
AKR1C3	NM_003739/	Aldo-keto reductase family 1	-6.4
MMP7	NM_002423	Matrix metallopeptidase 7	-6.37
S100A2	NM_005978	S100 Ca-binding protein A2	-6.33
MET	NM_000245	Oncogene MET	-5.92
SAA1	NM_000331	Serum amyloid A1	-5.81
C4BPA	NM_000715	Complement component 4 binding protein	-5.47
TRIB3	NM_021158	Tribbles homolog 3	-4.94

Results

Erlotinib sensitivity of cells isolated from NSCLC patients

Surgical samples of 41 patients with NSCLC at stages I-III were collected at La Paz University Hospital (Madrid, Spain). Patient history, histological classification and stage of the tumors are shown in **Table 1**. Cells were isolated from each surgical sample and their sensitivity to erlotinib (Tarceva®) determined. The results are summarized in the right column of **Table 1** that shows cell viability to a 10 μM concentration of erlotinib. DNA was extracted from each surgical sample and the presence of the more frequent EGFR activating mutations, small in-frame deletions in exon 19 of the gene and missense mutations in exon 21 (L858R) [5] and the more frequent mutation confering erlotinib resistance (exon 20, T790M [17]) were determined. Only two of the samples included small deletions in exon 19 (patients 29 and 31 in **Table 1**), the mutations most frequently found in the Spanish population [5]. These two samples were sensitive to erlotinib (60%-70% cell viability at 10 μM erlotinib, **Table 1**). 21 of the samples that did not have the studied mutations were resistant to erlotinib (more than 90% cell viability). The rest of them were sensitive, and a significant number (8) were very sensitive to the drug (less than 50% cell viability).

Gene expression profile

Since the patient samples that were more sensitive to erlotinib did not present the more frequent EGFR mutations, the gene expression profile of sensitive samples was compared with that of resistant ones to get information on the biological bases of their sensitivity. Thus, RNA was obtained from 4 resistant (15, 16, 17 and 19) and 3 sensitive samples (32, 35 and 38), converted to cDNA and hybridized to DNA microarrays containing 44,000 human gene probes. Sixteen genes were identified that were over-expressed in the resistant samples compared with the sensitive ones, considering a false discovery rate of less than 0.1 (**Table 2**). Gene ontology analyses indicated a significant enrichment in gene coding for proteins expressed in the extracellular region (9 genes, $P = 8.19 \times 10^{-5}$), involved in the formation of the extracellular matrix (4 genes, P = 0.0009), in collagen catabolic processes (2 genes, $P = 0.0059$) and in components of the basal plasma membrane (2 genes, $P = 0.0009$).

The expression of 3 of the genes identified was further analyzed by quantitative RT-PCR. The 7 samples used for the microarray were tested, as well as 2 additional samples sensitive to erlotinib but not highly sensitive (**Figure 1**). The analyzed samples showed a broad variation in expression levels; however, the average expression in the resistant, sensitive and highly sensi-

