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Research article

THE EXPRESSION OF COX-2, hTERT, MDM2, LATS2 AND S100A2 IN DIFFERENT TYPES OF NON-SMALL CELL LUNG CANCER (NSCLC)

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Abstract: Several studies have reported different expression levels of certain genes in NSCLC, mostly related to the stage and advancement of the tumours. We investigated 65 stage I-III NSCLC tumours: 32 adenocarcinomas (ADC), 26 squamous cell carcinomas (SCC) and 7 large cell carcinomas (LCC). Using the real-time reverse transcription polymerase chain reaction (RT-PCR), we analysed the expression of the COX-2, hTERT, MDM2, LATS2 and S100A2 genes and researched the relationships between the NSCLC types and the differences in expression levels. The differences in the expression levels of the LATS2, S100A2 and hTERT genes in different types of NSCLC are significant. hTERT and COX-2 were over-expressed and LATS2 under-expressed in all NSCLC. We also detected significant relative differences in the expression of LATS2 and MDM2, hTERT and MDM2 in different types of NSCLC. There was a significant difference in the average expression levels in S100A2 for ADC and SCC. Our study shows differences in the expression patterns within the NSCLC group, which may mimic the expression of the individual NSCLC type, and also new relationships in the expression levels for different NSCLC types.

Key words: COX-2, hTERT, MDM2, LATS2, S100A2, RT-PCR, Expression

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Abbreviations used: ADC – adenocarcinoma; CDK2 – cyclin-dependent kinase 2; cDNA – complementary DNA; COX-2 – cyclooxygenase 2; C_T – cycle threshold; DNA – deoxyribonucleic acid; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; hTERT – human telomerase reverse transcriptase; IHC – immunohistochemistry; LATS2 – homolog of large tumour suppressor 2, Drosophilae; LCC – large cell carcinoma; MDM2 – mouse double minute 2 homolog; mRNA – messenger RNA; NSCLC – non-small cell lung cancer; p21 – cyclin-dependent kinase inhibitor 1A; p53 – tumour protein p53; PCR – polymerase chain reaction; pTNM – pathological tumour-node-metastasis; RB – retinoblastoma; RNA – ribonucleic acid; r_s – Spearman's rank correlation coefficient; RT-PCR – real time PCR; S100A2 - S100 calcium binding protein A2; SCC – squamous cell carcinoma

INTRODUCTION

The non-small cell lung cancer (NSCLC) group includes adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC), which are major types of lung cancer with similar clinical and biological characteristics [1]. Various genes that undergo genetic alterations in lung cancer cells have been identified, and these alterations can be also shown as differences in the expression levels of the genes involved in lung cancerogenesis. The expression levels of the five genes included in this study were different in different types of NSCLC.

Telomerase itself can be expressed in up to 85% of NSCLC, and plays a critical role in maintaining cellular immortality [2-5]. In tumour cells, a telomerase complex with a catalytic subunit, telomerase reverse transcriptase (hTERT), enables telomere elongation [5]. The discovery that hTERT gene expression is very low or absent in somatic tissues, and is restricted to germ cells, stem cells and activated lymphocytes led to the discovery that hTERT is a limiting factor for telomerase activity [5]. Positive hTERT gene expression has been significantly associated with a worse survival rate, and has been suggested as an independent prognostic factor [2-6]. It was also published that telomerase over-expression may induce tumourigenesis in NSCLC [3, 4]. In 2004, it was published that hTERT expression is distinctive among histopathological classes of NSCLC, for example, being significantly lower in ADC than in SCC [7].

A major mechanism for the regulation of prostaglandin synthesis occurs at the level of cyclooxygenase, where the first rate-limiting step in the conversion of arachidonic acid to prostaglandins is catalyzed by two isoforms. COX-2 is associated with biological events such as injury, inflammation, and proliferation [8]. COX-2 is normally undetectable in lung tissue, and is induced by cytokines, growth factors, oncogenes and tumour promoters [9]. It plays a key role in lung cancer and is implicated in the modulation of apoptosis, the stimulation of angiogenesis and the modulation of the immune response, and in increased invasiveness [9]. There is considerable evidence of elevated COX-2 levels in NSCLC, and this is of importance in lung carcinogenesis [9-11]. It was published that higher levels of COX-2 are more related to ADC than to SCC [12-15], and over-expression of COX-2 has been proposed as a biomarker for biologically aggressive types of NSCLC and poorer survival [16-18]. The correlation between the higher expression of COX-2 and hTERT in NSCLC was already discussed and proposed as a prognostic marker [18].

