

AURKA mRNA expression is an independent predictor of poor prognosis in patients with non-small cell lung cancer

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Abstract. Deregulation of mitotic spindle genes has been reported to contribute to the development and progression of malignant tumours. The aim of the present study was to explore the association between the expression profiles of Aurora kinases (*AURKA*, *AURKB* and *AURKC*), cytoskeleton-associated protein 5 (*CKAP5*), discs large-associated protein 5 (*DLGAP5*), kinesin-like protein 11 (*KIF11*), microtubule nucleation factor (*TPX2*), monopolar spindle 1 kinase (*TTK*), and β -tubulins (*TUBB*) and (*TUBB3*) genes and clinicopathological characteristics in human non-small cell lung carcinoma (NSCLC). Reverse transcription-quantitative polymerase chain reaction-based RNA gene expression profiles of 132 NSCLC and 44 adjacent wild-type tissues were generated, and Cox's proportional hazard regression was used to examine associations. With the exception of *AURKC*, all genes exhibited increased expression in NSCLC tissues. Of the 10 genes examined, only *AURKA* was significantly associated with prognosis in NSCLC. Multivariate Cox's regression analysis demonstrated that *AURKA* mRNA expression [hazard ratio (HR), 1.81; 95% confidence interval (CI), 1.16-2.84; P=0.009], age (HR, 1.03; 95% CI, 1.00-1.06; P=0.020), pathological tumour stage 2 (HR, 2.43; 95% CI, 1.16-5.10; P=0.019) and involvement of distal nodes (pathological node stage 2) (HR, 3.14; 95% CI, 1.24-7.99; P=0.016) were independent predictors of poor prognosis in patients with NSCLC. Poor prognosis of patients with increased *AURKA* expression suggests that those patients may benefit from surrogate therapy with *AURKA* inhibitors.

Introduction

Lung cancer is the most common cause of cancer-associated mortality in the UK for both males and females (1), and >1/5 patients with cancer succumb to this malignancy worldwide (2). Non-small cell lung carcinoma (NSCLC) accounts for 80-85% of all cases of lung cancer, and develops through the accumulation of molecular alterations, which may serve as prognostic biomarkers for NSCLC outcome (3).

Mitotic spindle formation and the spindle checkpoint are critical for the maintenance of cell division and chromosome segregation (4). A number of mitotic spindle-associated proteins have been implicated in multiple malignancies, including lung cancer (5,6). Overexpression and gene amplification have been reported to contribute to the development and progression of malignant tumours for a number of mitotic spindle genes, including those involved in centrosome maturation [e.g., Aurora kinase (*AURK*)A, microtubule nucleation factor TPX2 (*TPX2*) and kinesin-like protein 11 (*KIF11*)] (7,8), microtubule formation [e.g., *AURKA*, cytoskeleton-associated protein 5 (*CKAP5*), tubulin β (*TUBB*) and *TUBB3*] (9-11), and chromosomal alignment and segregation [e.g., *AURKA*, *AURKB*, *AURKC*, discs large-associated protein 5 (*DLGAP5*) and TTK protein kinase (*TTK*)] (12-14). *AURKA* serves a central role in recruiting other mitotic spindle members (5). A number of previous studies conducted in lung cancer have investigated the prognostic value of various of the aforementioned genes, including *TPX2* (15), *AURKA* (16-18) and *AURKB* (18-21); however, the prognostic value of *AURKA* and *AURKB* remains a matter of debate. No information on the potential prognostic significance in human NSCLC has yet been provided for *DLGAP5*, *CKAP5* or *TTK*.

Personalised medicine relies on the utilisation of gene profiling (including expression, mutation and methylation) in combination with clinicopathological characteristics to provide an optimal management plan for the patient. Therefore, it is necessary to expand our efforts in investigating the association of particular molecular profiles with patient outcomes. The aim of the present study was to acquire a comprehensive expression profile of mitotic spindle-associated genes (*AURKA*, *AURKB*, *AURKC*, *CKAP5*, *DLGAP5*, *KIF11*, *TPX2*, *TTK*, *TUBB* and *TUBB3*) in NSCLC and to investigate the

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potential associations with clinicopathological characteristics and patient survival rates.

