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PKM2 as a biomarker for chemosensitivity to front-line platinum-based chemotherapy in patients with metastatic non-small-cell lung cancer

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Background: Tumour cells exclusively express the embryonic M2 isoform of pyruvate kinase (PKM2). PKM2 expression levels have been correlated with the effect of platinum compounds in cancer cell lines and xenograft models. The potential predictive role of PKM2 in patients with metastatic/advanced non-small-cell lung cancer (NSCLC) receiving platinum-based chemotherapy as first-line was investigated.

Methods: Quantitative real-time PCR was used to assess the expression of *PKM2* in tumour samples from 148 and 157 NSCLC patients in the training and the validation set, respectively. All patients received front-line platinum-based chemotherapy. *PKM2* mRNA expression was also analysed in a control group of 85 NSCLC patients treated with non-platinum containing regimens.

Results: In the training set, high *PKM2* mRNA levels were associated with decreased progression-free survival (PFS; 4.9 months *vs* 6.4, $P=0.006$), overall survival (OS; 10.1 *vs* 17.0 months, $P=0.01$) and disease control rate (DCR; 57.7% *vs* 74.3%; $P=0.021$) compared to patients with low *PKM2* levels. In the validation set, high *PKM2* mRNA levels were also associated with deceased PFS (3.7 *vs* 5.9 months, $P=0.006$), OS (8.3 *vs* 16.8 months, $P=0.003$) and DCR (57.7% *vs* 70.9%; $P=0.049$) compared to those with low *PKM2* mRNA levels. There was no correlation between the *PKM2* mRNA levels and the PFS (5.6 *vs* 5.9, $P=0.43$) or the OS (9.8 *vs* 10.1, $P=0.51$) in the control group. Multivariate analysis revealed high *PKM2* mRNA expression as an independent predictive factor for the poor patients' outcome.

Conclusions: *PKM2* expression may be a predictive biomarker of platinum sensitivity in advanced NSCLC patients treated with platinum-based chemotherapy.

Systemic platinum-based chemotherapy remains the mainstay for the treatment of advanced non-small-cell lung cancer (NSCLC) since it improves survival, symptom control and quality of life compared to best supportive care (Schiller *et al*, 2002). Despite these advances, response to front-line chemotherapy remains poor since patients experience disease progression on an average of 4–6 months from the treatment initiation, and 1-year survival rate is $<45\%$ (Fossella *et al*, 2003).

Cisplatin exerts its cytotoxic effect by reacting with DNA, causing inter- and intra-strand DNA crosslinks that result in the formation of DNA adducts (Ferry *et al*, 2000). A major limitation of cisplatin efficacy is due to intrinsic or acquired resistance by

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tumour cells. Multiple mechanisms contribute to cisplatin resistance including decreased drug accumulation due to decreased cellular uptake and increased cellular efflux, increased drug detoxification by thiols and increased DNA repair (Kelland, 2007). In the last decade numerous studies have focused on molecules that are components of the pathways that regulate resistance mechanisms and their potential role as factors that could predict the response to cisplatin treatment.

Metabolic requirements in cancer cells are fundamentally different from those in normal differentiated adult cells since they are characterised by increased glucose uptake and lactate production, regardless of oxygen availability (Levine and Puzio-Kuter, 2010). This altered metabolic phenotype known as 'aerobic glycolysis' or 'Warburg effect' is likely preferred by the tumour cells to efficiently convert glucose into the macromolecules needed for tumour growth (Warburg, 1956). One of the most established key regulators of aerobic glycolysis is the embryonic M2 isoform of pyruvate kinase (PKM2), which is preferentially expressed in cancer and in all rapidly proliferating cells (Christofk *et al*, 2008a). Pyruvate kinase catalyses the last step of glycolysis by the formation of pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. PKM2 activity is allosterically regulated by binding of fructose 1,6-biphosphate, and also by interactions with tyrosine-phosphorylated proteins in response to growth signals and by post-translational modifications (Christofk *et al*, 2008b; Lv *et al*, 2011; Anastasiou *et al*, 2011, 2012). PKM2 can switch from a highly-active tetrameric form to a low-active dimeric form with low affinity to PEP (Mazurek *et al*, 2005; Christofk *et al*, 2008b).

