Research Paper

Towards Prognostic Profiling of Non-Small Cell Lung Cancer: New Perspectives on the Relevance of Polo-Like Kinase 1 Expression, the *TP53* Mutation Status and Hypoxia

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Abstract

Background: Currently, prognosis of non-small cell lung cancer (NSCLC) patients is based on clinicopathological factors, including TNM stage. However, there are considerable differences in patient outcome within a similar staging group, even when patients received identical treatments. In order to improve prognostic predictions and to guide treatment options, additional parameters influencing outcome are required. Polo-like kinase 1 (Plk1), a master regulator of mitotic cell division and the DNA damage response, is considered as a new potential biomarker in this research area. While several studies reported Plk1 overexpression in a broad range of human malignancies, inconsistent results were published regarding the clinical significance hereof. A prognostic panel, consisting of Plk1 and additional biomarkers that are related to the Plk1 pathway, might further improve prediction of patient prognosis.

Methods: In this study, we evaluated for the first time the prognostic value of Plk1 mRNA and protein expression in combination with the *TP53* mutation status (next generation sequencing), induction of apoptotic cell death (immunohistochemistry for cleaved caspase 3) and hypoxia (immunohistochemistry for carbonic anhydrase IX (CA IX)) in 98 NSCLC adenocarcinoma patients.

Results: Both Plk1 mRNA and protein expression and CA IX protein levels were upregulated in the majority of tumor samples. Plk1 mRNA and protein expression levels were higher in *TP53* mutant samples, suggesting that Plk1 overexpression is, at least partially, the result of loss of functional p53 (<0.05). Interestingly, the outcome of patients with both Plk1 mRNA and CA IX protein overexpression, who also harbored a *TP53* mutation, was much worse than that of patients with aberrant expression of only one of the three markers (p=0.001).

Conclusion: The combined evaluation of Plk1 mRNA expression, CA IX protein expression and *TP53* mutations shows promise as a prognostic panel in NSCLC patients. Moreover, these results pave the way for new combination strategies with Plk1 inhibitors.

Key words: Polo-like kinase 1, Carbonic anhydrase IX, *TP53*, Non-small cell lung cancer, Prognostic biomarker, Hypoxia.

Introduction

Non-small cell lung cancer (NSCLC), accounting for an estimated 75-85% of all lung neoplasms, is the leading cause of cancer-related death worldwide [1]. Over the last decades, molecular profiling studies revealed that both genetic and molecular changes underlie this malignancy, leading to the development of individualized and more selective treatment approaches [2]. However, clinical efficacy of these targeted therapies in long-term treatments is often limited by the emergence of resistance mechanisms. Therefore, numerous new potential targets, including several cell cycle kinases, are under investigation in order to further improve patient survival [3, 4].

The Polo-like kinase (Plk) family, which consists of five members (Plk1-5), represents a family of highly conserved serine/threonine kinases that play crucial roles in cell cycle processes [5]. Plk1, the first identified member of the Plk family, mediates several mitotic events, including mitotic entry, microtubule nucleation, centrosome maturation, kinetochore assembly and regulation of cytokinesis (reviewed by Zitouni *et al.* [6] and Van den Bossche *et al.* [7]). In addition, Plk1 also plays a role in DNA damage response by regulating (i) G_2/M DNA damage checkpoint maintenance and recovery and (ii) DNA repair through phosphorylation of the RAD51 recombinase to facilitate homologous recombination [8]. Both Plk1 mRNA and protein levels are tightly regulated in time, with low levels during interphase and high expression in mitosis. Aberrant Plk1 activity may result in overriding cell cycle checkpoints, leading to genomic instability [9]. To date, Plk1 has been found to be overexpressed in a broad range of tumor types. In some malignancies, high expression levels were correlated with genetic instability and poor patient prognosis [10-19]. Moreover, high tumor mitotic rate and risk of metastasis have been associated with high Plk1 levels, implying a role for Plk1 in aggressive cancers and the potential for its use as a prognostic marker and therapeutic target [20].

Another crucial regulator of the cell cycle is p53, which ensures regulation of the response to cellular stress signals by induction of cell cycle arrest, senescence or apoptosis in order to prevent malignant transformation. In addition to activating the expression of a wide range of genes involved in these protective processes, p53 also represses the expression of many genes required for cell cycle progression and survival, including Plk1. Hence, it became clear that the Plk1 pathway and p53 are cross-linked [21]. In the presence of DNA damage during the G_2/M phase, the Plk1 promotor is downregulated by p53, leading to mitotic arrest and the induction of apoptotic cell death. As such, loss of p53 function may result in deregulated Plk1 levels [21]. Furthermore, Sur *et al.* reported that p53 deficient cells become more dependent on Plk1 under stress conditions, suggesting that the p53 status might be a predictive marker for successfully targeting Plk1 [22]. Meanwhile, Plk1 also negatively regulates p53, thereby inhibiting its pro-apoptotic function [21]. However, the exact relationship between Plk1 expression levels, the *TP53* status and the induction of apoptotic cell death still needs to be further elucidated.

Reduced oxygen levels (i.e. hypoxia) is a common characteristic of human solid tumors and initiates a complicated biological response, involving numerous pathways that influence the properties of malignant cells: from proliferation and the induction of cell death to angiogenesis, metastatic spread and the selection of cells with more aggressive phenotypes [23-25]. Consequently, hypoxic tumor regions often contain viable cells that are more resistant to chemo- and/or radiotherapy. These adoptive mechanisms are under strict regulation of the hypoxia inducible factor 1 (HIF-1α). Recently, Ward *et al.* published that Plk1 and Plk4 expression become deregulated under reduced oxygen tension through epigenetic modifications in their promoter regions in a cell-specific p53-dependent manner [26]. However, the exact mechanism controlling Plk1 expression under reduced oxygen conditions is still unknown, making it interesting to further elucidate the involvement of p53 and Plk1 in the hypoxia signaling pathway.

In the present study, we performed a detailed analysis of Plk1 expression at both the transcript and protein level in 98 primary NSCLC (adenocarcinoma) tumor tissue samples compared to adjacent normal lung tissue samples and we correlated our findings with clinicopathological parameters. Furthermore, we investigated for the first time the relationship between Plk1 and the *TP53* gene mutation status, induction of apoptotic cell death and the occurrence of hypoxia.