The MDM2 oncogene, mostly over-expressed in NSCLC, is the main negative regulator of p53 [19-21]. As such, it promotes cell survival and cell cycle progression by binding the amino-terminal transactivation domain of the p53 tumour suppressor protein. It is known that MDM2 also inhibits RB function via ubiquitin-dependent degradation [22, 23]. That indicates that the MDM2 gene is involved in tumourigenesis in at least two different pathways. A recent report states that the MDM2 gene can be down-regulated by small interfering RNA [24],

and is also absent from lung cancer cell lines, and that the regulation of MDM2 is not defined only by p53 status [25]. Amplification of the MDM2 gene occurs at lower frequencies (5 to 15%) in NSCLC than in other cancers [26]. Down-regulation of MDM2 expression has also been found in primary lung tumours, mostly in SCC, and is related to an alternative pathway to NSCLC pathogenesis [27]. MDM2 mRNA expression was determined as a favourable prognostic factor in NSCLC [28].

LATS2 has a role in cell cycle regulation, the maintenance of mitotic fidelity and genomic stability, and the promotion of apoptosis through the down-regulation of the anti-apoptotic proteins of the Bcl family [29-32]. LATS2 itself is a target for positive transcriptional regulation by p53, showing the LATS2-MDM2-p53 pathway as critical for maintaining the proper chromosome number [32]. It has been implicated in the induction of p53-dependent senescence as a response to Ras activation [33], and also implemented in a novel pathway in which, by interacting with Ajuba, LATS2 modulates centrosome modulation [34]. Another gene that is involved in tumourigenesis and has been recently researched in more detail is S100A2, a small calcium-binding protein, one of the S100 proteins. It binds and activates p53 [35] and is implicated in COX-2 expression in the human malignant squamous carcinoma cell line [36]. S100A2 may have an impact on the progression of cancerogenesis [36]. The results of expression analysis of the S100A2 gene in NSCLC are somewhat contradictory. Early studies suggested that S100A2 expression is suppressed at an early stage of lung carcinogenesis [37], but later data indicated that it might be strongly expressed in the majority of NSCLC tumours [37, 38]. In the most recent studies, it was proposed that over-expression of the S100A2 gene in stage I NSCLC indicates a poor prognosis, and may be used to identify patients with early-stage NSCLC [39], or even as a predictor of distant metastasis [40]. There could also be differences in the levels of expression according to the NSCLC type [38].

We present here data on the COX-2, hTERT, MDM2, LATS2 and S100A2 expression levels in NSCLC, and how they relate to the NSCLC type. Differences in the expression of the genes included in our study in primary NSCLC are either already known (hTERT, COX-2) or have been a matter for discussion (S100A2, MDM2, LATS2), but there had yet to be research done on the relationship between the expression of two or more genes taking into account that those genes cooperate in lung tumourigenesis. We also found NSCLC type-specific relationships between the deregulation of expression and genes. The genes were chosen on the basis of deregulation in NSCLC and other cancers, but the levels of expression and the overall relationships were contra-indicatory or had not been studied according to the NSCLC types.

MATERIALS AND METHODS

Patients and tissue samples

The study material was surgical specimens from 65 patients with primary non-small cell lung carcinoma (32 adenocarcinoma (ADC), 26 squamous cell carcinoma (SCC), 7 large cell carcinoma (LCC)) with a known stage of NSCLC at diagnosis (classified according to the American Joint Committee for Cancer Staging System). Fifty seven (87.7%) patients were male and 8 (12.3%) were female, with ages ranging from 53 to 79 years (mean ± standard deviation; 65.7 ± 8.0 years). Fifty nine out of the 65 patients defined themselves as being smokers (90.8%; the information about smoking status was provided by the physicians), 54 of them male (94.7%) and 5 female (62.5%). None of the tumours were metastatic and all were stage I-III according to the pTNM classification. The lung resectates, which had been examined by an expert pathologist, and adjacent macroscopically normal lung tissue (obtained several centimetres from the tumour whenever possible and used as a control) were snap-frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

RNA extraction and the analysis of total RNA

RNA for expression analysis was extracted with TRI® reagent and chloroform. After extraction, the total RNA was cleaned with a Purelink™ Micro-to-Midi System (Invitrogen, USA). The quality and quantity of the RNA was checked on a Bioanalyzer 2100 (Agilent, USA) using an RNA 6000 Nano Labchip (Agilent, USA), and an RNA 6000 ladder as a reference (Ambion, USA).

cDNA synthesis and gene expression detection

First strand cDNA was synthesized using MultiScribe™ reverse transcriptase (Applied BioSystems, USA), with random hexamers, according to the manufacturer's instructions.