Recently, several studies have reported that PKM2 is more than a regulator of aerobic glycolysis suggesting multiple non-metabolic functions with diverse implications during tumourigenesis (Harris *et al*, 2011; Luo and Semenza, 2012; Tamada *et al*, 2012b). PKM2 has been described to act as a transcriptional co-activator and as a protein kinase (Luo *et al*, 2011; Yang *et al*, 2011, 2012; Gao *et al*, 2012), and has also been found to play a role in the control of reactive oxygen species (ROS) concentrations and glutathione antioxidant protection (Anastasiou *et al*, 2011; Tamada *et al*, 2012a). The latter appears to imply a possible role of PKM2 to cisplatin resistance.

There are few *in vitro* studies investigating the tumoural expression of PKM2 and the effect of platinum compounds in cancer cell lines, but the results still remain confusing. Decreased PKM2 protein and activity was found in cisplatin- and oxaliplatin-resistant gastric and colorectal cell lines, respectively (Yoo *et al*, 2004; Martinez-Balibrea *et al*, 2009). In contrast, the combination of siRNA targeting the PKM2 and cisplatin increased apoptosis and decreased tumour volume in a lung cancer xenograft model (Guo *et al*, 2010). In the present study, we investigated the predictive significance of *PKM2* mRNA expression in tumours from NSCLC patients treated with front-line platinum-based chemotherapy and provided evidence of its potential role as a predictive biomarker.

MATERIALS AND METHODS

Patient population. Formalin-fixed, paraffin-embedded (FFPE) tissues from 148 consecutive patients with histologically confirmed stage IIIB (with pleural effusion) and IV NSCLC who were treated with front-line platinum-based chemotherapy were retrospectively collected and analysed (training set). The validation set consisted of 157 NSCLC patients with unresectable stage IIIB (with pleural effusion) or IV from an independent cohort of patients receiving platinum-based chemotherapy doublets in the front-line setting. Furthermore, 85 NSCLC patients who were treated with front-line non-platinum-based doublets were enrolled (control group). The used platinum-based regimens in the three cohorts of patients are presented in Table 1. All the above mentioned patients received front-line treatment in the context of two randomised trials conducted by the Hellenic Oncology Research Group (Georgoulias *et al*, 2001, 2005). The study was approved by the Ethics and Scientific Committees of the University General Hospital of Heraklion. All patients gave their written informed consent for the use of their tissue for translational research.

Specimen selection and RNA extraction. Formalin-fixed, paraffin-embedded tumour sections were examined by a pathologist (EL) in order to identify the most appropriate tumour areas for dissection. Serial sections of 5 μm were prepared and stained with Nuclear Fast Red (Sigma-Aldrich, St Lious, MO, USA). In the case of samples with $<80\%$ tumour cells, an Eppendorf piezoelectric microdissector (Eppendorf, Hamburg, Germany) was used to procure only malignant cells. TRIzol LS (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction, followed by DNase (DNA-free, Ambion, Austin, TX, USA) treatment in order to avoid genomic DNA contamination (Papadaki *et al*, 2009).

mRNA expression analysis. Reverse transcription and RT–qPCR have been described elsewhere (Papadaki *et al*, 2009). Briefly, 200 ng of total RNA were used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). Relative cDNA quantification for *PKM2* and *β-actin* and *phosphoglycerate kinase 1* (*PGK*) as internal controls was performed using the ABI Prism 7900 HT Sequence Detection System (AB, Foster City, CA, USA).

The primers and 5′-labelled fluorescent reporter dye (6FAM) probe sets were designed using the Primer Express 2.0 Software (AB) according to the Ref Seq NM_002654 for *PKM2* and were as follows: *PKM2*, 5′-GCCATAATCGTCCTCACCAAGT-3′ (forward), 5′-GCACGTGGGCGGTATCTG-3′ (reverse) and 5′-CAGGTCTGCTCACCAGG-3′ (probe). The primers and probe sequences for both housekeeping genes, *β-actin* and *PGK* have been reported elsewhere (Saridaki *et al*, 2011). Comparative Ct method was used for gene expression analysis using both *β-actin* and *PGK* as reference genes and commercial RNA (Stratagene, La Jolla, CA, USA) as calibrators. Final expression values were determined as follows: $2^{-(\Delta CT\ \text{sample} - \Delta CT\ \text{calibrator})}$, where ΔCT values of the calibrator and sample were determined by subtracting the CT value of the target gene from the mean value of both reference genes. In all experiments, only triplicates with a s.d. of the CT value <0.25 were accepted. In addition, genomic DNA contamination was excluded by including non-reverse-transcribed RNA as a control for each sample.