Materials and Methods

Patients and tissue specimens

Formaldehyde fixed-paraffin embedded (FFPE) samples from NSCLC adenocarcinoma tissues were obtained from 98 patients who underwent surgery at the Antwerp University Hospital (Belgium). The patients' main clinical and pathological parameters

are summarized in **Table 1**. FFPE samples from adjacent normal lung tissue from 16 NSCLC patients were used as control samples. Tissue specimens were obtained from the Antwerp University Hospital Tumorbank and fixed in 4% formaldehyde for 6-18h and paraffin embedded on a routine basis. The study was approved by the Ethics committee of the Antwerp University Hospital.

Table 1. Patient characteristics.

Patient characteristic	Number of patients (%)
Gender	
Female	64 (65.3%)
Male	34 (34.7%)
Smoking behavior	
Non-smoker	7 (7.1%)
Smoker	76 (77.6%)
Unknown	15 (15.3%)
Clinical stage	
I	43 (43.9%)
II	26 (26.5%)
III	22 (22.4%)
IV	7 (7.1%)
Tumor classification	
T1	32 (32.7%)
T2	38 (38.8%)
T3	22 (22.4%)
T4	6 (6.1%)
N0	66 (67.3%)
N1	19 (19.4%)
N2	13 (13.3%)
M0	90 (91.8%)
M1	8 (8.2%)
Metastasis during follow-up	
No	71 (72.4%)
Yes	27 (27.6%)
Differentiation	
Poor	28 (28.6%)
Moderate	34 (34.7%)
Well	36 (36.7%)
Neo-adjuvant therapy	
No	81 (82.7%)
Yes	17 (17.3%)
Adjuvant therapy	
No	50 (51.0%)
Yes	40 (40.8%)
Unknown	8 (8.2%)

Overview of the clinicopathological parameters of patients.

Plk1 mRNA expression levels: quantitative PCR

Plk1 mRNA expression levels were successfully determined on 66 NSCLC samples and 4 corresponding normal lung tissue samples. The remaining 32 tumor samples were excluded from the study due to insufficient RNA yields or high Ct values. RNA was isolated from 5-10 FFPE tissue slides (10 µm thick), depending on the size of the tumor region. Tumor regions were determined based on hematoxylin/eosin stained reference slides and macrodissection was performed to achieve a

minimum of 80% tumor cells. RNA was extracted using the RNeasy FFPE isolation kit (Qiagen, Venlo, The Netherlands), according to the manufacturers' instructions. RNA concentrations and purity were measured as described previously [27]. Subsequently, reverse transcription was performed using the High Capacity RNA-to-cDNA kit (ThermoFisher Scientific, Nepean, Canada) and real-time PCR was performed using the TaqMan Gene Expression Master Mix on a LightCycler480 instrument (Roche, Vilvoorde, Belgium). The hs00153444_m1 (Plk1) TaqMan gene expression assay was used as target of interest (ThermoFisher Scientific) and hs00609296_g1 (HMBS), hs00984230_m1 (B2M), hs01122445_g1 (YWHAZ) were used as housekeeping genes for normalization. Relative gene expression levels were calculated according to the Ct method of the qbasePLUS software (Biogazelle, Zwijnaarde, Belgium) and plotted against normal tissue.

Protein expression levels: immunohistochemistry

Five µm-thick FFPE sections were prepared and subjected to heat-induced antigen retrieval by incubation in a 10mM citrate buffer (pH 9.0 for Plk1 and CA IX, pH 6.0 for cleaved caspase 3) for 20 minutes at 97°C. Subsequently, the endogenous peroxidase activity was quenched by incubating the slides in peroxidase blocking buffer (DAKO, Glostrup, Denmark) for 10 minutes. Incubations with primary monoclonal antibodies were performed as follows: anti-Plk1 (Cell Signaling Technology, Leiden, The Netherlands, 4513S, 1:50), anti-CA IX (Abcam, Cambridge, United Kingdom, 108351, 1:350) or anti-cleaved caspase 3 (Cell Signaling Technology, 95795, 1:250), all for 1 hour at room temperature. After a washing step, bound antibody was detected with the Envision FLEX+ detection kit (DAKO) using 3,3'-diaminobenzidine chromogen solution (DAKO), according to the manufacturers' instructions. Negative controls were prepared by substituting antibody diluent for primary antibody. Positive controls were included in each staining run and consisted of A549 cells (Plk1), stomach tissue (CA IX) and tonsil tissue (cleaved caspase 3).

Evaluation of immunohistochemical tissue staining

Biopsy specimens were scored by three independent investigators and reviewed by an experienced pathologist. To account for heterogeneity of Plk1 and CA IX expression, an immunoreactivity scoring (IRS) system was applied. Staining intensity was designated as either not existent (0), weak (1), moderate (2) or intense (3). For Plk1 expression, the

percentage positive tumor cells was scored 0 to 4 (0%, 1-5%, 6-29%, 30-59% and > 60% of tumor cells stained). For CA IX expression, the percentage positive cells was scored 0 to 4 (0%, 1-19%, 20-39%, 40-59%, 60-79% and > 80% of tumor cells stained). Afterwards, the overall IRS score was calculated by multiplication of these two parameters. Cases were grouped as Plk1/CA IX negative (IRS 0), weak Plk1/CA IX positive (IRS 1-3), moderate Plk1/CA IX positive (IRS 4-6) or strong Plk1/CA IX positive (IRS 7-15). For cleaved caspase 3 staining, positivity was assigned when at least 5% of the tumor cells showed staining.

TP53 mutation status analysis: Next generation sequencing

For *TP53* mutation status analysis, DNA was obtained from 5-10 FFPE tissue slides (5 µm thick). Tumor regions were determined based on hematoxylin/eosin stained reference slides by a pathologist and enriched by macrodissection. DNA isolation was performed using the QIAmp DNA FFPE tissue kit (Qiagen), according to the manufacturer's instructions. DNA concentrations and purity were determined as described previously [27]. Subsequently, *TP53* next generation sequencing was performed on 69 genomic tumor DNA samples using the commercially available *TP53* MASTR™ with MID for Illumina Miseq kit (Multiplicom, Niel, Belgium), according to the manufacturer's instructions. Twenty-nine samples were excluded because they did not pass the quality control steps of the *TP53* MASTR™ kit protocol. Sequencing was performed on a MiSeq Sequencing system with a MiSeq® Reagent Kit v2 (500 cycles) (Illumina, San Diego, USA). Using the MUT-*TP53* 2.0 tool, inactivating *TP53* mutations were identified which were used for statistical analysis [28].