Materials and methods

Patients and samples. The present study was undertaken within the context of the Liverpool Lung Project (22). Appropriate ethical approval from the Liverpool Research Ethics Committee, ref 157/97, was obtained and all patients provided written informed consent. A total of 132 frozen surgical tumour samples, collected between January 1999 and December 2005 at Liverpool Heart and Chest Hospital (Liverpool, UK), were available from patients with primary NSCLC, 56 from adenocarcinoma (AdC) and 76 from squamous cell carcinoma of the lung (SqCCL). In addition, 44 paired non-tumour surgical lung samples (20 from patients with AdC and 24 from patients with SqCCL) were analysed. The median age of the patients was 67 years (range, 45-82 years); 56 of the patients were female and 77 were male. The majority of the specimens were of the pathological tumour (pT)2 stage (n=101), whereas the pT1 and pT3/4 groups comprised 19 and 12 patients, respectively. The HBEC-3KT cell line (23) used as a calibrator was provided by Professor John Minna and Professor Adi Gazdar.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from primary lung tumour tissue (ten 20- μ m thick sections per specimen) using a Direct-zol™ RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA), according to the manufacturer's protocol. The quality and quantity of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and 200 ng RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Predesigned 6-carboxyfluorescein-labelled TaqMan Gene Expression Assays (Thermo Fisher Scientific, Inc.) were employed, according to the manufacturer's protocol, to analyse mRNA expression: *AURKA*, Hs01582072_m1; *AURKB*, Hs00945858_g1; *AURKC*, Hs00152930_m1; *CKAP5*, Hs01120723_m1; *DLGAP5*, Hs00207323_m1, *KIF11*, Hs00189698_m1; *TPX2*, Hs00201616_m1; *TTK*, Hs01009870_m1; *TUBB*, Hs00962419_g1; and *TUBB3*, Hs00964962_g1, with a 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein-labelled β -actin (*ACTB*) TaqMan Gene Expression Assay (cat. no. 4326315E; Thermo Fisher Scientific, Inc.) serving as an endogenous control. RNA from human bronchial epithelial cells (HBEC-3KT) was used as technical calibrator. Three technical replicates were performed for every qPCR assay. Thermocycling conditions were 95°C for 10 min (activation), 45 cycles of 95°C for 15 sec (denaturation), 60°C for 1 min (annealing and extension) on a Life Technologies StepOnePlus Real-Time PCR System. mRNA levels were expressed as relative quantification (RQ) values, which were calculated as $RQ=2^{-\Delta\Delta Cq}$ (24). Quantification cycle (Cq) values were determined using StepOne software (version 1.2; Thermo Fisher Scientific, Inc.) and normalised to the corresponding Cq value for the endogenous control *ACTB*, generating ΔCq values ($\Delta Cq=Cq$ target-Cq *ACTB*). Sample ΔCq values were further normalised against an immortalised bronchial

epithelial cell line HBEC-3KT (23) calibrator using the formula: $\Delta\Delta Cq=(\Delta Cq$ sample- ΔCq HBEC-3KT).