Immunohistochemistry and staining evaluation. Approximately, 4 μm-thick FFPE tissue sections were stained with haematoxylin and eosin and histopathologically verified by a pathologist. The primary antibody used for PKM2 staining was directed against the specific sequence of exon 9 that is unique to PKM2 (rabbit polyclonal Ab, cat. no. 3198, Cell Signaling, Danvers, MA, USA; dilution 1 : 600). Immunostaining was performed using Ultra Vision LP Quanto Detection System HRP Polymer (Thermo Fisher Scientific, Fremont, CA, USA). Sections stained for PKM2 were previously treated in Tris-EDTA buffer (pH 9.0) for 13 min. Staining evaluation was performed by two independent pathologists (EL and AK) blinded to each other's scores and to each patient's clinical information. Staining of PKM2 was scored as the product of the intensity on a scale of 0–5: low and 5.1–8: high expression, as described elsewhere (Yang *et al*, 2011).

Study design and statistical analysis. Progression-free survival (PFS) and overall survival (OS) were calculated from the start of treatment to the first documented disease progression or death,

Table 1. Characteristics of patients and tumours in the training, validation and control set

	Training		Validation		Control	
Feature	***N***	**%**	***N***	**%**	***N***	**%**
	148		157		85	
Gender						
Male	121	82	135	86	78	91
Female	27	18	22	14	7	8
Median age (range) years	60 (34–78)		61 (31–80)		62 (37–79)	
Tumour type						
Squamous	44	30	44	28	17	20
Non-squamous	104	70	113	72	68	80
ECOG PS						
0–1	127	86	135	86	80	94
2	21	14	21	14	5	6
Stage						
IIIB (wet)	38	25	41	26	24	28
IV	110	75	116	74	61	72
Platinum-based first-line						
Platinum + docetaxel	110	74	111	71	NA	
Platinum + docetaxel + Avastin	18	12	22	14	NA	
Platinum + gemcitabine	15	10	17	11	NA	
Platinum + pemetrexed	5	4	7	4	NA	
Non-platinum-based first-line						
Docetaxel + gemcitabine	NA		NA		85	100
Post-progression treatment	100	68	105	67	55	65
EGFR mutational status						
EGFR wt	27		34		10	
EGFR mut	4		4		1	
EGFR uknown	117		119		74	
PKM2 mRNA expression						
Median (range)	12.77 (0.34–71.88)		NA		12.73 (0.36–70.34)	
High expression	74	50	78	49	42	49
Low expression	74	50	79	51	43	51

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group Performance Status; NA = not applicable.

respectively. Objective responses were recorded according to the RECIST criteria (Therasse *et al*, 2000). Cutoff points were calculated according to the median value for the mRNA expression. Samples with mRNA expression above or equal to the median were considered as samples with high expression, while those with value below the median as samples with low expression. All the laboratory analyses were performed blinding to the clinical data.

The potential association between baseline characteristics, response and gene expression levels were compared with either the two-sided Fisher's exact test or the chi-square test for categorical variables and the Kruskal–Wallis test for continuous variables. The normality of continuous variables was verified with the Kolmogorov–Smirnov test. The association of risk factors with time-to-event end points was analysed with the log-rank test and the Kaplan–Meier method was used to plot the corresponding time-to-progression and survival curves. A univariate Cox regression analysis, with hazard ratios (HRs) and 95% confidence intervals (CIs), was used to assess the association between each potential prognostic factor and survival and time to progression. These factors were then included in a multivariate Cox proportional hazards regression model with a stepwise procedure (both forward and backward) to evaluate the independent significance of different variables on survival and time to progression. Statistical significance was set at $P = 0.05$.

RESULTS

Patients' characteristics and clinical features. The main clinical characteristics and gene mRNA levels of PKM2 in the three cohorts of patients are summarised in Table 1. Among the three groups analysed, the median age ranged from 60 to 62 years old and patients were predominantly males. All three groups were predominantly constituted from non-squamous type of tumours and most patients had stage IV disease. The EGFR mutation status of the tested patients is also shown in Table 1.