Statistical analysis

All statistical analyses were performed using SPSS v23.0 software and significance was recorded if P < 0.05. The correlation between Plk1 mRNA and protein expression was investigated using a Spearman rank correlation test. Associations between Plk1 expression, *TP53* mutations and cleaved caspase 3 staining were analysed using Kruskall Wallis test; a Spearman rank correlation test was used to identify the correlation between Plk1 and CA IX. The association with clinicopathological parameters was determined using Kruskall Wallis test or Mann Witney U-test for continuous variables (i.e. Plk1 and CA IX expression) and Fisher's exact test or χ^2 analysis for categorical variables (i.e. cleaved caspase 3 expression and *TP53* status). Differences in overall survival (OS) and progression free survival (PFS) between patient groups were determined by Kaplan-Meier analysis and univariate Cox regression. A multivariate Cox proportional-hazard model was conducted to identify independent prognostic markers, presented as hazard ratio (HR) and 95% confidence interval (CI).

Results

Plk1 mRNA and protein expression in NSCLC adenocarcinoma samples

Quantitative RT-PCR was performed to determine the relative Plk1 mRNA expression level in 66 NSCLC adenocarcinoma samples and 4 corresponding healthy/normal lung tissue samples. Plk1 mRNA expression was detected in all specimens, including healthy lung tissue samples. Interestingly, NSCLC tissues expressed a significantly higher level of Plk1 mRNA than adjacent healthy lung tissues (P = 0.007) **(Table 2)**. The average Plk1 mRNA expression in tumor samples was 5.73-fold higher (range 0.742–18.846) compared to normal samples.

In addition to the Plk1 mRNA expression level, immunohistochemical analysis was performed to evaluate Plk1 protein expression in 95 NSCLC tumor samples and 16 healthy lung specimens from the same patients. **Figure 1** shows representative Plk1 immunostaining patterns, with Plk1 expression located in both the nucleus and the cytoplasm. The Plk1 protein level was significantly higher in NSCLC tissues compared to control samples (P < 0.01) **(Table 2)**. While in healthy control samples, no (n=10, 62.5%) or weak (n=6, 37.5%) positive staining was detected, NSCLC adenocarcinomas showed weak (n=33, 34.7%), moderate (n=32, 33.7%) or strong (n=30, 31.6%) Plk1 expression. Both intensity and percentage of Plk1 positive cells varied widely within a tumor section and between tumor samples. A significant correlation was demonstrated between Plk1 expression at the mRNA and protein level (P < 0.001, R^2 = 0.497).

Associations between Plk1 expression and clinicopathological parameters

Table 2 summarizes the Plk1 mRNA and protein expression data linked to the patients' clinicopathological parameters. Overall, Plk1 mRNA expression showed no association with age, gender, clinical stage, pathological stage or metastasis (P > 0.368). Interestingly, Plk1 mRNA expression was associated with differentiation grade (P = 0.004), being predominantly present in poorly differentiated tumor samples. In addition, significantly higher Plk1 mRNA expression levels were observed in the group of

smokers versus non-smokers (P = 0.001). No statistically significant associations were found between Plk1 protein expression levels and clinicopathological characteristics (P > 0.084).

Hypoxia, apoptotic cell death and *TP53* status in NSCLC

Hypoxic regions and the presence of apoptotic cells were determined by immunohistochemical staining using antibodies against the CA IX and cleaved caspase 3 proteins, respectively **(Figure 1)**. As shown in **Table 3**, hypoxia was observed in 96% of all neoplastic tissues, with CA IX expression ranging from weak/moderate (60%) to strong (36%). Only 4 NSCLC samples were negative for the hypoxia marker. In the healthy control group, 14 patients (88%) were negative for CA IX, while weak CA IX expression was observed in only 2 patients (12%). As such, hypoxic regions were significantly more prevalent in tumor samples compared to healthy tissue (P < 0.001).

Cleaved caspase 3 was ubiquitously expressed in both NSCLC and control samples. No difference in expression level between normal versus malignant tissues was observed for this apoptotic marker (P = 0.427) **(Table 3)**.

In order to detect mutations in the *TP53* gene, next generation sequencing was successfully performed on 69 NSCLC samples. A total of 33 mutations, consisting of 29 missense mutations and 4 nonsense mutations, were detected in 29 patients (40.0%). One patient harbored both a nonsense and missense mutation (R196X/R110L), one patient showed 2 missense mutations (V157F/V172D) and one patient had three missense mutations (G245C/G266V/R280G) in the *TP53* gene. In addition, one insertion (c.1162_1163insT) was identified. For statistical analysis, samples harboring a *TP53* nonsense or missense mutation, affecting p53's normal function, were classified as *TP53* mutant using the MUT-*TP53* 2.0 tool.

Table 2. Association between Plk1 expression levels and clinicopathological parameters of NSCLC patients.

Patient characteristic	Plk1 mRNA expression		Plk1 protein expression			
	Mean ± SD	P-value	Negative/weak	Moderate	Strong	P-value
Tissue						
Control sample	1.51 ± 0.70	0.007	16 (100%)	0 (0.0%)	0 (0.0%)	< 0.001
Tumor sample	5.73 ± 4.40		33 (34.7%)	32 (33.7%)	30 (31.6%)	
Age						
< 60 years	5.23 ± 3.79	0.368	21 (63.6%)	12 (37.5%)	13 (43.3%)	0.084
> 60 years	6.21 ± 4.91		12 (36.4%)	20 (62.5%)	17 (56.7%)	
Gender						
Female	6.06 ± 4.58	0.379	22 (66.7%)	22 (68.8%)	20 (66.7%)	0.979
Male	5.03 ± 4.00		11 (33.3%)	10 (31.3%)	10 (33.3%)	
Smoking behavior						
Non-smoker	2.51 ± 1.34	0.001	3 (12.0%)	2 (7.1%)	2 (7.1%)	0.772
Smoker	5.89 ± 4.50		22 (88.0%)	26 (92.9%)	26 (92.9%)	
Clinical stage						
I	5.69 ± 4.63	0.812	15 (45.5%)	12 (37.5%)	16 (53.3%)	0.666
II	5.09 ± 3.21		8 (24.2%)	10 (31.3%)	7 (23.3%)	
III	6.20 ± 4.98		9 (27.3%)	8 (25.0%)	4 (13.3%)	
IV	7.17 ± 6.21		1 (3.0%)	2 (6.3%)	3 (10.0%)	
Tumor classification						
T1	5.88 ± 5.11	0.949	8 (24.2%)	9 (28.1%)	13 (43.3%)	0.289
T2	5.91 ± 4.08		14 (42.4%)	14 (43.8%)	10 (33.3%)	
T3	5.16 ± 4.04		9 (27.3%)	5 (15.6%)	7 (23.3%)	
T4	6.15 ± 4.62		2 (6.1%)	4 (12.5%)	0 (0.0%)	
N0	5.63 ± 4.36	0.959	23 (69.7%)	22 (68.8%)	20 (66.7%)	0.999
N1	6.02 ± 4.51		6 (18.2%)	6 (18.8%)	6 (20.0%)	
N2	5.83 ± 4.91		4 (12.1%)	6 (20.0%)	4 (13.3%)	
M0	5.63 ± 4.34	0.513	32 (97.0%)	29 (90.6%)	27 (90.0%)	0.496
M1	6.98 ± 5.40		1 (3.0%)	3 (9.4%)	3 (10.0%)	
Metastasis during follow-up						
No	6.13 ± 4.60	0.426	17 (51.5%)	15 (46.9%)	18 (60.0%)	0.577
Yes	5.26 ± 4.16		16 (48.5%)	17 (53.1%)	12 (40.0%)	
Differentiation						
Poor	8.13 ± 5.46	0.004	8 (24.2%)	8 (25.0%)	10 (33.3%)	0.779
Moderate	5.63 ± 3.65		10 (30.3%)	13 (40.6%)	10 (33.3%)	
Well	3.90 ± 3.07		15 (45.5%)	11 (34.4%)	10 (33.3%)	