Statistical analysis. Gene expression in tumour and adjacent wild-type tissues were compared using the Wilcoxon non-parametric test. The study characteristics were examined using descriptive statistics. Categorical variables were compared using a χ^2 test and continuous variables were examined using a Mann-Whitney U test. Overall survival time was calculated from the date of surgery to the date of mortality or last follow-up date. Overexpression for a tumour sample was designated as >95% reference interval [mean \pm (2x standard deviation)] of wild-type tissues. Postoperative univariate survival analysis was explored using Kaplan-Meier estimator curves for all the categorical predictors. Tests of equality across strata were also conducted to evaluate the suitability of including potential predictors in the final multivariate model. For the categorical variables, a log-rank test of equality across strata was used, and a univariate Cox's proportional hazard regression was used to analyse continuous variables to examine the differences in survival rate. Variables with $P<0.25$ in the univariate analysis were selected for inclusion in the final multivariate model as previously suggested (25). A multivariate Cox's proportional hazard model was used to examine the association between mRNA expression and other relevant prognostic factors. All statistical analyses were performed using IBM® SPSS® statistical software (version 22.0; IBM SPSS, Armonk, NY, USA) and Stata® (version 13.1; StataCorp LLC, College Station, TX, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Gene expression analysis. RT-qPCR analysis revealed that, with the exception of *AURKC*, the mRNA expression levels of all the genes examined in the present study (*AURKA*, *AURKB*, *AURKC*, *DLGAP5*, *CKAP5*, *KIF11*, *TPX2*, *TTK*, *TUBB* and *TUBB3*) were significantly upregulated in NSCLC tissues compared with those in wild-type adjacent lung tissues ($P<0.0001$; Fig. 1). Comparison between histology types (Fig. 2) revealed that the mRNA expression of seven genes was significantly increased in SqCCL compared with that in AdC tissues ($P<0.001$ for *AURKA*, *AURKB*, *DLGAP5*, *TPX2*, *TTK* and *TUBB*; $P=0.001$ for *KIF11*).

Survival analysis. There was no association between the mRNA expression of any of the genes evaluated with age, sex, pathological stage or nodal status (Table I). Potential associations between the expression level of the target genes and overall survival rate were examined. In univariate analysis, pathological stage, nodal status and *AURKA* mRNA expression were predictors of overall survival rate (Table II). Most importantly, multivariate analysis demonstrated that *AURKA* mRNA expression [hazard ratio (HR), 1.81; 95% confidence interval (CI) 1.16-2.84; $P=0.009$] independently predicts poor prognosis in patients with NSCLC upon adjusting for age, pT2 and involvement of distal nodes (pathological node stage 2) (Table II). This observation was consistent with the Kaplan-Meier estimator curve (Fig. 3). The association with prognosis remained significant even when SqCCL and AdC