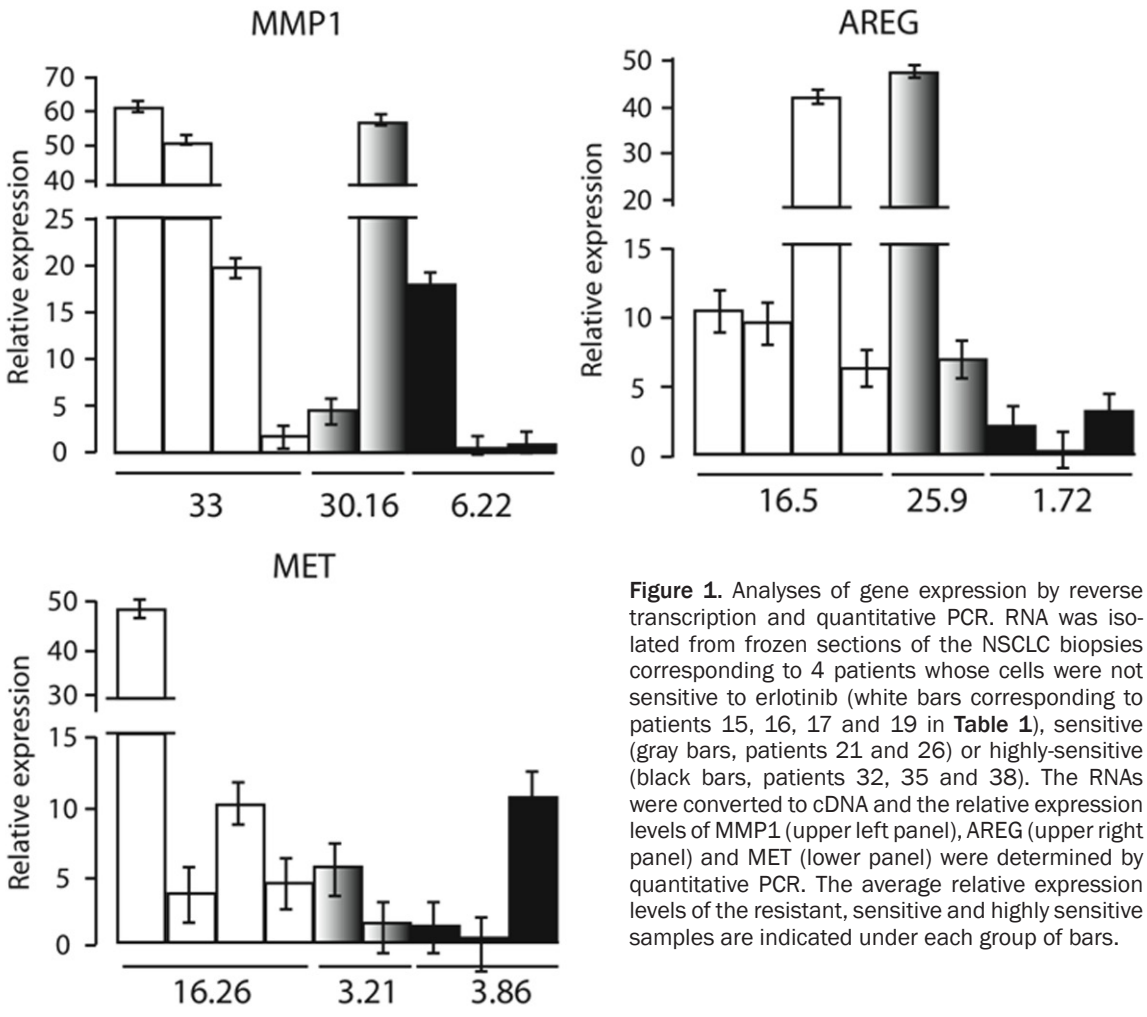

Figure 1. Analyses of gene expression by reverse transcription and quantitative PCR. RNA was isolated from frozen sections of the NSCLC biopsies corresponding to 4 patients whose cells were not sensitive to erlotinib (white bars corresponding to patients 15, 16, 17 and 19 in **Table 1**), sensitive (gray bars, patients 21 and 26) or highly-sensitive (black bars, patients 32, 35 and 38). The RNAs were converted to cDNA and the relative expression levels of MMP1 (upper left panel), AREG (upper right panel) and MET (lower panel) were determined by quantitative PCR. The average relative expression levels of the resistant, sensitive and highly sensitive samples are indicated under each group of bars.

tive samples (shown under the graphs in **Figure 1**) was in agreement with the data obtained in the microarray analyses. The highly sensitive samples expressed lower levels of the 3 genes while samples with intermediate sensitivity expressed lower levels of MET, but similar levels of MMP1 and AREG mRNAs than the resistant samples.

Comparative analyses in NCI60 cancer cell lines

To further test if the expression of these 16 genes was related to erlotinib sensitivity we analyzed their expression in the NCI60 series of cancer cell lines. These cell lines have been broadly used for functional and pharmacological studies. Their genotype and gene expression profiles have been determined [18] and are publicly available through the NCI60 database (http://discover.nci.nih.gov/cellminer). We focused the study on the 21 NCI60 cell lines derived from tumors typically treated with erlotinib (breast cancer, colon cancer and NSCLC). In this database, erlotinib response is expressed as the negative logarithm of the IC50 molar concentration, thus increasing with the sensitivity of the sample. Since the genes identified have lower expression in more sensitive cells, a negative correlation between gene expression and erlotinib response was expected. Seven of the 16 genes showed a significant negative correlation (correlation coefficient, R, lower than -0.3), excluding the NSCLC EKVX and H322M cell lines, as will be discussed in Section 4. Because a wide variability in the expression of each gene had been observed in the patient samples (**Figure 1**), we considered that the average expression of the genes could be more relevant than the expression of each