PKM2 mRNA expression and patients' outcome. In total, 390 NSCLC patients were treated with front-line platinum and/or non-platinum containing doublets in the context of two randomised trials conducted by the Hellenic Oncology Research Group. Among the 305 patients treated with platinum containing doublets, 148 and 157 of them were analysed as the training and the validation set, respectively. The remaining 85 platinum-naive patients constituted the control set (Figure 1). *PKM2* mRNA expression was successfully assessed in all of the samples analysed and the level of expression was associated with PFS and OS.

The median mRNA expression level was 12.77 (minimum, maximum: 0.34, 71.88) for the training set and the same cutoff was used for the analysis of the validation and control sets. In 30 tumour

specimens, which were randomly selected from the training and the validation sets that were stained for PKM2 protein expression by immunohistochemistry, there was no correlation between PKM2 mRNA and protein expression (Spearman's test, $P=0.275$; data not shown). Moreover, there was no significant correlation between the *PKM2* mRNA expression and the patients' age, gender, tumour histology, stage and PS (all *P*-values >0.05).

In the training set, patients with high tumoural *PKM2* mRNA levels had significantly shorter median PFS (4.9 *vs* 6.4 months; $P=0.006$; Figure 2A) compared to patients with low tumoural *PKM2* mRNA levels. Similarly, patients with high mRNA expression of *PKM2* were significantly associated with decreased median OS (10.1 *vs* 17.0; $P=0.01$; Figure 3A) compared to the patients with low expression levels. On the contrary, there was no significant correlation between the *PKM2* mRNA levels and the objective response to cisplatin-based chemotherapy ($P=0.497$; Table 2). However, when the analysis was performed according to the clinical benefit (DCR) a significant correlation was observed between *PKM2* mRNA expression levels and DCR (Table 2; 74.3% and 57.7% DCR for patients with low and high *PKM2* mRNA expression, respectively; $P=0.021$).

Results in the validation set were similar to those in the training set. Median PFS was significantly decreased in patients with high mRNA expression of *PKM2* (3.7 *vs* 5.9 months; $P=0.006$; Figure 2B) in comparison with those with low mRNA levels. Furthermore, patients with high *PKM2* mRNA levels had significantly decreased median OS (8.3 *vs* 16.8; $P=0.003$; Figure 3B) as compared with those whose tumours had low *PKM2* mRNA levels. Similarly to the training set, although there was no significant correlation between *PKM2* mRNA levels and objective response rate ($P=0.390$; Table 2) in the validation set, a marginal correlation with the DCR (70.9% *vs* 57.7%; $P=0.049$; Table 2) was observed.

Finally, the analysis of the whole group of patients (after combining the training and the validation sets) clearly revealed that high *PKM2* mRNA expression was associated with decreased PFS (3.9 *vs* 6.3 months; $P=0.001$), OS (9.6 *vs* 16.8 months; $P<0.001$) and DCR (57.2% *vs* 72.5%; $P=0.014$).

The expression values of *PKM2* mRNA in the control set were quite similar with that recorded in the training set since the median mRNA expression level for the control set was 12.73 (minimum, maximum: 0.46, 72.17) and no significant difference was observed in comparison with that of the training set ($P=0.057$). Despite that, and as opposed to the patients treated with platinum-based regimens both in the training and validation sets, there was no difference in terms of PFS (5.6 *vs* 5.9; $P=0.43$; Figure 2C) and OS (9.8 *vs* 10.1; $P=0.51$; Figure 3C) among patients with low and high *PKM2* mRNA expression.

Univariate and multivariate analysis. Univariate analysis in the whole group of patients enrolled in both the training and validation sets revealed that patients with high tumoural *PKM2* mRNA expression levels (HR: 1.89, 95% CI: 1.48–2.27; $P=0.003$), PS of 2 (HR: 2.64, 95% CI: 1.89–3.17; $P=0.001$) as well as stage IV (HR: 1.77, 95% CI: 1.11–2.31; $P=0.03$) were significantly associated with decreased PFS, whereas age >70 years ($P=0.77$), gender ($P=0.51$), histology ($P=0.61$) and tumour differentiation ($P=0.14$) did not show any significant correlation with the PFS (Table 3). Similarly, high *PKM2* mRNA expression (HR: 1.93, 95% CI: 1.54–2.46; $P=0.002$) and PS of 2 (HR: 2.87, 95% CI: 1.92–3.44; $P<0.001$) were significantly associated with decreased OS. In contrast, age >70 years ($P=0.23$), gender ($P=0.48$), stage IV ($P=0.17$), histology ($P=0.94$) and tumour differentiation ($P=0.31$) were not significantly associated with decreased OS (Table 3).