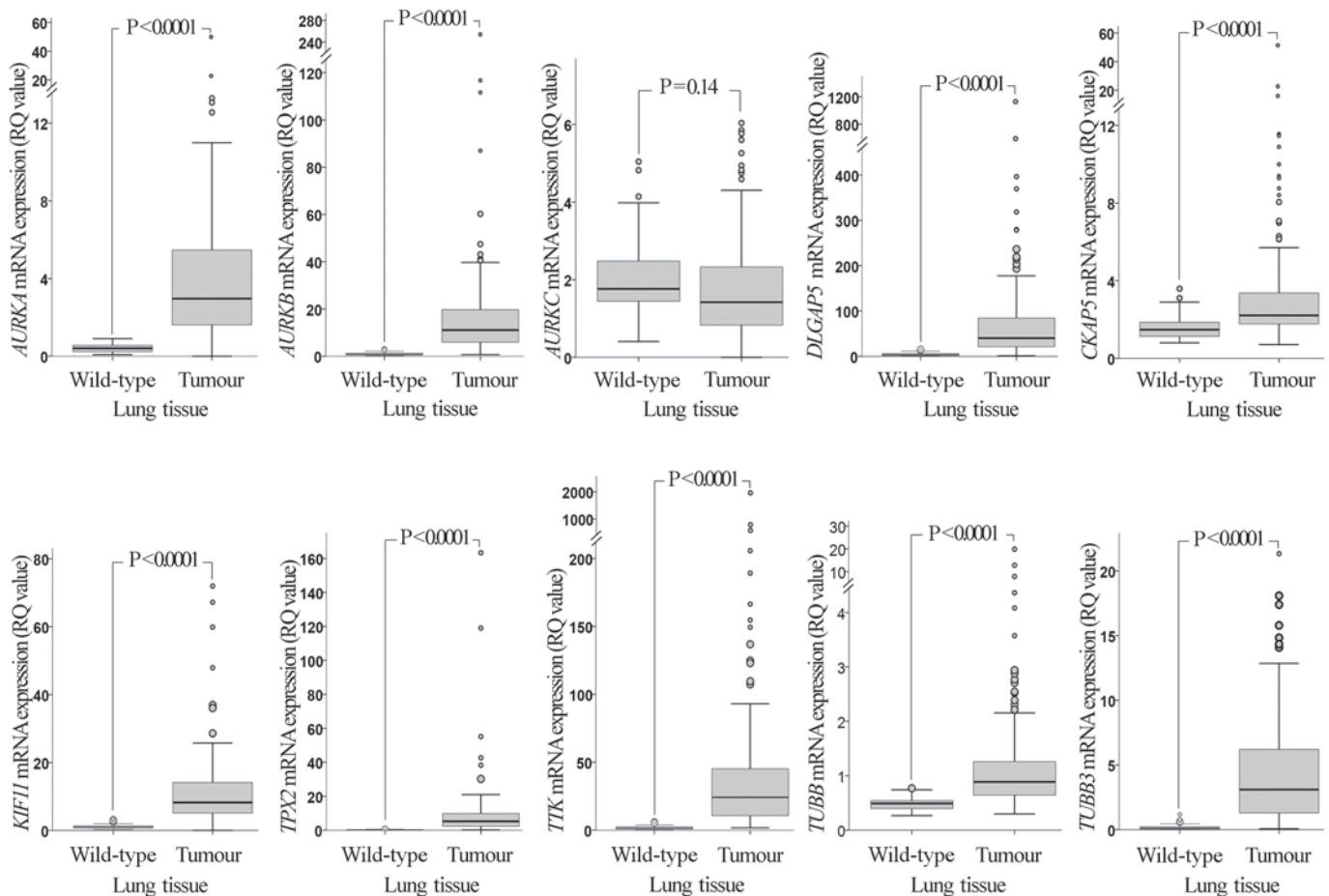


Figure 1. Comparative mRNA expression of the examined genes in NSCLC tissues and adjacent normal tissues. The mRNA expression levels of the genes in NSCLC tumours are significantly higher than those in adjacent normal tissues, with the exception of *AURKC*. P-values were calculated using a Mann-Whitney U test and adjusted for multiple comparisons by Bonferroni correction. RQ values were calculated using RNA from the non-tumorigenic immortalised human bronchial epithelial cell line HBEC-3KT as a calibrator. Larger circles represent outlier values ($>1.5x$ interquartile range); smaller circles represent extreme values ($>3x$ interquartile range). NSCLC, non-small cell lung cancer; RQ, relative quantification; *AURK*, Aurora kinase; *DLGAP5*, discs large-associated protein 5; *CKAP5*, cytoskeleton-associated protein 5; *KIF11*, kinesin-like protein 11; *TPX2*, microtubule nucleation factor TPX2; *TTK*, TTK protein kinase; *TUBB*, tubulin β .

tissues were tested separately ($P=0.025$ and $P=0.029$, respectively; Fig. 3).

Discussion

Spindle formation is a key process for cell proliferation (8). It is well known that spindle assembly aberrations lead to aneuploidy and are extensively involved in the development of cancer (26). Thus, it was hypothesised that the expression of genes associated with this process may be indicative of the aggressiveness of a tumour and therefore may exhibit prognostic value.

In the present study, the mRNA expression of the *AURKA*, *AURKB*, *AURKC*, *CKAP5*, *DLGAP5*, *KIF11*, *TPX2*, *TTK*, *TUBB* and *TUBB3* genes was investigated in a large cohort of human NSCLC tissues, and potential associations between expression profiles and clinicopathological characteristics, including survival rates, were evaluated. All genes, with the exception of *AURKC*, were overexpressed in the malignant tissues in comparison with adjacent wild-type tissues. These results possibly reflect the requirement for increased mitotic spindle genes expression to cope with the increased replication

rate of cancer cells (27,28). However, the important clinical question is whether the overexpression of any of these genes is able to confer a selective advantage on cancer cells and increase their invasive properties. The results of the present study confirm that up-regulation of mitotic spindle genes is a common abnormality in NSCLC and further support a role for the maintenance of a tumorigenic phenotype (5,29). The results of the present study demonstrated that, of the 10 genes examined, only *AURKA* overexpression was associated with poor prognosis, which suggests that this gene has a particular contribution to a more aggressive phenotype. It is notable that multivariate Cox's regression analysis identified *AURKA* mRNA expression as an independent predictor of poor prognosis in patients with NSCLC.