Upper row: Plk1 expression in control samples versus tumor samples. Other rows: associations between Plk1 expression in tumor samples and clinicopathological parameters of NSCLC patients. Relative Plk1 mRNA expression levels were calculated according to the Ct method of the qbasePLUS software and plotted against normal tissue. For Plk1 protein expression, an IRS score was calculated by multiplying the staining intensity score and % positive cells group. Cases were grouped as Plk1 negative (IRS 0), weak Plk1 positive (IRS 1-3), moderate Plk1 positive (IRS 4-6) or strong Plk1 positive (IRS 7-12).
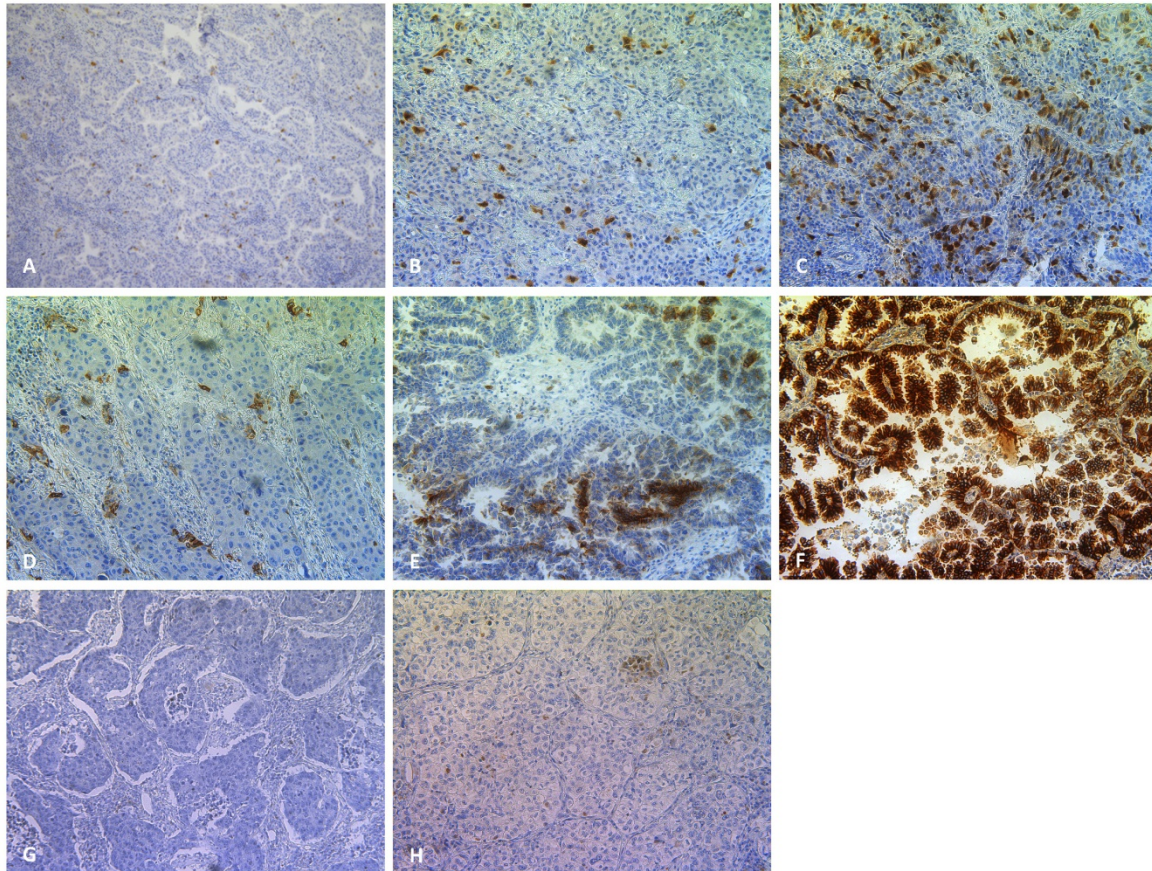
Figure 1. Plk1 (200x), CA IX (200x) and cleaved caspase 3 (200x) staining in primary NSCLC. Representative sections of NSCLC tumor samples classified as (A) weak, (B) moderate, (C) strong Plk1 expression. A weak, moderate and strong CA IX positive tumor sample is shown in (D),(E) and (F), respectively. A cleaved caspase 3 negative and positive tumor sample is shown in (G) and (H), respectively.

Association between *TP53* status, apoptotic cell death, hypoxia and clinicopathological parameters

The patients' clinicopathological parameters were associated with CA IX expression, cleaved caspase 3 expression and the *TP53* mutation status **(Table 3)**. A trend towards an association between CA IX immunostaining and differentiation grade was observed (P = 0.074). Well differentiated tumors expressed higher levels of CA IX compared to well differentiated tumor tissues. No associations were found between the hypoxic marker and age, gender, smoking behavior, clinical stage, pathological stage, lymph node invasion and metastasis (P > 0.124).

Concerning cleaved caspase 3 expression, a trend for an association could only be established with clinical stage (P = 0.064). Apoptotic cell death was not associated with age, gender, smoking behavior, TNM stage, metastasis or differentiation status (P > 0.111).

