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

C Papadaki1, M Sfakianaki1, E Lagoudaki2, G Giagkas1, G Ioannidis1, M Trypaki1, E Tsakalaki1, A Voutsina1, A Koutsopoulos2, D Mavroudís1,3, V Georgoulías1,3 and J Souglakos*,1,3

1Laboratory of Tumor Cell Biology, School of Medicine, University of Crete, Heraklion, 71003 Crete, Greece; 2Department of Pathology, University General Hospital of Heraklion, Heraklion, 71110 Crete, Greece and 3Department of Medical Oncology, University General Hospital of Heraklion, Heraklion, 71110 Crete, Greece

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
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; Anastassiou 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 (Anastassiou 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-Balbrea 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 (Georgoulis 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 Louis, MO, USA). In the case of samples with <80% tumour cells, an Eppendorf pieceoelectric 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’-GCTATAATCGTGTCTACCAAGT-3’ (forward), 5’-GCAGTGGGCGATCTG-3’ (reverse) and 5’-CAGGTCTGCTACGG-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^-ΔΔCt (sample – ACT calibrator) where ΔΔCt 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 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,
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

| Feature                              | Training | %  | Validation | %  | Control | %  |
|--------------------------------------|----------|----|------------|----|---------|----|
| 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                                |          |    |            |    |         |    |
| IIB (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 unknown                         | 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.
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.

**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

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

**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).
PKM2 mRNA expression in NSCLC

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

| PKM2 mRNA expression | ORR, N (%) | DCR, N (%) |
|----------------------|------------|------------|
| Training             |            |            |
| High                 | 32 (43.2)  | 42 (56.8)  | 0.497       |
| Low                  | 31 (41.9)  | 43 (58.1)  | 55 (74.3)  | 19 (25.7) | 0.021 |
| Validation           |            |            |
| High                 | 25 (32.1)  | 53 (67.9)  | 0.390       |
| Low                  | 28 (35.4)  | 51 (64.6)  | 56 (70.9)  | 23 (29.1) | 0.049 |
| Whole population     |            |            |
| High                 | 57 (37.5)  | 95 (62.5)  | 0.590       |
| Low                  | 59 (38.6)  | 94 (61.4)  | 111 (72.5) | 42 (27.5) | 0.014 |

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.

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).
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, 2008). 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 (Cys358); 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).

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 (Cys358); 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).

### 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.
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 tumoral 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 transcriptional or post-transcriptional, could modulate anticancer-drug cytotoxicity.

ACKNOWLEDGEMENTS

This work was partially supported by a research grant from the Hellenic Society for Medical Oncology (HeSMO) and the Cretan Association for Biomedical Research (CABR).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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