The overexpression of *AURKA* in NSCLC has been demonstrated previously (16,17). Consistent with these previous studies, it was observed in the present study that *AURKA* mRNA overexpression was increased in SqCCL compared with that in AdC tissue. However, the prognostic value of *AURKA* in lung cancer has not yet been established. In contrast to the study of Tang *et al* (30), the prognostic significance of *AURKA* expression in the present study appears to hold true

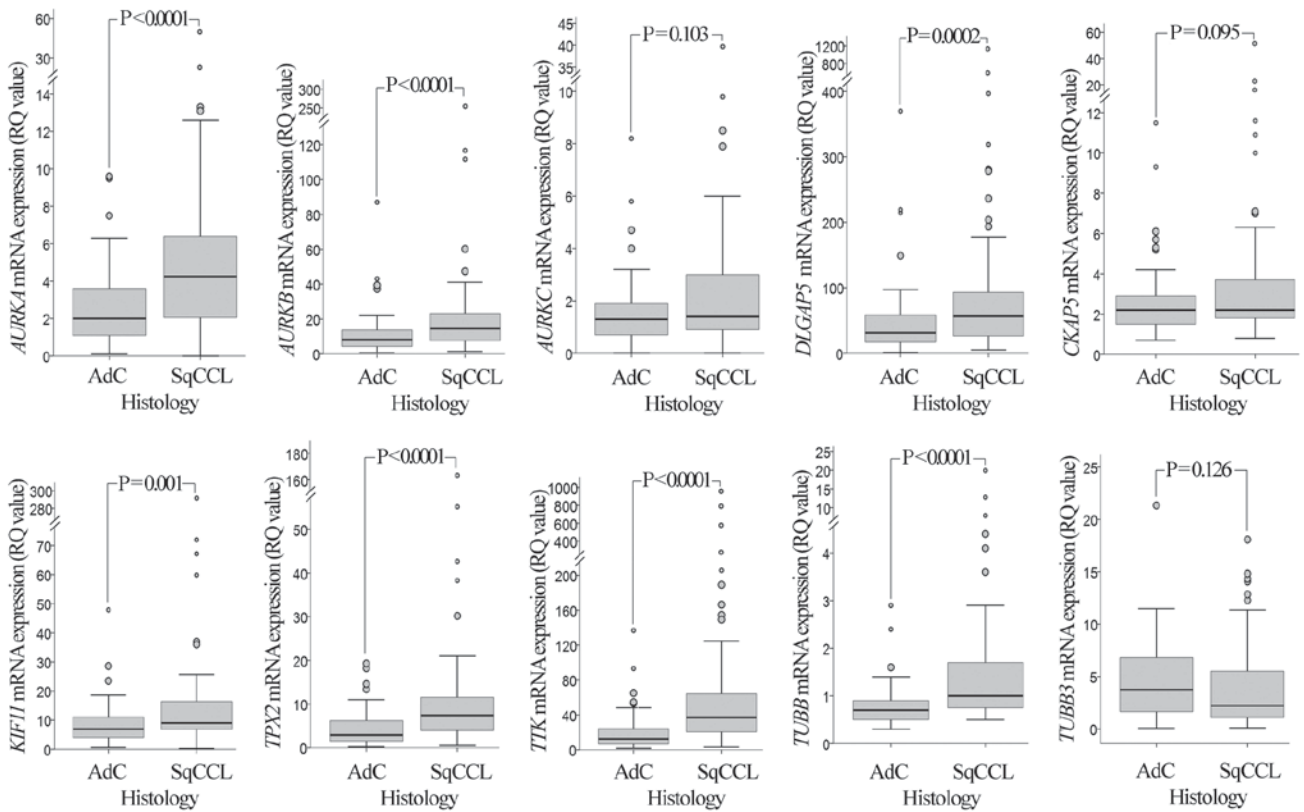


Figure 2. mRNA expression of *AURKA*, *AURKB*, *AURKC*, *DLGAP5*, *CKAP5*, *KIF11*, *TPX2*, *TTK*, *TUBB* and *TUBB3* genes in SqCCL and AdC of the lung. Comparison between histology types demonstrated that the mRNA expression levels of the *AURKA*, *AURKB*, *DLGAP5*, *KIF11*, *TPX2*, *TTK* and *TUBB* genes in SqCCL tumours are significantly higher than those in AdC tumours. P-values were calculated using a Mann-Whitney U test and adjusted for multiple comparisons by Bonferroni correction. RQ values were calculated using RNA from the non-tumorigenic immortalised human bronchial epithelial cell line HBEC-3KT as a calibrator. Larger circles represent outlier values ($>1.5x$ interquartile range); smaller circles represent extreme values ($>3x$ interquartile range). *AURK*, Aurora kinase; *DLGAP5*, discs large-associated protein 5; *CKAP5*, cytoskeleton-associated protein 5; *KIF11*, kinesin-like protein 11; *TPX2*, microtubule nucleation factor TPX2; *TTK*, TTK protein kinase; *TUBB*, tubulin β ; SqCCL, squamous cell carcinoma of the lung; AdC, adenocarcinoma; RQ, relative quantification.