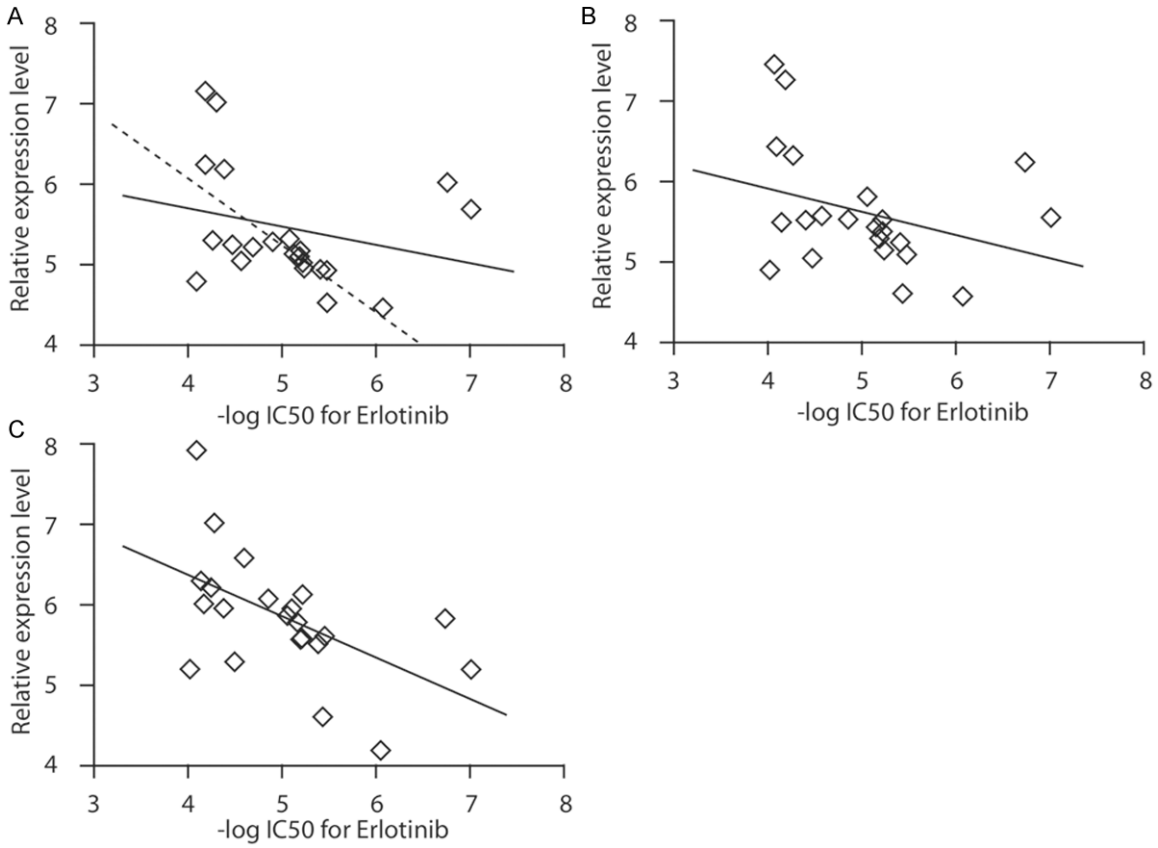

Figure 2. Correlation between gene expression levels and erlotinib activity in NSCLC, breast cancer and colon cancer NCI60 cell lines. Relative expression levels of the indicated genes in 9 NSCLC, 5 breast cancer and 7 colon cancer cell lines, were obtained from the NCI60 database. The same source was used to determine the erlotinib activity in these cell lines, expressed as the negative logarithm of the IC50 concentration. The correlation between relative gene expression levels (Y-axis) and erlotinib activity (X-axis) is represented for each cell line. A. Correlation of the average relative expression levels of the genes UGT1A6, TRIB3, MET, MMP7, COL17A1, LCN2 and PTPRZ1 and erlotinib activity for each cell line. The solid line indicates the correlation found considering all the cell lines (R = -0.265), whereas the broken line indicates the correlation obtained excluding the two cell lines shown at the right of the graph (NSCLC cell lines EKVX and H322M; R=-0.615). B. Correlation of the average relative expression level of the genes UGT1A6, TRIB3, MET, MMP7 and COL17A1 and erlotinib activity. The solid line indicates the correlation found for all the cell lines (R=-0.328). C. Correlation of the average relative expression level of the genes UGT1A6, TRIB3 and MET and erlotinib activity. The solid line indicates the correlation found for all the cell lines (R = -0.529).

Table 3. Clinical history of the analyzed TCGA patients with NSCLC

Barcode patient	Sex	Histological type	Stage	Smoker	Days to death
TCGA-05-4402	F	Adenocarcinoma	IV	No	244
TCGA-22-5480	F	Squamous cell carcinoma	IA	Yes	2170
TCGA-53-7624	F	Adenocarcinoma	IV	Yes	1043
TCGA-60-2715	M	Squamous cell carcinoma	IA	Yes	1075
TCGA-62-A46S	M	Papillary adenocarcinoma	IB	Yes	1653

individually. The correlation observed with the average expression of these 7 genes: LCN2, MET, MMP7, PTPRZ1, TRIB3, UGT1A6 and COL17A1; is shown in **Figure 2A**, solid line (R = -0.27). Omitting the NSCLC cell lines EKVX and H322M (the 2 samples to the right in **Figure 2A**) the correlation coefficient was -0.615 (broken line in **Figure 2A**). If all cell lines are considered, 5 genes showed a negative correlation between erlotinib response and gene expression: MET, TRIB3, UGT1A6, MMP7 and COL17A1. A correlation coefficient of -0.328 was observed considering the average expression of these 5 genes (**Figure 2B**). When the analyses were further focused on the