The expression of the COX-2, hTERT, MDM2, LATS2 and S100A2 genes in the tumours relative to the normal adjacent tissues was measured using quantitative real-time PCR (RT-PCR) based on the TaqMan® fluorescence methodology.

We used a ready mixture of probes and primers specific for COX-2 (Assay-on-Demand™, Hs0015133_m1), hTERT (Assay-on-Demand™, Hs99999022_m1), S100A2 (Assay-on-Demand™, Hs00195582_m1), LATS2 (Assay-on-Demand™, Hs00324396_m1) and MDM2 (Assay-on-Demand™, Hs01066938_m1), with Pre-Developed TaqMan Assay Reagents Human GAPDH (20x) mRNA as the endogenous control gene (all Applied Biosystems, USA).

The reactions were performed with a TaqMan Universal PCR Master Mix (Applied BioSystems, USA) in a 20-µl reaction volume. All the reactions were performed in triplicate and included a negative control. Quantification was done using the ABI Prism 7900 sequence detection system (Applied Biosystems, USA). The cycling conditions were: 2 min at 50ºC, 10 min at 95ºC, and 40 cycles of 15 s at 95ºC and 1 min at 60ºC.

The relative quantification of the mRNA levels of the target genes (the quantity of transcripts of the target in the tumours relative to that in normal tissues) was determined using the $\Delta\Delta C_T$ (ΔC_T tumour -ΔC_T normal) method. If the $\Delta\Delta C_T$ was significantly (2σ) higher or lower than zero, the gene was considered to be statistically significantly over- or under-expressed.
The relative quantification of the mRNA levels of the target genes in the tumours was determined using ΔC_T (the difference between threshold cycles of the tested and reference genes) and represented as fold change on graphs ($2^{-\Delta\Delta CT}$).

Statistics
The magnitudes and directions of the associations between the intervals or ratio scale variables were determined using Spearman's rank correlation coefficient (r_s) and comparisons between two independent samples with the Mann-Whitney U test, as appropriate. To determine the associations between the average expressions in the tumour relative to healthy lung tissue and to different types of NSCLC, Student's t test was used. A value of $p < 0.05$ was considered statistically significant for all analyses. All the statistical analyses were performed using SPSS ver.14 (SPSS Inc., USA).

RESULTS

The expression of the COX-2 gene was elevated in 60%, and that of hTERT in 55.3% of the analysed lung tumours. All of the analysed lung tumours had the LATS2 gene down-regulated. The expression of the MDM2 gene was down-regulated in 67.7% of the analysed lung tumours (84.6% SCC, 58.4% ADC and 42.9% LCC), but the level of expression was not significant. The expression of the S100A2 gene was up-regulated in 77.8% of SCC and down-regulated in 87.5% of ADC. There were more tumours with a down-regulated S100A2 gene in ADC than in SCC (87.5% in ADC compared to 14.3% in SCC). The Mann-Whitney U test was also performed to differentiate between genders. hTERT and COX-2 expression was found to be significantly higher in women ($p < 0.05$); the expression of all the other tested genes was not related to the gender of the patients ($p > 0.05$).
There were also differences in the levels of expression. Comparisons of the average expression levels of the COX-2, hTERT, LATS2, MDM2 and S100A2 mRNAs in different types of NSCLC revealed differences in the expression levels for different types of NSCLC (Fig. 1). The average COX-2 and hTERT mRNA levels were significant for all the NSCLC types, and for lung tumours overall (Student's t test, $p < 0.05$, Fig. 1). The expression of the COX-2 and hTERT genes was significantly higher in ADC related to in SCC and LCC (Student's t test, $p < 0.05$; Fig. 1). LATS2 was significantly down-regulated both in different NSCLC types and overall in the analysed lung tumours (Student's t test, $p < 0.05$, Fig. 1). The MDM2 and S100A2 mRNA levels were not significantly different in the tumours relative to the adjacent healthy tissue, but