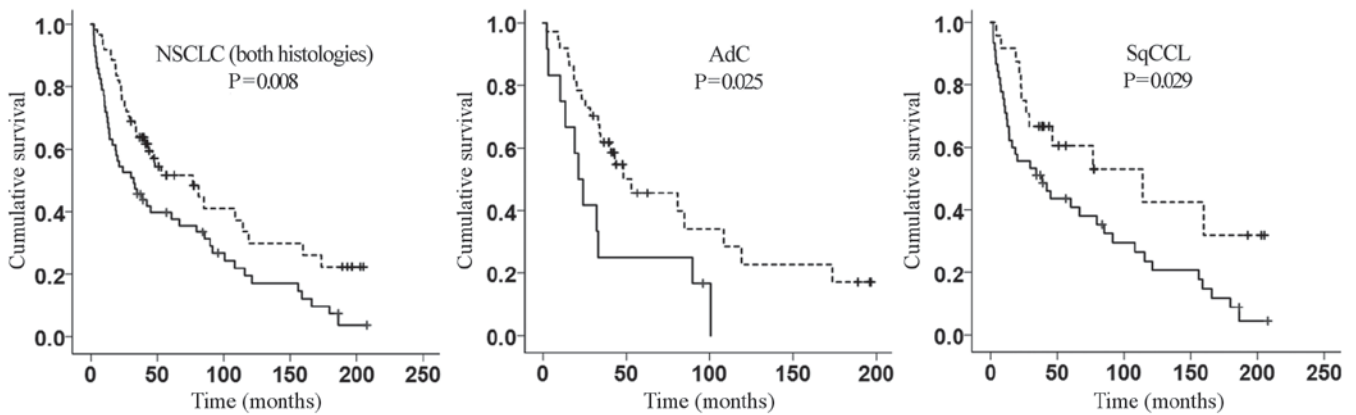


Figure 3. Kaplan-Meier estimator curves of cumulative survival of patients with NSCLC dichotomised by 95% reference interval of *AURKA* mRNA expression in wild-type tissues. The P-values were calculated using a log-rank (Mantel-Cox) test. Increased *AURKA* expression (unbroken line) is associated with decreased survival time. The correlation between *AURKA* expression and cumulative survival is significant in AdC and SqCCL. Decreased *AURKA* expression (broken line) is associated with increased survival time. NSCLC, non-small cell lung cancer; AdC, adenocarcinoma; SqCCL, squamous cell carcinoma of the lung.

for both histological subtypes. There is a lack of consensus on this issue, with previous studies debating on the prognostic significance of *AURKA* in SqCCL (17,18). Furthermore, perimembrane immunohistochemical staining was demonstrated to be a marked predictor of poor prognosis in patients with

SqCCL, but not in patients with AdC (16), whereas microarray data analysis demonstrated that *AURKA* mRNA overexpression is associated with poor prognosis in patients with AdC, but not in patients with SqCCL (30). The reported differences are possibly due to dissimilarities in the study design,

Table I. Clinicopathological characteristics of the study patients in association with *AURKA* mRNA expression profile.