No associations were found between clinicopathological features and *TP53* status (P > 0.367).

Correlation between Plk1 expression, hypoxia, apoptotic cell death and *TP53* status

As previous studies reported that Plk1 phosphorylates p53, resulting in the inhibition of its pro-apoptotic function, we investigated the relationship between (i) Plk1 expression, (ii) *TP53* mutations and (iii) induction of apoptotic cell death in NSCLC samples. Plk1 was significantly upregulated at both the mRNA and protein level in *TP53* mutated samples compared to *TP53* wild type NSCLC tissues (P = 0.050 and P = 0.049 for Plk1 mRNA and protein expression, respectively). The mean Plk1 mRNA expression level was 1.47-fold higher in *TP53* mutated samples compared to *TP53* wild type samples. For Plk1 protein expression, samples with mutant *TP53* showed a mean IRS score of 5.97, while in *TP53* wild type samples the mean IRS value was 4.25. As such, elevated Plk1 expression levels could be, at least in part, due to a loss of p53 function. More apoptosis was induced in NSCLC samples with higher Plk1 mRNA expression levels compared to weak Plk1 mRNA expressing tissues (P = 0.015). No association was found between Plk1 protein expression and cleaved caspase 3 staining (P = 0.959).

Table 3. Association between CA IX expression, cleaved caspase 3 expression, *TP53* mutation status and clinicopathological parameters of NSCLC patients.

Patient characteristic	CA IX protein expression				Cleaved caspase 3 protein expression			TP53 gene mutation analysis		
	-	+	++	P-value	-	+	P-value	WT	MT	P-value
Tissue										
Control sample	14 (88.0%)	2 (12.0%)	0 (0.0%)	< 0.001	10 (62.5%)	6 (37.5%)	0.427	/	/	/
Tumor sample	4 (4.3%)	56 (59.6%)	34 (36.2%)		49 (50.5%)	48 (49.5%)		40 (58.0%)	29 (42.0%)	
Age										
< 60 years	2 (50.0%)	28 (50.0%)	17 (50.0%)	1.000	24 (49.0%)	23 (47.9%)	1.000	19 (47.5%)	14 (48.3%)	1.000
> 60 years	2 (50.0%)	28 (50.0%)	17 (50.0%)		25 (51.0%)	25 (52.1%)		21 (52.5%)	15 (51.7%)	
Gender										
Female	2 (50.0%)	38 (67.9%)	20 (58.8%)	0.579	34 (69.4%)	30 (62.5%)	0.525	28 (70.0%)	21 (72.4%)	1.000
Male	2 (50.0%)	18 (32.1%)	14 (41.2%)		15 (30.6%)	18 (37.5%)		12 (30.0%)	8 (27.6%)	
Smoking behavior										
Non-smoker	0 (0.0%)	2 (4.2%)	5 (17.2%)	0.124	5 (11.9%)	2 (4.9%)	0.433	4 (12.5%)	1 (3.8%)	0.367
Smoker	3 (100%)	46 (95.8%)	24 (82.8%)		37 (88.1%)	39 (95.1%)		28 (87.5%)	25 (96.2%)	
Clinical stage										
I	3 (75.0%)	25 (44.6%)	13 (38.2%)	0.247	18 (36.7%)	25 (52.1%)	0.064	16 (40.0%)	11 (37.9%)	0.990
II	0 (0.0%)	18 (32.1%)	8 (23.5%)		17 (34.7%)	9 (18.8%)		11 (27.5%)	9 (31.0%)	
III	0 0.0%)	11 (19.6%)	10 (29.4%)		9 (18.4%)	13 (27.1%)		9 (22.5%)	6 (20.7%)	
IV	1 (25.0%)	2 (3.6%)	3 (8.8%)		5 (10.2%)	1 (2.1%)		4 (10.0%)	3 (10.3%)	
Tumor classification										
T1	1 (25.0%)	21 (37.5%)	10 (29.4%)	0.562	12 (24.5%)	20 (41.7%)	0.321	13 (32.5%)	7 (24.1%)	0.796
T2	3 (75.0%)	18 (32.1%)	15 (44.1%)		22 (44.9%)	15 (31.3%)		15 (37.5%)	11 (37.9%)	
T3	0 (0.0%)	14 (25.0%)	6 (17.6%)		12 (24.5%)	10 (20.8%)		10 (25.0%)	10 (34.5%)	
T4	0 (0.0%)	3 (5.4%)	3 (8.8%)		3 (6.1%)	3 (6.3%)		2 (5.0%)	1 (3.4%)	
N0	3 (75.0%)	41 (73.2%)	19 (55.9%)	0.349	31 (63.3%)	35 (72.9%)	0.315	27 (67.5%)	19 (65.5%)	0.720
N1	0 (0.0%)	9 (16.1%)	10 (29.4%)		12 (24.5%)	6 (12.5%)		7 (17.5%)	7 (24.1%)	
N2	1 (25.0%)	6 (10.7%)	5 (14.7%)		6 (12.2%)	7 (14.5%)		6 (15.0%)	3 (10.3%)	
M0	3 (75.0%)	53 (94.6%)	31 (91.2%)	0.327	43 (87.8%)	47 (97.9%)	0.111	36 (90.0%)	26 (89.7%)	1.000
M1	1 (25.0%)	3 (5.4%)	3 (8.8%)		6 (12.2%)	1 (2.1%)		4 (10.0%)	3 (10.3%)	
Differentiation										
Poor	0 (0.0%)	17 (30.4%)	8 (23.5%)	0.074	10 (20.4%)	17 (35.4%)	0.155	11 (27.5%)	8 (27.6%)	0.893
Moderate	3 (75.0%)	22 (39.3%)	8 (23.5%)		21 (42.9%)	13 (27.1%)		13 (32.5%)	8 (27.6%)	
Well	1 (25.0%)	17 (30.4%)	18 (62.9%)		18 (36.7%)	18 (37.5%)		16 (40.0%)	13 (44.8%)	
Metastasis during follow-up										
No	2 (50.0%)	33 (58.9%)	13 (38.2%)	0.163	23 (46.9%)	28 (58.3%)	0.312	19 (47.5%)	16 (55.2%)	0.628
Yes	2 (50.0%)	23 (41.1%)	21 (61.8%)		26 (53.1%)	20 (41.7%)		21 (52.5%)	13 (44.8%)	

Upper row: CA IX expression, cleaved caspase 3 expression and *TP53* mutations in control samples versus tumor samples. Other rows: associations between CA IX expression, cleaved caspase 3 expression or *TP53* mutations in tumor samples and clinicopathological parameters of NSCLC patients. For CA IX staining: - negative, + weak/moderate positive, ++ strong positive. For cleaved caspase 3 staining: - < 5% positive cells, + > 5% positive cells. For *TP53* status: WT wild type, MT mutant.