Multivariate Cox regression analysis revealed that high tumoural *PKM2* mRNA expression (HR: 1.81, 95% CI: 1.40–2.38; $P=0.002$) as well as PS of 2 (HR: 3.97, 95% CI: 2.77–4.16; $P<0.001$) emerged as independent predictive factors for decreased PFS (Table 4). Similarly, high PKM2 mRNA levels (HR: 1.97, 95% CI: 1.45–2.46; $P=0.001$) and PS of 2 (HR: 4.01, 95% CI: 3.56–5.06; $P<0.001$; Table 4) were revealed as independent predictive factors for shorter OS.

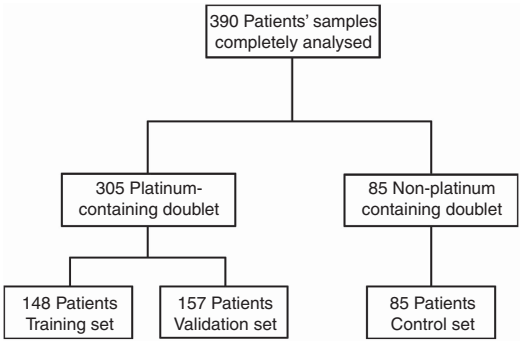

Figure 1. Flow chart of NSCLC patients analysed for *PKM2* mRNA expression.

DISCUSSION

In the current study, we investigated the role of tumoural *PKM2* mRNA expression levels as a predictive factor in the outcome of metastatic NSCLC patients treated with front-line platinum-based

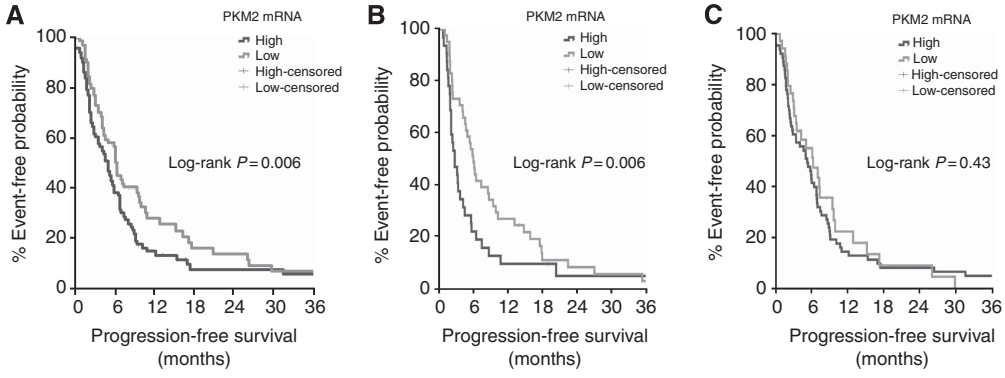

Figure 2. (**A**) PFS according to *PKM2* mRNA expression in the training set. (**B**) PFS according to *PKM2* mRNA expression in the validation set. (**C**) PFS according to *PKM2* mRNA expression in the control set. Higher levels of PKM2 mRNA were associated with decreased PFS in the training (**A**) and in validation set (**B**) but not in the control group (**C**).