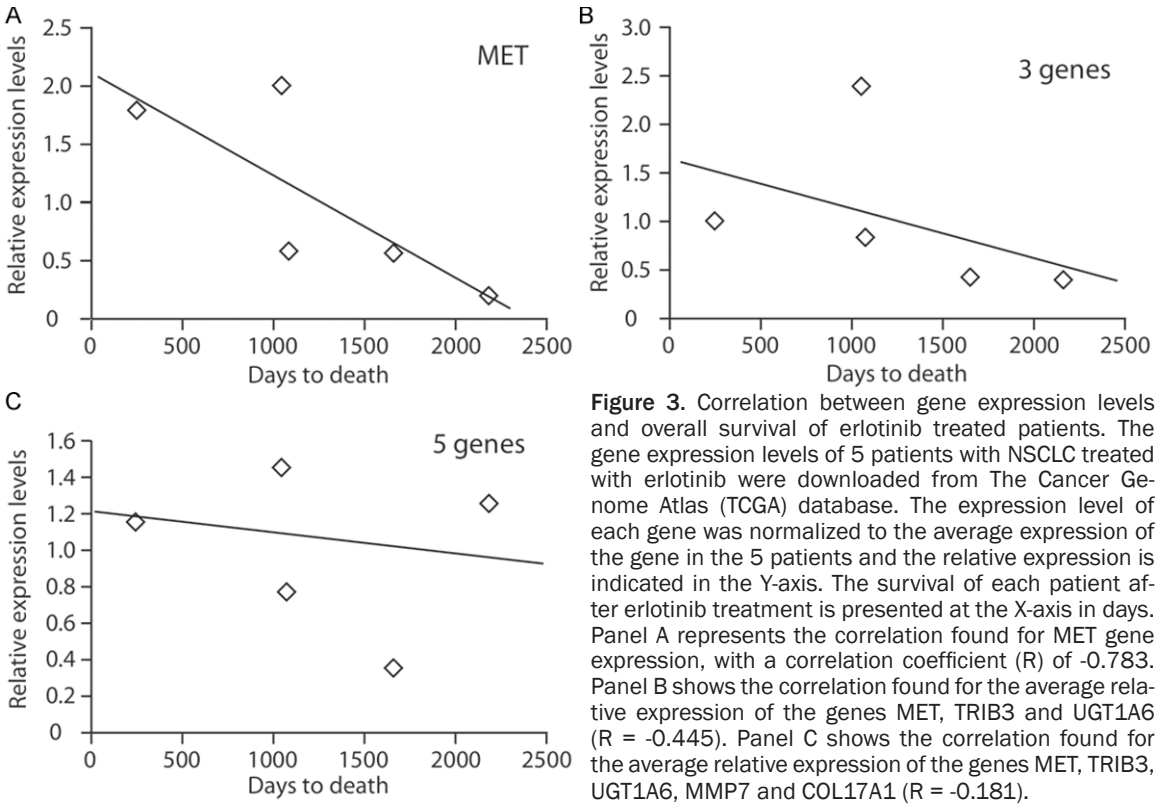

Figure 3. Correlation between gene expression levels and overall survival of erlotinib treated patients. The gene expression levels of 5 patients with NSCLC treated with erlotinib were downloaded from The Cancer Genome Atlas (TCGA) database. The expression level of each gene was normalized to the average expression of the gene in the 5 patients and the relative expression is indicated in the Y-axis. The survival of each patient after erlotinib treatment is presented at the X-axis in days. Panel A represents the correlation found for MET gene expression, with a correlation coefficient (R) of -0.783. Panel B shows the correlation found for the average relative expression of the genes MET, TRIB3 and UGT1A6 (R = -0.445). Panel C shows the correlation found for the average relative expression of the genes MET, TRIB3, UGT1A6, MMP7 and COL17A1 (R = -0.181).

3 genes that showed a correlation coefficient less than -0.3 (MET, TRIB3 and UGT1A6), their average expression gave a correlation coefficient of -0.529 (**Figure 2C**).

Comparative analyses at the cancer genome atlas database

The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/) is a collaborative project that catalogues genomic data for over 20 types of cancer. This database contains information concerning 5 patients with lung adenocarcinoma and squamous cell lung carcinoma treated with erlotinib, (**Table 3**). The gene expression data from these patients, obtained in RNA-Seq experiments [19, 20], were correlated with their survival after erlotinib treatment (**Figure 3**). Considering the 7 genes selected using the NCI60 cell lines, the strongest correlation was between MET expression and overall patient survival (R = -0.783) (**Figure 3A**). The average expression of the 3 genes characterized in Section 3.3 (MET, TRIB3, UGT1A6) also demonstrated a significant correlation (R = -0.445) (**Figure 3B**). When the average expression of the 5 genes described above was compared (**Figure 3C**), the correlation coefficient decreased to -0.181, due to the high expression levels of the COL17A1 gene in one of the patients. When the group of 7 genes was considered, the correlation coefficient was -0.206.