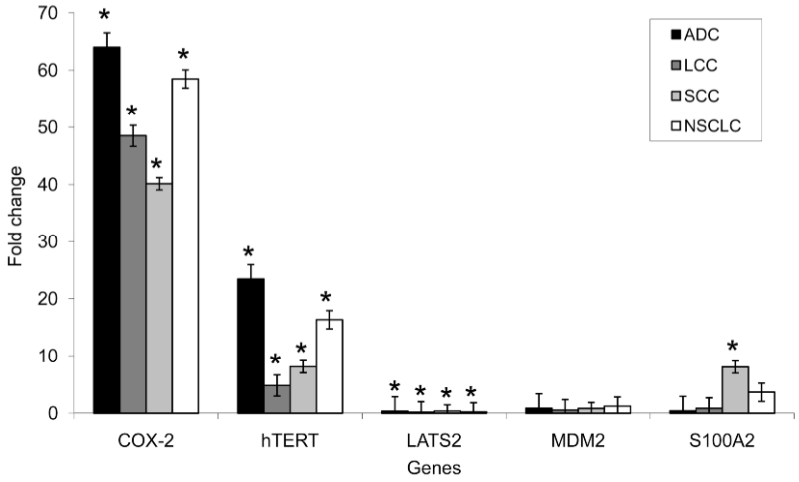

Fig. 1. The expression levels of COX-2, hTERT, LATS2, MDM2 and S100A2 mRNA in different types of NSCLC. The expressions of the hTERT and COX-2 genes were significantly higher in all the types of NSCLC (Student's t test, $p < 0.05$), and that of LATS2 was lower (Student's t test, $p < 0.05$). The expression of S100A2 was significantly higher for SCC, and lower but not significant in ADC and LCC (Student's t test, $p < 0.05$). The expression of MDM2 was lower in the tumours but not significantly so (Student's t test, $p < 0.05$). The bars represent the average fold change between the expression of the target genes in normal versus NSCLC tumour tissue, determined using the $\Delta\Delta C_T$ (ΔC_T normal -ΔC_T tumour) method. The fold change ($2^{-\Delta\Delta CT}$) is a relative comparison of the gene expression of the target genes in normal lung tissue versus tumour tissue normalized to an endogenous reference. The error bars are the average standard deviation of the tested NSCLC samples. * indicates significant differences in the expression levels of the analysed genes in separate tumour types.

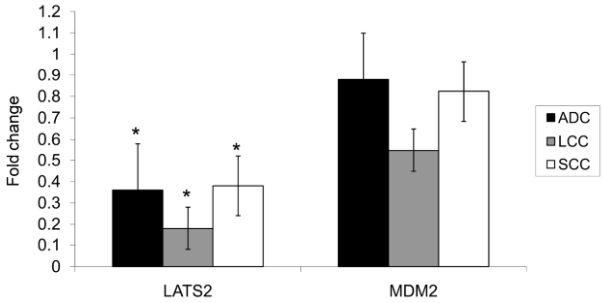

Fig. 2. The normalized average expression levels of LATS2 and MDM2 mRNA in tumours and adjacent normal lung tissue. The levels of mRNA of both genes were lower in the tumours, and the relationship was significant for LATS2 (Student's t test, $p < 0.05$). The fold change ($2^{-\Delta\Delta CT}$) is a relative comparison of the gene expression of the target genes in normal lung tissue versus tumour tissue normalized to an endogenous reference. The error bars represent the average standard deviation of the tested NSCLC samples. * indicates significant differences in the expression levels of the analysed genes in separate tumour types.