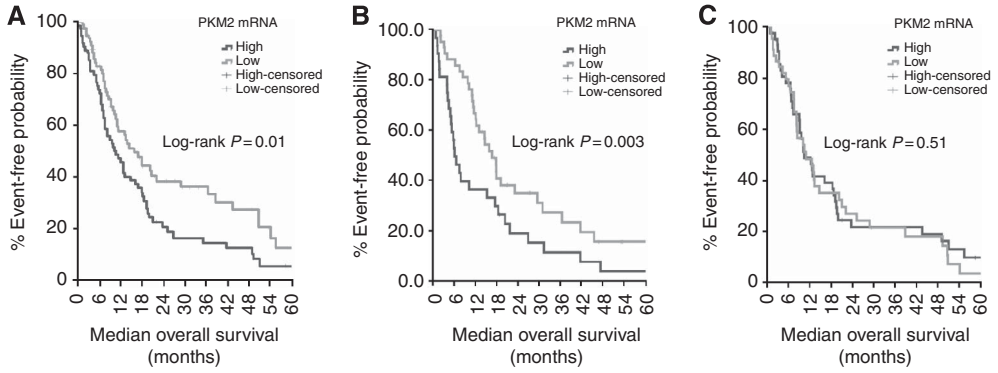

Figure 3. (**A**) OS according to *PKM2* mRNA expression in the training set. (**B**) OS according to *PKM2* mRNA expression in the validation set. (**C**) OS according to *PKM2* mRNA expression in the control set. Higher levels of PKM2 mRNA were associated with decreased OS in the training (**A**) and validation set (**B**) but not in the control group (**C**).

Table 2. ORR and DCR according to *PKM2* mRNA expression in the training, validation set and whole population

	ORR, *N* (%)			DCR, *N* (%)		
PKM2 mRNA expression	**CR + PR**	**SD + PD**	***P***	**CR + PR + SD**	**PD**	***P***
Training						
High	32 (43.2)	42 (56.8)	0.497	42 (57.7)	32 (42.3)	0.021
Low	31 (41.9)	43 (58.1)		55 (74.3)	19 (25.7)	
Validation						
High	25 (32.1)	53 (67.9)	0.390	45 (57.7)	33 (42.3)	0.049
Low	28 (35.4)	51 (64.6)		56 (70.9)	23 (29.1)	
Whole population						
High	57 (37.5)	95 (62.5)	0.590	87 (57.2)	65 (42.3)	0.014
Low	59 (38.6)	94 (61.4)		111 (72.5)	42 (27.5)	

Abbreviations: CR = complete response; DCR = disease control rate; ORR = objective response rate; PD = progressive disease; PR = partial response; SD = stable disease.

Table 3. Univariate analysis for PFS and OS

	Hazard ratio	95% CI	*P*-value
PFS			
PKM2 expression (high *vs* low)	1.89	1.48–2.27	0.003
PS (2 *vs* 0–1)	2.64	1.89–3.17	0.001
Age (>70 *vs* ⩽70 years)	1.24	0.87–1.62	0.77
Gender (male *vs* female)	1.19	0.84–1.41	0.51
Stage (IV *vs* IIIB)	1.77	1.11–2.31	0.03
Histology (squamous *vs* non-squamous)	1.32	0.85–1.54	0.61
Tumour differentiation (low *vs* well-moderate)	1.41	0.90–1.77	0.14
OS			
PKM2 expression (high *vs* low)	1.93	1.54–2.46	0.002
PS (2 *vs* 0–1)	2.87	1.92–3.44	<0.001
Age (>70 *vs* ⩽70 years)	1.35	0.91–1.88	0.23
Gender (male *vs* female)	1.16	0.81–1.33	0.48
Stage (IV *vs* IIIB)	1.45	0.96–1.85	0.17
Histology (squamous *vs* non-squamous)	1.17	0.89–1.28	0.94
Tumour differentiation (low *vs* well-moderate)	1.32	0.87–1.32	0.31

Abbreviations: CI = confidence interval; OS = overall survival; PFS = progression-free survival; PS = performance status.

chemotherapy. In a training set of 148 patients, those with tumours having low mRNA levels of *PKM2* presented significantly higher PFS ($P = 0.006$) and OS ($P = 0.01$) and DCR ($P = 0.021$) as well. Our results were confirmed in an independent cohort of 157 patients who have also been treated with cisplatin-based chemotherapy in the first-line setting. Patients with low *PKM2* mRNA levels attained statistically significant increase of PFS ($P = 0.006$) and OS ($P = 0.003$) and higher DCR ($P = 0.049$) as well. Unlike the results in the training and validation set, in the control group of 85 patients, who did not received platinum-based chemotherapy, *PKM2* mRNA levels were not correlated with PFS ($P = 0.43$) and OS ($P = 0.51$). This observation clearly suggests that the predictive value of *PKM2* mRNA levels is mainly related to the platinum compounds. This association could not be attributed to a possible effect of tyrosine kinase inhibitors administered to our patients since the number of patients who received this anti-EGFR treatment was very low. Furthermore, multivariate analysis revealed that high *PKM2* mRNA expression was an independent predictive factor for shorter PFS and decreased OS in both the training and validation sets.