In our study population, no correlation was found between Plk1 mRNA/protein expression and CA IX expression (P > 0.602) (R² = 0.029 and 0.054, respectively). In addition, oxygen deficiency was not correlated with the presence of *TP53* mutations or cleaved caspase 3 staining (P > 0.455).

Correlation between Plk1, CA IX, apoptotic cell death, *TP53* mutation status and survival

Follow up data were available for all 98 NSCLC patients. At the end of the observation period, 41 patients (41.8%) were deceased and 34 patients (34.7%) showed disease progression. Kaplan-Meier analysis was used to assess OS and PFS probability for Plk1 expression, CA IX expression (i.e. hypoxia), cleaved caspase 3 expression (i.e. apoptosis induction) and *TP53* mutation status **(Figure 2)**. For the Kaplan-Meier plot, NSCLC tissues were divided into a low Plk1 mRNA expression group (n = 35, 53.0%) and a high Plk1 mRNA expression group (n = 31, 47.0%), based on the median Plk1 mRNA expression in tumor samples (i.e. 4.55-fold overexpression). The mean OS was reduced from 1942 ± 139 days in the low positive Plk1 mRNA group to 1861 ± 223 days in the high positive Plk1 mRNA group **(Figure 2A)**. As such, Plk1 mRNA expression was a significant predictor of worse OS (P = 0.001, HR = 1.150) **(Table 4)**. No statistically significant difference in OS was observed between NSCLC patients with and without Plk1 protein overexpression (P = 0.616, HR = 1.024) **(Figure 2B)**. Interestingly, our data also demonstrated that both hypoxia and *TP53* mutations are correlated with OS of NSCLC patients **(Figure 2C and 2E)**. Patients with strong CA IX scores lived significantly shorter than patients with negative or low CA IX expression levels in their tumor samples (P = 0.002, HR = 2.560) and *TP53* mutations were also clearly correlated with a significantly worse OS (P = 0.004, HR = 3.240) **(Table**

4). Cleaved caspase 3 expression levels had no impact on OS of NSCLC patients (P = 0.667, HR = 1.146) **(Figure 2D)**.

Univariate analysis indicated that, besides Plk1 mRNA expression, CA IX protein expression and *TP53* mutations, clinical stage (P = 0.006, HR = 2.470), lymph node metastasis (P = 0.050, HR = 1.466) and distant metastasis (P = 0.002, HR = 3.635) were also significant predictors of worse OS **(Table 4)**. In addition to univariate analysis, we fitted a Cox proportional hazard model with Plk1 mRNA expression as predictor for OS and accounting for potential confounders (i.e. CA IX expression, *TP53* status, clinical stage, lymph node metastasis and distant metastasis). Using the stepwise backward model, CA IX expression (P = 0.010, HR = 6.484), *TP53* mutations (P = 0.011, HR = 5.766) and clinical tumor stage (P = 0.038, HR = 5.680) were assigned as independent prognostic markers for OS **(Table 4)**. A strong trend was observed for the effect of Plk1 mRNA expression on OS in NSCLC patients, with a HR of 1.126 (P = 0.075) **(Table 4)**.

Interestingly, patients harboring a *TP53* mutation together with a Plk1 mRNA overexpression showed a significantly shorter OS time compared to patients with a *TP53* mutation or Plk1 overexpression alone (P = 0.003) **(Figure 2F)**. This effect was further enhanced when CA IX positivity was also taken into account.
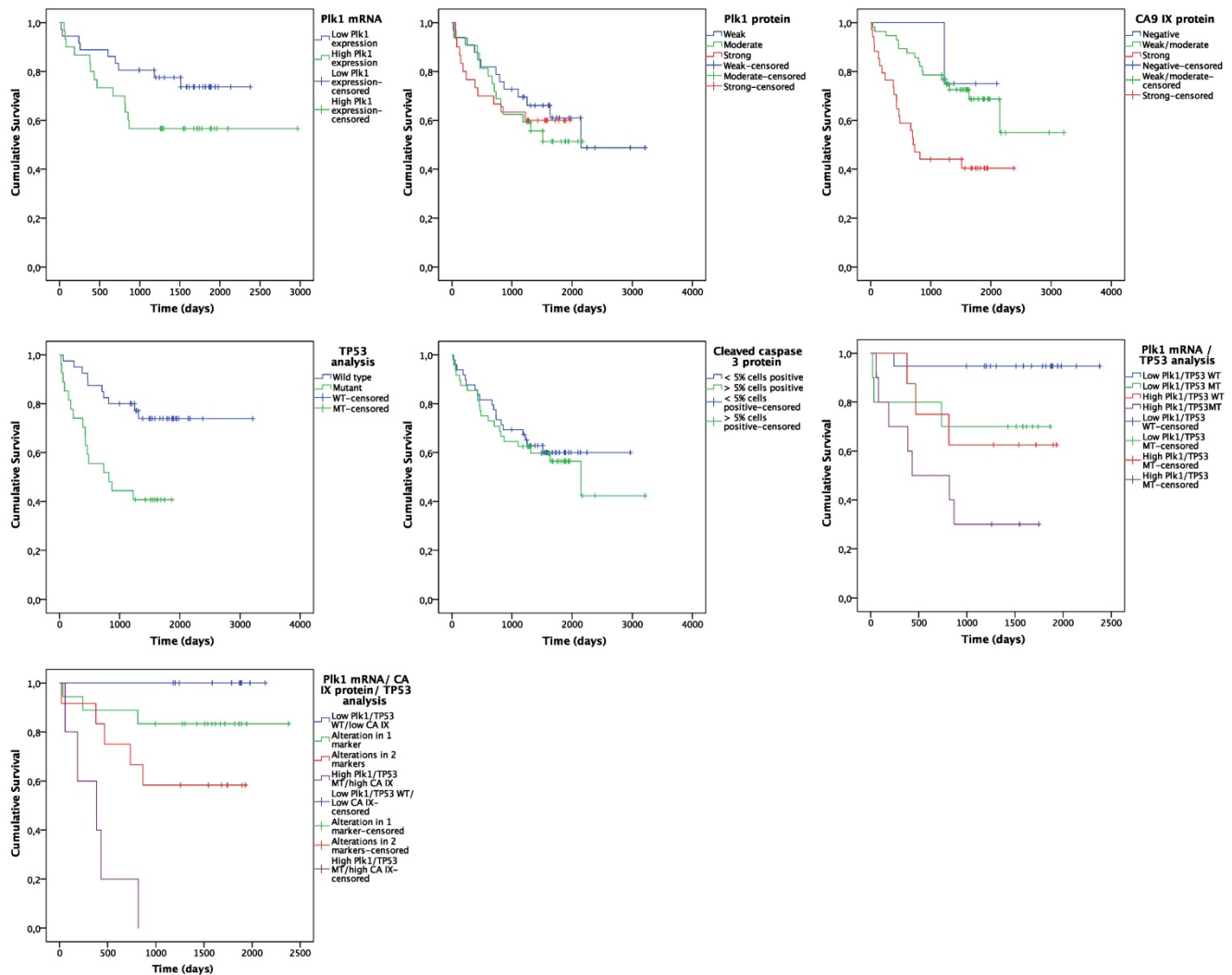
Figure 2. Kaplan-Meier curves for OS. Curves are based on (A) Plk1 mRNA expression, (B) Plk1 protein expression, (C) CA IX protein expression, (D) *TP53* mutations, (E) cleaved caspase 3 expression, (F) combination of Plk1 mRNA expression and *TP53* mutations and (G) combination of Plk1 mRNA expression, CA IX protein expression and *TP53* mutations.