Discussion

The sensitivity to erlotinib of cells isolated from 41 NSCLC surgical samples from patients who had not received any previous treatment was determined. Because activating EGFR mutations are considered the most useful predictive marker of erlotinib response [8, 21-23], the presence of the more common mutations was tested. Two of the patients presented small deletions in EGFR exon 19, and their cells were sensitive to erlotinib, as expected. However, cells from 8 patients who did not present the studied mutations were even more sensitive to erlotinib. These patients did not show any distinctive differences in sex, histology, tumor grade or smoking habits. Their cells could present other non-analyzed EGFR mutations, given that an increasing number of mutations are being described [2]; however, the relatively high number of sensitive samples makes this possibility unlikely because these other mutations

are present in a low proportion of NSCLC patients. In agreement with this observation, Jazieh et al. [9] recently described patients who respond to tyrosine kinase inhibitors who do not present EGFR mutations.

The identification of patients who respond to erlotinib in the absence of EFGR mutations could be of great therapeutic interest. Therefore, the gene expression profiles of sensitive and resistant patients were compared to find possible markers of the EFGR-non-mutated erlotinib-sensitive patients. Sixteen genes expressed at lower levels in sensitive samples were found. Many of them are involved in cancer-related processes. Among them, the expression of MMP1 [24], MMP7 [25], SAA1 [26], S100A2 [27] and AKR1C3 [28] was related to cell invasion and cancer metastasis. PIGR [29], TRIB3 [30], LCN2 [31] and IGF2 [32] expression was related to cell proliferation. PTPRZ1 [33], AREG (EGFR ligand [34]), TRIB3 [30] and MET [35] proteins participate in cell signaling pathways related to cancer. In addition, UGT1A6 [36], C4BP [37], AREG [38] and LCN2 [39] expression has been related to NSCLC cell response to chemotherapy and radiotherapy. In general terms, the lower expression of these genes would predict lower invasive and metastatic capacity, increased sensitivity to chemotherapy and increased proliferative capacity of the more sensitive patient cells. At least 3 of the proteins encoded by these genes, C4BP [40], IGF2 [41] and SAA1 [26], are present in serum and could easily be detected and quantified.

The results obtained using patient cells were further tested on the NCI60 group of cancer cell lines. The analysis was focused on the cancer types that receive erlotinib treatment (breast cancer, colon cancer and NSCLC). The average expression of a group of 7 genes identified in our analyses showed correlation with erlotinib sensitivity ($R = -0.265$). The correlation coefficient increased to -0.615 if the NSCLC cell lines EKVX and H322M were excluded from the analyses. These two cell lines are highly sensitive to erlotinib and express average levels of the 7 genes analyzed. However, the EFGR gene is not mutated or overexpressed in these two cell lines, and were included in subsequent analyses. The correlation coefficient increased when the expression of the 5 ($R = -0.328$) or 3 ($R = -0.529$) more related genes was considered. These results are therefore in good agreement with the results obtained from the patient cells.

The 3 genes showing the strongest correlation with erlotinib sensitivity are UGT1A6, TRIB3 and MET. UGT1A6 codes for a UDP glucuronosyltransferase with detoxifying activity and increased expression could result in erlotinib modification and degradation or decreased activity of the drug. Polymorphisms in this gene have been previously associated with lung cancer [42]. TRIB3-encoded protein activates the Notch signaling pathway, and upregulation of this gene in patients with NSCLC has been related to increased metastasis and poor prognoses [43]. MET codes for the hepatocyte growth factor receptor [44]. MET is considered as an oncogene because it is mutated in an increasing proportion of patients with NSCLC [2]. In addition, MET amplification is one of the mechanisms that induces resistance to erlotinib and other TKI drugs [45, 46]. The data reported in this article are in agreement with these observations and would further indicate that low expression levels of MET could predict increased erlotinib sensitivity in NSCLC tumors.

The group of 5 genes also includes MMP7 and COL17A1. MMP7 codes for an extracellular metalloproteinase whose overexpression correlates with NSCLC tumor proliferation and poor prognosis [47]. Polymorphisms in the promoter region are associated with metastasis [24]. COL17A1 codes for the alpha subunit of type XVII collagen. This protein has not been previously related to cancer biology. The group of 7 genes also includes LCN2 and PTPRZ1. LCN2 codes for lipocalin 2, and high expression levels of this protein have been related to radioresistance [39]. PTPRZ1 codes for the pleiotrophin receptor and is considered oncogenic in small cell lung cancer [33].