Table 4. Multivariate analysis for PFS and OS

	Hazard ratio	95% CI	*P*-value
PFS			
PKM2 expression (high *vs* low)	1.81	1.40–2.38	0.002
PS (2 *vs* 0–1)	3.97	2.77–4.16	<0.001
Stage (IV *vs* IIIB)	1.34	0.96–1.88	0.13
OS			
PKM2 expression (high *vs* low)	1.97	1.45–2.46	0.001
PS (2 *vs* 0–1)	4.01	3.56–5.06	<0.001

Abbreviations: CI = confidence interval; OS = overall survival; PFS = progression-free survival; PS = performance status.

PKM2 is one of the four isoforms of pyruvate kinase, the enzyme that catalyses the formation of pyruvate and ATP from PEP and ADP (Mazurek, 2010). The four isoforms of pyruvate kinase are encoded by two genes that are expressed in a cell- and tissue-specific manner. The *L* and *R* isoenzymes, derived from the *PKLR* gene are expressed in the liver and red blood cells, respectively (Noguchi *et al*, 1987). The *PKM* gene encodes the M1- and M2-type isoenzymes (Noguchi *et al*, 1986). It consists of 12 exons, of which 9 and 10 are alternatively spliced in a mutually exclusive manner to give rise to the *PKM1* and *PKM2* isoforms, respectively (Noguchi *et al*, 1986). Alternative splicing in *PKM* gene is regulated by heterogeneous nuclear ribonucleoproteins hnRNP, under the control of c-Myc (Clower *et al*, 2010; David *et al*, 2010). From the four isoforms of pyruvate kinase, cancer cells exclusively express the M2 isoform (Christofk *et al*, 2008a). *PKM2* but not *PKM1* is necessary for aerobic glycolysis since the replacement of *PKM2* by *PKM1* reduced the capacity of tumour cell lines to develop into a tumour (Christofk *et al*, 2008a). *PKM2* is negatively regulated in response to growth factors by binding to tyrosine-phosphorylated proteins (Christofk *et al*, 2008b; Hitosugi *et al*, 2009). Phosphorylation of *PKM2* results in the formation of its inactive dimeric form that enables the diversion of glycolytic intermediates into anabolic pathways (Hitosugi *et al*, 2009; Mazurek, 2010). Also, *PKM2* enzymatic activity can be modulated by a variety of post-translational modifications such as acetylation, sumoylation, ubiquitination and oxidation (Anastasiou *et al*, 2011; Lv *et al*, 2011; Luo and Semenza, 2012; Yang and Lu, 2013). The lack of significant correlation between *PKM2* mRNA and protein expression observed in the current study could be explained on the basis of post-translational modifications mentioned above. It is obvious that IHC and mRNA expression analysis in a larger cohort of patients would confirm this observation.

The role of PKM2 to modulate the cytotoxicity of cisplatin and its derivatives is as yet not fully explored. Proteomic analysis showed that *PKM2* is downregulated in oxaliplatin-resistant cell lines, while high mRNA expression was associated with higher response rate in oxaliplatin-treated colorectal cancer patients (Martinez-Balibrea *et al*, 2009). In the same line of evidence it was shown that *PKM2* protein and activity were lower in cisplatin-resistant human gastric carcinoma cell lines (Yoo *et al*, 2004). In contrast, our results are in the opposite direction, since low *PKM2* mRNA levels were associated with better outcome of NSCLC patients both in the training and validation set. This evidence is in agreement with previous results from our laboratory on SCLC patients treated with platinum-based chemotherapy, since patients with low expression levels of *PKM2* attained significantly better PFS and OS (Karachaliou *et al*, 2013). Accordingly, results from previous studies showed that inhibition of *PKM2* mRNA expression by siRNA targeting in combination with chemotherapeutic agents, significantly increased apoptosis and decreased tumour volume in xenograft models (Guo *et al*, 2010).