Clinicopathological characteristic	Total number of patients (%) 124 (100)	High expression of Aurora-A mRNA (n=59)	Low expression of Aurora-A mRNA (n=65)	P-value
Mean age, years (standard deviation)	66.5 (8.5)	65.9 (8.5)	67.5 (8.5)	0.223 ^a
Gender				0.180 ^b
Male	70 (56.5)	37 (52.9)	33 (47.1)	
Female	54 (43.5)	22 (40.7)	32 (59.3)	
Histology				<0.001 ^b
Adenocarcinoma	52 (41.9)	13 (25.0)	39 (75.0)	
Squamous cell carcinoma	72 (58.1)	46 (63.9)	26 (36.1)	
Tumour stage				0.513 ^b
1	19 (15.3)	7 (36.8)	12 (63.2)	
2	91 (73.3)	45 (49.5)	46 (50.6)	
≥3	12 (9.6)	7 (58.3)	5 (41.7)	
Nodal status				0.975 ^b
0	68 (54.8)	32 (47.1)	36 (52.9)	
1	38 (30.6)	18 (47.4)	20 (52.6)	
2	18 (14.6)	9 (50.0)	9 (50.0)	

^aMann-Whitney U test; ^b χ^2 test. *AURK*, Aurora kinase.

Table II. Univariate and multivariate Cox's proportional hazard regression analyses of potential predictors of overall survival among the study patients.

Covariates	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
<i>AURKA</i> mRNA	1.79 (1.16-2.77)	0.009	1.81 (1.16-2.84)	0.009
Age	1.02 (1.00-1.05)	0.066	1.03 (1.00-1.06)	0.020
Tumour stage				
1	Reference	Reference	Reference	Reference
2	2.82 (1.35-5.86)	0.006	2.43 (1.16-5.10)	0.019
≥3	3.80 (1.42-10.15)	0.008	1.39 (0.38-5.09)	0.623
Nodal status				
0	Reference	Reference	Reference	Reference
1	1.62 (1.03-2.55)	0.037	1.45 (0.90-2.34)	0.128
2	2.55 (1.35-4.84)	0.004	3.14 (1.24-7.99)	0.016

HR, hazard ratio; CI, confidence interval; *AURK*, Aurora kinase.

measurement of *AURKA* expression and the small study size, which decreases statistical significance. It is imperative that a large multicentre study is undertaken to determine a definitive explanation of these discrepancies.

AURKA overexpression may serve an important role in cancer aggressiveness through a range of underlying molecular mechanisms. Elevated levels of *AURKA* perturb mitotic spindle formation and therefore cytokinesis due to centrosome amplification, leading to chromosomal instability and

consequently aneuploidy or polyploidy (5,31). *AURKA* overexpression also inactivates several tumour-suppressor genes, including *p53* (32). The association between *AURKA* overexpression and *p53* mutation, as well as advanced tumour grade and advanced cancer stage, was also reported in patients with hepatocellular carcinoma (33), and with clinically aggressive disease and decreased survival rates in patients with ovarian cancer (34). These *AURKA*-associated events (the perturbation of spindle formation and inactivation of tumour-suppressor

genes by elevated *AURKA* expression) may explain the association identified between up-regulated *AURKA* expression and poor outcome of patients with NSCLC. Nonetheless, the hypothesis that up-regulated *AURKA* expression contributes to a poor survival outcome in lung cancer has been debated, presumably because NSCLC represents a set of heterogeneous malignancies, with various outcomes, even among those with the same clinicopathological features (35). The results of the present study provide evidence to support the prognostic role of *AURKA* expression in patients with NSCLC and highlight the requirement for a large multicentre clinical study which will take into consideration further parameters, including therapeutic regimens. Most importantly, the results of the present study suggest that NSCLC patients may benefit from therapy with *AURKA* inhibitors and this requires validation in a prospective clinical study.

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