The results obtained were further tested using the information available in the TCGA database on the gene expression profile and clinical history of cancer patients. The overall survival of 5 NSCLC patients treated with erlotinib was compared with the expression profile of the genes described above. A negative correlation was found between the expression of the groups of the 5 and 3 genes identified and the survival of the erlotinib-treated patients with correlation

coefficients of -0.181 and -0.445, respectively. However, the strongest correlation was found between MET expression and overall survival (R = -0.783). It is of interest that these data, obtained from the clinical treatment of patients, are in good agreement with the data obtained from cells isolated from untreated patients and in cancer cell lines.

In summary, this article identifies 16 genes whose expression appears to be related to erlotinib sensitivity in patients with NSCLC who do not carry the more common EGFR mutations. This group can be further narrowed to 7, 5 or 3 genes whose expression is more closely related to erlotinib sensitivity in the NCI60 repertoire of the NSCLC, breast cancer and colon cancer cell lines. The analysis of the data available for 5 patients in the TCGA database was in agreement with these results, whereas the more significant association was found between MET expression and overall survival after erlotinib treatment. These observations would require confirmation in a larger cohort of patient samples to ascertain the clinical utility of the expression of these genes as a predictive biomarker of erlotinib activity.

Acknowledgements

We thank J.C. Oliveros for assistance with the analyses of the DNA microarray data and Roche for providing erlotinib. This study was supported by the Fondo para la Investigacion Sanitaria (FIS) grants PI12/00386, PI11/00949, PI11/00537 and PI12/01463, all of them supported by Fondo Europeo de Desarrollo Regional (FEDER) funds. IIC was supported by the "Miguel Servet" program (CP 08/000689).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Leandro Sastre or Rosario Perona, Instituto de Investigaciones Biomédicas CSIC/UAM; Biomarkers and Experimental Therapeutics in Cancer, IdiPaz 28029, Madrid, Spain, Instituto de Investigaciones Biomédicas, CSIC/UAM, C/Arturo Duperier 4. Tel: 34-915854437; Fax: 34-915854401; E-mail: lsastre@iib.uam.es (LS); rperona@iib.uam.es (RP)

References

[1] Siegel R, Naishadham D and Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

[2] The-Cancer-Genome-Atlas-Research-Network. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014; 511: 543-550.

[3] Mok TS. Personalized medicine in lung cancer: what we need to know. Nat Rev Clin Oncol 2011; 8: 661-668.

[4] Yewale C, Baradia D, Vhora I, Patil S and Misra A. Epidermal growth factor receptor targeting in cancer: a review of trends and strategies. Biomaterials 2013; 34: 8690-8707.

[5] Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, Majem M, Lopez-Vivanco G, Isla D, Provencio M, Insa A, Massuti B, Gonzalez-Larriba JL, Paz-Ares L, Bover I, Garcia-Campelo R, Moreno MA, Catot S, Rolfo C, Reguart N, Palmero R, Sanchez JM, Bastus R, Mayo C, Bertran-Alamillo J, Molina MA, Sanchez JJ and Taron M. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009; 361: 958-967.

[6] Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, Zhang S, Wang J, Zhou S, Ren S, Lu S, Zhang L, Hu C, Luo Y, Chen L, Ye M, Huang J, Zhi X, Zhang Y, Xiu Q, Ma J and You C. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. Lancet Oncol 2011; 12: 735-742.

[7] Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, Garcia-Gomez R, Pallares C, Sanchez JM, Porta R, Cobo M, Garrido P, Longo F, Moran T, Insa A, De Marinis F, Corre R, Bover I, Illiano A, Dansin E, de Castro J, Milella M, Reguart N, Altavilla G, Jimenez U, Provencio M, Moreno MA, Terrasa J, Munoz-Langa J, Valdivia J, Isla D, Domine M, Molinier O, Mazieres J, Baize N, Garcia-Campelo R, Robinet G, Rodriguez-Abreu D, Lopez-Vivanco G, Gebbia V, Ferrera-Delgado L, Bombaron P, Bernabe R, Bearz A, Artal A, Cortesi E, Rolfo C, Sanchez-Ronco M, Drozdowskyj A, Queralt C, de Aguirre I, Ramirez JL, Sanchez JJ, Molina MA, Taron M and Paz-Ares L. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012; 13: 239-246.

[8] Thunnissen E, van der Oord K and den Bakker M. Prognostic and predictive biomarkers in lung cancer. A review. Virchows Arch 2014; 464: 347-358.

[9] Jazieh AR, Al Sudairy R, Abu-Shraie N, Al Suwairi W, Ferwana M and Murad MH. Erlotinib in wild type epidermal growth factor receptor non-small cell lung cancer: A systematic review. Ann Thorac Med 2013; 8: 204-208.