Several mechanisms of resistance to platinum compounds, either intrinsically or acquired, have been described. Decreased membrane transport, increased cytoplasmic detoxification, increased DNA repair activity and increased tolerance to DNA damage are the major mechanisms that can contribute to cisplatin resistance (Siddik, 2003; Kelland, 2007). Excision repair complementation group 1 (ERCC1) and breast cancer susceptibility gene 1 (BRCA1) that participate in DNA-repair pathways have been considered as potential predictive factors, since their level of expression seems to be correlated and influence cisplatin efficacy in a variety of solid tumours (Postel-Vinay *et al*, 2012). It is also well established that platinum analogues interact with sulfur-containing thiomolecules such as glutathione and metallothionein leading to its inactivation and subsequent failure from binding to DNA (Siddik, 2003; Yang *et al*, 2006; Kelland, 2007). Also, studies in preclinical models and tumour samples suggest that elevated levels of glutathione or glutathione-related enzymes are associated with limited cisplatin efficacy (Godwin *et al*, 1992; Yang *et al*, 2005, 2006). Furthermore, glutathione as an antioxidant offers protection from ROS that are known to accumulate and induce apoptosis after radiotherapy and chemotherapeutic drug treatment (Bragado *et al*, 2007; Choi *et al*, 2007). Recent studies suggest that *PKM2* might play a role in the control of glutathione and ROS concentrations, implying a possible role to cisplatin resistance. In human lung cancer cells, increased concentrations of ROS can inhibit *PKM2* activity through oxidation of cysteine 358 (Cys^{358}; Anastasiou *et al*, 2011). This resulted in the diversion of glucose intermediates into pentose phosphate pathway (PPP), which produces nicotinamide adenine dinucleotide phosphate (NADPH). Nicotinamide adenine dinucleotide phosphate provides reducing equivalents for the reduction of oxidised GSH (GSSG) to reduced GSH, thereby increasing ROS detoxification (Anastasiou *et al*, 2011). The above regulation mechanism of ROS concentrations by *PKM2* seems to be specific since an oxidation-resistant mutant form of *PKM2* failed to confer antioxidant response (Anastasiou *et al*, 2011). Additionally, PKM2 has been described to regulate ROS accumulation by interacting with the cell-surface marker of stem cells, CD44 (Tamada *et al*, 2012a, b). This interaction promotes glycolysis and increases the flux to PPP resulting to the production of NADPH and to the subsequent increase of reduced GSH and decrease ROS accumulation (Tamada *et al*, 2012a). The role of CD44 to inhibit ROS accumulation in cancer cells has also been addressed earlier (Ishimoto *et al*, 2011). It is questionable if the observed association between tumoural *PKM2* mRNA expression and the poor patient's outcome in our study could be explained on the basis of the resistance mechanisms described above. Preliminary data from our laboratory strengthens this possibility since they demonstrate a strong positive correlation between *PKM2* and CD44 expression in ovarian tumour specimens from patients treated with cisplatin in front line (data not shown).

Tumour cells have multiple ways to regulate *PKM2* both in transcriptional and post-transcriptional level, in order to ensure anabolic metabolism and survival under hypoxic conditions, *PKM2* acting in a positive feedback loop interacts with *HIF1α* through the prolyl hydroxylase 3 and promotes the activation of *HIF1α* regulated genes (Luo *et al*, 2011). This positive feedback loop maintains expression of *PKM2* and other glycolytic enzymes in high levels. Accordingly, the above mechanism seems to be biologically relevant since preliminary data from our laboratory have demonstrated that there is a strong positive correlation between *PKM2* and *HIF1α* mRNA expression in NSCLC tumour samples (data not shown). A strong positive correlation also exist between *PKM2* and *c-Myc* mRNA expression (data not shown). Therefore, as argued by our data, measuring *PKM2* mRNA levels by RT–PCR in clinical samples seem to be reasonable.

Although our results are retrospectively originated, in the best of our knowledge this study is the first one providing evidence for the

predictive significance of a biomarker by validating the results in an independent cohort of patients. Also, unpublished data from our laboratory have evaluated the predictive significance of *PKM2* in other tumour types treated with cisplatin or its derivatives. However, this evidence has to be interpreted with caution and any clinical relevance of the tumoural *PKM2* mRNA expression should be validated prospectively. Furthermore, it is an interesting issue to investigate whether *PKM2* mRNA are influenced by chemotherapy by using a rebiopsy in the metastatic tumour. In addition, it remains a challenge that has to be answered using *in vitro* models, to elucidate at which level of *PKM2* regulation, either transcriptional or post-transcriptional, could modulate anticancer-drug cytotoxicity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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