Table 4. Survival analysis of NSCLC patients.

	Univariate survival analysis			Multivariate Cox regression		
	HR	P-value	95% CI	HR	P-value	95% CI
Plk1 mRNA expression	1.150	0.001	1.055 – 1.253	1.126	0.075	0.988 – 1.283
CA IX expression	2.560	0.002	1.396 – 4.675	6.484	0.010	1.566 – 26.838
TP53 status	3.240	0.004	1.456 – 7.098	5.766	0.011	1.506 – 22.079
Clinical stage	2.470	0.006	1.263 – 4.879	5.680	0.038	1.104 – 29.225
Lymph node metastasis	1.466	0.050	1.000 – 2.140			
Distant metastasis	3.635	0.002	1.521 – 7.866			

HR: Hazard Ratio, 95% CI: 95% confidence interval.

In addition, we investigated the predictive value of Plk1 expression in combination with hypoxia and the *TP53* status in our study population. Treatment data showed that 54.1% of the NSCLC patients received neo-adjuvant and/or adjuvant therapy (i.e. plantinum-based chemotherapy and/or radiotherapy). In both the non-treated and treated group, overall survival was negatively influenced by the biomarker panel (P < 0.030).

No significant effect on PFS was observed according to the patients' Plk1 mRNA/protein expression level, oxygen deficiency, apoptotic cell death or *TP53* status, not even when the parameters were combined (P > 0.199).

Discussion

Currently, prognosis of patients with NSCLC is based on specific clinicopathological factors, including histologic tumor type, differentiation status and the presence of metastases. Although TNM stage is being considered as the most important prognostic parameter, there are considerable differences in patient outcome within a similar staging group, even when patients received identical treatments [10, 29]. In order to improve prognostic predictions and to guide treatment options, the identification of additional parameters influencing outcome is required. In recent years, a growing spectrum of proteins that are related to cell proliferation, apoptosis and genomic instability (e.g. KRAS, EGFR, p53, CDKs) have been studied as new potential biomarkers for tumor progression [29, 30]. Nevertheless, inconsistent results were reported in literature and not a single protein marker has been validated for clinical use. Therefore, in the present study, we investigated Plk1 expression levels in relation to *TP53* mutations, apoptosis (cleaved caspase 3 expression) and the presence of hypoxic tumor regions (CA IX protein expression) to determine their prognostic potential in NSCLC.

As Plk1 plays a crucial role in cell cycle progression and the cellular stress response, Plk1 overexpression is considered as a prognostic marker and potential target for anti-cancer therapy in various human malignancies [11, 17, 19, 31-34]. To the best of our knowledge, we are the first to investigate the Plk1 expression level in combination with *TP53* mutations and hypoxia in NSCLC. Plk1 upregulation was reported at both the mRNA and protein level in the majority of NSCLC adenocarcinoma samples. The mean Plk1 mRNA expression level was 5.73-fold higher (range 0.742–18.846) in tumor samples compared to healthy lung tissue and was significantly more frequent observed in poorly differentiated neoplasms. Interestingly, the group of non-smokers showed higher Plk1 mRNA levels compared to the non-smokers in our study population. In the study of Wolf *et al.*, the normalized Plk1 mRNA expression after Northern blot analysis ranged between 0.08 and 0.11 density units for normal lung tissues, while in NSCLC adenocarcinoma samples this range was between 0.02 and 7.10 [10]. In addition, immunostaining showed Plk1 protein overexpression in 64% of the NSCLC patients. This result is in line with the study of Wang *et al.*, who also reported elevated Plk1 protein levels in 58% of the NSCLC patients [11]. In our study, Plk1 positivity was mainly located in the nucleus and showed high inter-and intratumor variability in both % of positive cells and staining intensity. This heterogeneity could reflect, at least in part, different proliferation rates of the cells. It is also plausible that the most intensely stained cells are those in the G_2/M phase or that Plk1 upregulation is caused by different mechanisms [12, 17].

No significant relationship between Plk1 protein levels and clinicopathological factors could be established. In contrast, Wang *et al.* showed correlations between Plk1 protein expression and poor tumor differentiation, advanced clinical stage and lymph node metastases. They included NSCLC samples of different histological types, while we only investigated adenocarcinomas [11]. Plk1 overexpression is also reported in a variety of other human malignancies, including head and neck carcinoma, melanoma, colorectal cancer, hepatocellular carcinoma, bladder carcinoma, ovarian cancer and glioblastoma. In these studies, high Plk1 expression levels were mainly correlated with clinical stage, histological grade and (lymph node) metastasis [14, 16, 19, 31, 34-36]. However, other research groups were not able to reproduce these correlations [10, 12, 13, 37, 38]. These conflicting data suggest that Plk1 plays critical roles in tumor development and progression, but that Plk1 expression should not be considered as the sole prognostic factor. As such, a prognostic panel, consisting of Plk1 and additional

molecular biomarkers, might further improve prediction of prognosis.