[10] Perez-Soler R. The role of erlotinib (Tarceva, OSI 774) in the treatment of non-small cell lung cancer. Clin Cancer Res 2004; 10: 4238s-4240s.
[11] Lopez-Ayllon BD, Moncho-Amor V, Abarrategi A, de C, Il, Castro-Carpeno J, Belda-Iniesta C, Perona R and Sastre L. Cancer stem cells and cisplatin-resistant cells isolated from non-small-lung cancer cell lines constitute related cell populations. Cancer Med 2014; 3: 1099-1111.
[12] Molina-Vila MA, Bertran-Alamillo J, Reguart N, Taron M, Castella E, Llatjos M, Costa C, Mayo C, Pradas A, Queralt C, Botia M, Perez-Cano M, Carrasco E, Tomas M, Mate JL, Moran T and Rosell R. A sensitive method for detecting EGFR mutations in non-small cell lung cancer samples with few tumor cells. J Thorac Oncol 2008; 3: 1224-1235.
[13] Breitling R, Armengaud P, Amtmann A and Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 2004; 573: 83-92.
[14] Oliveros J. FIESTA at BioinfoCP. An interactive server for analyzing DNA microarray experiments with replicates. http://bioinfogp.cnb.csic.es/tools/FIESTA. 2007.
[15] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM and Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000; 25: 25-29.
[16] Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R and Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 2001; 25: 386-401.
[17] Godin-Heymann N, Ulkus L, Brannigan BW, McDermott U, Lamb J, Maheswaran S, Settleman J and Haber DA. The T790M "gatekeeper" mutation in EGFR mediates resistance to low concentrations of an irreversible EGFR inhibitor. Mol Cancer Ther 2008; 7: 874-879.
[18] Abaan OD, Polley EC, Davis SR, Zhu YJ, Bilke S, Walker RL, Pineda M, Gindin Y, Jiang Y, Reinhold WC, Holbeck SL, Simon RM, Doroshow JH, Pommier Y and Meltzer PS. The exomes of the NCI-60 panel: a genomic resource for cancer biology and systems pharmacology. Cancer Res 2013; 73: 4372-4382.
[19] Wang K, Singh D, Zeng Z, Coleman SJ, Huang Y, Savich GL, He X, Mieczkowski P, Grimm SA, Perou CM, MacLeod JN, Chiang DY, Prins JF and Liu J. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. Nucleic Acids Res 2010; 38: e178.
[20] Li B and Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011; 12: 323.
[21] Peters S, Adjei AA, Gridelli C, Reck M, Kerr K and Felip E. Metastatic non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2012; 23 Suppl 7: vii56-64.
[22] Keedy VL, Temin S, Somerfield MR, Beasley MB, Johnson DH, McShane LM, Milton DT, Strawn JR, Wakelee HA and Giaccone G. American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) Mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. J Clin Oncol 2011; 29: 2121-2127.
[23] Korpanty GJ, Graham DM, Vincent MD and Leighl NB. Biomarkers That Currently Affect Clinical Practice in Lung Cancer: EGFR, ALK, MET, ROS-1, and KRAS. Front Oncol 2014; 4: 204.
[24] Liu D, Guo H, Li Y, Xu X, Yang K and Bai Y. Association between polymorphisms in the promoter regions of matrix metalloproteinases (MMPs) and risk of cancer metastasis: a meta-analysis. PLoS One 2012; 7: e31251.
[25] Yue W, Sun Q, Landreneau R, Wu C, Siegfried JM, Yu J and Zhang L. Fibulin-5 suppresses lung cancer invasion by inhibiting matrix metalloproteinase-7 expression. Cancer Res 2009; 69: 6339-6346.
[26] Hansen MT, Forst B, Cremers N, Quagliata L, Ambartsumian N, Grum-Schwensen B, Klingelhofer J, Abdul-Al A, Herrmann P, Osterland M, Stein U, Nielsen GH, Scherer PE, Lukanidin E, Sleeman JP and Grigorian M. A link between inflammation and metastasis: serum amyloid A1 and A3 induce metastasis, and are targets of metastasis-inducing S100A4. Oncogene 2015; 34: 424-435.
[27] Naz S, Ranganathan P, Bodapati P, Shastry AH, Mishra LN and Kondaiah P. Regulation of S100A2 expression by TGF-beta-induced MEK/ERK signalling and its role in cell migration/invasion. Biochem J 2012; 447: 81-91.
[28] Wu CH, Ko JL, Chen SC, Lin YW, Han CP, Yang TY, Chien MH and Wang PH. Clinical implications of aldo-keto reductase family 1 member C3 and its relationship with lipocalin 2 in cancer of the uterine cervix. Gynecol Oncol 2014; 132: 474-482.
[29] Ocak S, Pedchenko TV, Chen H, Harris FT, Qian J, Polosukhin V, Pilette C, Sibille Y, Gonzalez AL and Massion PP. Loss of polymeric immunoglobulin receptor expression is associated with lung tumourigenesis. Eur Respir J 2012; 39: 1171-1180.

[30] Izrailit J, Berman HK, Datti A, Wrana JL and Reedijk M. High throughput kinase inhibitor screens reveal TRB3 and MAPK-ERK/TGFbeta pathways as fundamental Notch regulators in breast cancer. Proc Natl Acad Sci U S A 2013; 110: 1714-1719.

[31] Berger T, Cheung CC, Elia AJ and Mak TW. Disruption of the Lcn2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. Proc Natl Acad Sci U S A 2010; 107: 2995-3000.

[32] Zhu CQ, Popova SN, Brown ER, Barsyte-Lovejoy D, Navab R, Shih W, Li M, Lu M, Jurisica I, Penn LZ, Gullberg D and Tsao MS. Integrin alpha 11 regulates IGF2 expression in fibroblasts to enhance tumorigenicity of human non-small-cell lung cancer cells. Proc Natl Acad Sci U S A 2007; 104: 11754-11759.

[33] Makinoshima H, Ishii G, Kojima M, Fujii S, Higuchi Y, Kuwata T and Ochiai A. PTPRZ1 regulates calmodulin phosphorylation and tumor progression in small-cell lung carcinoma. BMC Cancer 2012; 12: 537.

[34] Busser B, Sancey L, Brambilla E, Coll JL and Hurbin A. The multiple roles of amphiregulin in human cancer. Biochim Biophys Acta 2011; 1816: 119-131.

[35] Sadiq AA and Salgia R. MET as a possible target for non-small-cell lung cancer. J Clin Oncol 2013; 31: 1089-1096.

[36] Inoue K, Sonobe M, Kawamura Y, Etoh T, Takagi M, Matsumura T, Kikuyama M, Kimura M, Minami S, Utsuki H, Yamazaki T, Suzuki T, Tsuji D, Hayashi H and Itoh K. Polymorphisms of the UDP-glucuronosyl transferase 1A genes are associated with adverse events in cancer patients receiving irinotecan-based chemotherapy. Tohoku J Exp Med 2013; 229: 107-114.

[37] Cai XW, Shedden KA, Yuan SH, Davis MA, Xu LY, Xie CY, Fu XL, Lawrence TS, Lubman DM and Kong FM. Baseline plasma proteomic analysis to identify biomarkers that predict radiation-induced lung toxicity in patients receiving radiation for non-small cell lung cancer. J Thorac Oncol 2011; 6: 1073-1078.

[38] Busser B, Sancey L, Josserand V, Niang C, Khochbin S, Favrot MC, Coll JL and Hurbin A. Amphiregulin promotes resistance to gefitinib in nonsmall cell lung cancer cells by regulating Ku70 acetylation. Mol Ther 2010; 18: 536-543.

[39] Shiiba M, Saito K, Fushimi K, Ishigami T, Shinozuka K, Nakashima D, Kouzu Y, Koike H, Kasamatsu A, Sakamoto Y, Ogawara K, Uzawa K, Takiguchi Y and Tanzawa H. Lipocalin-2 is associated with radioresistance in oral cancer and lung cancer cells. Int J Oncol 2013; 42: 1197-1204.

[40] Luo X, Liu Y, Wang R, Hu H, Zeng R and Chen H. A high-quality secretome of A549 cells aided the discovery of C4b-binding protein as a novel serum biomarker for non-small cell lung cancer. J Proteomics 2011; 74: 528-538.

[41] Livingstone C. IGF2 and cancer. Endocr Relat Cancer 2013; 20: R321-339.

[42] Kua LF, Ross S, Lee SC, Mimura K, Kono K, Goh BC and Yong WP. UGT1A6 polymorphisms modulated lung cancer risk in a Chinese population. PLoS One 2012; 7: e42873.

[43] Zhou H, Luo Y, Chen JH, Hu J, Luo YZ, Wang W, Zeng Y and Xiao L. Knockdown of TRB3 induces apoptosis in human lung adenocarcinoma cells through regulation of Notch 1 expression. Mol Med Rep 2013; 8: 47-52.

[44] Ma PC, Maulik G, Christensen J and Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev 2003; 22: 309-325.

[45] Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC and Janne PA. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007; 316: 1039-1043.

[46] Xu L, Kikuchi E, Xu C, Ebi H, Ercan D, Cheng KA, Padera R, Engelman JA, Janne PA, Shapiro GI, Shimamura T and Wong KK. Combined EGFR/MET or EGFR/HSP90 inhibition is effective in the treatment of lung cancers codriven by mutant EGFR containing T790M and MET. Cancer Res 2012; 72: 3302-3311.

[47] Liu D, Nakano J, Ishikawa S, Yokomise H, Ueno M, Kadota K, Urushihara M and Huang CL. Overexpression of matrix metalloproteinase-7 (MMP-7) correlates with tumor proliferation, and a poor prognosis in non-small cell lung cancer. Lung Cancer 2007; 58: 384-391.