In order to define this 'golden' combination, it is crucial to understand the exact mechanism by which deregulated Plk1 levels contribute to carcinogenesis. At first, it was thought that Plk1 overexpression was a consequence of enhanced mitotic activity in neoplastic cells, rather than a cause of oncogenic transformation. However, Plk1 positivity was observed in much more tumor cells than those expected to be in G_2/M phase. Subsequently, amplification of region 16p12, situated at the locus of the human *Plk1* gene, was described in gastric, breast, colorectal and esophageal cancer. However, not all Plk1 overexpressing tumors showed *Plk1* gene amplification, indicating alternative mechanisms [14, 17]. Furthermore, it was reported that the Plk1 pathway and p53 signaling are highly cross-linked. P53 is regarded as the guardian of the genome and represses transcription and activity of Plk1 in a cell cycle specific manner and in response to DNA damage. Meanwhile, it has also been proven that p53 is a target of Plk1, with Plk1 inhibiting its tumorsuppressor function [21]. Since *TP53* is the most frequently altered gene in human cancer, it was hypothesized that the majority of Plk1 overexpression might be due to the loss of functional p53. Indeed, in women with breast cancer, it was demonstrated that there was a 3.5 greater likelihood of having detectable Plk1 immunostaining if breast tumor samples harbor a mutation in the *TP53* gene [12]. In agreement with these observations, our results also indicated more Plk1 overexpression in *TP53* mutated samples compared to wild type NSCLC tissues. Plk1 expression has also been reported to be significantly associated with detectable p53 protein expression, which is associated with mutant *TP53* [39, 40].

Next, we investigated the relationship between Plk1, the *TP53* status and induction of apoptosis. Our results showed more cleaved caspase 3 immunostaining in tumor samples with higher Plk1 mRNA levels, possibly due to p53 independent pathways, since no correlation was found between the *TP53* status and apoptosis. Intuitively, less apoptosis would be expected in cancer cells with high Plk1 expression levels, so additional experiments are warranted to further elucidate the underlying molecular mechanisms.

Concerning the link between Plk1 and hypoxia, Ward *et al.* recently reported a p53 dependent epigenetic modification of Plk1 under hypoxic conditions in hepatocellular and osteocarcinoma cell lines. They proposed a model in which wild type p53 leads to downregulation of Plk1 transcription under hypoxia by recruiting DNA methyltransferases (DNMTs) and/or histone deacetylases (HDACs).

Consequently, absence of functional p53 led to Plk1 upregulation under reduced oxygen conditions [26]. In our immunohistochemical study, normal lung tissues stained negative to moderate for the hypoxic marker CA IX, while weak to very strong positivity was observed in NSCLC samples. These results are in accordance with other studies, reporting CA IX overexpression in NSCLC [41-45].

It has been shown in a variety of cancer types that quantification of Plk1 expression, CA IX expression, cleaved caspase 3 expression or the occurrence of *TP53* mutations had a prognostic value as single biomarker [41, 42, 44-49]. In agreement with these data, our study demonstrated that Plk1 mRNA expression, the presence of hypoxic tumor regions and mutant *TP53* were significant predictors of worse overall survival in the univariate analysis. However, no significant difference in overall survival between NSCLC patients with and without Plk1 protein overexpression was found. Contrarily, a significant correlation was demonstrated between Plk1 mRNA and protein expression, although this was not absolute as indicated by the correlation coefficient (R^2 = 0.479). As described above, strong intratumor heterogeneity in Plk1 protein expression was observed. For mRNA expression analysis, only tumor regions with a minimum of 80% tumor cells were included. As such, a global view of Plk1 mRNA expression was obtained. Using multivariate analysis, correcting for CA IX expression, *TP53* mutations, clinical stage, lymph node metastasis and distant metastasis, hypoxic regions, *TP53* mutations and clinical tumor stage were assigned as independent prognostic markers for overall survival. Furthermore, a strong trend towards Plk1 mRNA expression as independent prognostic marker for NSCLC adenocarcinoma patients was detected.

Interestingly, elevated Plk1 mRNA levels combined with a *TP53* mutation were associated with worse patient outcome, much more so than in patients harboring either one. This is in line with the studies of Kanjai *et al.* and King *et al.*, who reported a significantly worse prognosis of patients with both Plk1- and p53-positive tumors, compared to Plk1- and p53- negative or Plk1 negative and p53 positive tumors [12, 39, 40]. Interestingly, this effect was even stronger when CA IX positivity was also taken into account. Patients with high Plk1 mRNA and CA IX protein expression levels and harboring a mutation in the *TP53* gene had a significantly shorter survival time than those with aberrant expression patterns in only one or two of these parameters. When patients were stratified by treatment strategy, overall survival was also negatively influenced by Plk1 mRNA overexpression in combination with hypoxia and the

presence of a *TP53* mutation. This result suggests that patients with aberrant expression of the three markers would not benefit from standard (neo)adjuvant therapy.

As a result, our study paves the way for new combination strategies with Plk1 inhibitors. The observed Plk1 overexpression could be, at least partially, the result of loss of functional p53. Mutant *TP53* cells are able to evade apoptosis and progress into more malignant variants that are resistant to conventional treatments. Previous studies reported that reactivation of mutant p53, using small molecules (such as APR-246 (PRIMA-1^{MET})) that restore p53's wild-type conformation, results in the induction of the p53 dependent apoptotic pathway [50]. Moreover, it has been published that the efficacy of PRIMA-1^{MET} is enhanced under hypoxic conditions [51]. Combination treatments between Plk1 inhibitors and reactivators of p53 could therefore result in Plk1 downregulation and activation of the apoptotic pathway under both normal and reduced oxygen conditions.

In conclusion, we found that Plk1 and CA IX levels were frequently upregulated in NSCLC adenocarcinoma samples when compared to normal lung tissue. Moreover, the combined evaluation of Plk1 mRNA expression, CA IX protein expression and *TP53* mutations shows promise as a prognostic panel in NSCLC adenocarcinoma patients. Importantly, these results will have to be confirmed in a larger study population in order to assess the true potential and applicability of this biomarkerpanel.

Abbreviations

Plk1: Polo-like kinase 1; CA IX: Carbonic anhydrase IX; NSCLC: Non-small cell lung cancer; HIF-1: Hypoxia inducible factor 1α; FFPE: Formaldehyde fixed-paraffin embedded; IRS: Immunoreactivity scoring system; OS: Overall survival; PFS: Progression free survival; HR: Hazard ratio; CI: Confidence interval; DNMTs: DNA methyltransferases; HDACs: Histone deacetylases.

Acknowledgements

Competing Interests

The authors have declared that no competing interest exists.

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