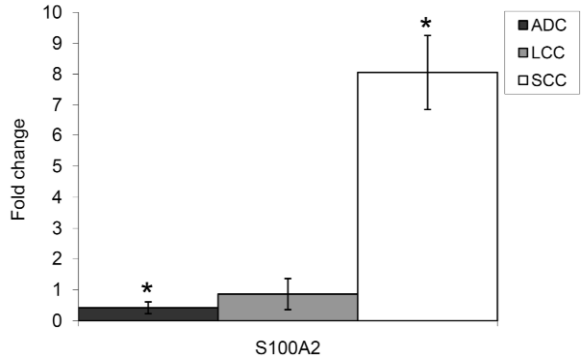

Fig. 3. The normalized average expression levels of S100A2 mRNA in different types of NSCLC. The average expression levels of the S100A2 gene were significantly higher in SCC and lower in ADC (Student's t test, $p < 0.05$) when comparing tumours. The overall expression of the S100A2 gene in the tumours and adjacent normal lung tissue was not significant, mostly due to over expression in SCC and under expression in ADC. The fold change ($2^{-\Delta\Delta CT}$) is a relative comparison of the gene expression of the target genes in normal lung tissue versus tumour tissue normalized to an endogenous reference. The error bars represent the average standard deviation of the tested NSCLC samples. * indicates significant differences in the expression levels of the analysed genes in separate tumour types.

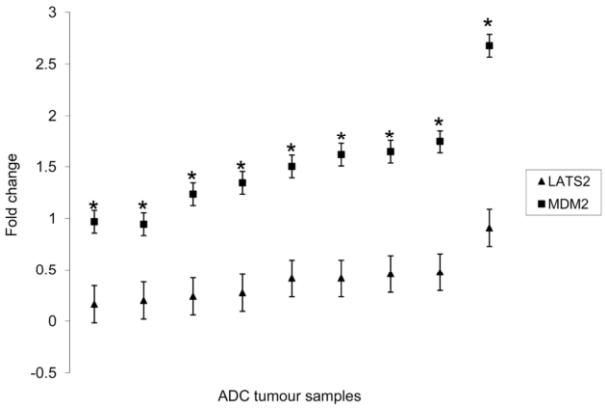

Fig. 4. The correlation between the lowered LATS2 and MDM2 gene expression in ADC tumours (MDM2 and LATS2; $r_s = 0.722$, $p = 0.008$). The correlation is significant for ADC, but not for the other tumour types. The fold change ($2^{-\Delta\Delta CT}$) is a relative comparison of the gene expression of the target genes in normal lung tissue versus tumour tissue normalized to an endogenous reference. ADC – adenocarcinoma tumour samples. The error bars define the standard deviation of triplicates for every sample. * indicates significant differences between the lowered LATS2 and MDM2 in the tested ADC tumours.

when analysed separately, we discovered that the S100A2 gene is significantly down-regulated in ADC (Student's t test, $p < 0.05$) and significantly up-regulated in SCC (Student's t test, $p < 0.05$; Fig. 3). MDM2 was generally down-regulated, but the relationship was not significant (Student's t test, $p > 0.05$). Analyzing the different expression levels of the genes in tumours also revealed that down-regulation of MDM2 correlates with down-regulation of LATS2, and this change is borderline significant for ADC and SCC (Student's t test, $p = 0.05$; Figs 2 and 4).

Spearman's rank correlation

The higher expression of COX-2 and hTERT was significant for all the types of NSCLC and for NSCLC overall ($r_s = 0.539$, $p < 0.001$). A significant positive correlation was found between the higher expression of the COX-2 and hTERT genes ($r_s = 0.337$, $p < 0.001$). A positive correlation was also found between the lowered expression in the MDM2 and LATS2 genes ($r_s = 0.466$, $p = 0.016$) in NSCLC; all the other correlations were not significant when comparing the $\Delta\Delta C_T$ (ΔC_T normal - ΔC_T tumour) of the analysed genes.

Further analysis was done on all three types of NSCLC separately, and it revealed several interesting correlations in the expression of the analysed genes.

We proved positive correlations between the higher expression of the COX-2 and hTERT genes and the lower expression of the MDM2 and LATS2 genes in ADC (COX-2 and hTERT; $r_s = 0.34$, $p = 0.48$; MDM2 and LATS2; $r_s = 0.722$, $p = 0.008$, Figs 2 and 4). The correlation of the expression of the COX-2 and hTERT genes was also significantly positive for SCC (higher expression of COX-2 and hTERT; $r_s = 0.489$, $p = 0.01$) and near significance for LCC ($r_s = 0.45$, $p = 0.05$). In ADC, we also detected a correlation between the lowered expression of the MDM2 and S100A2 genes ($r_s = 0.678$, $p = 0.045$) and between the higher expression of hTERT and lowered expression of MDM2 ($r_s = -0.738$, $p = 0.037$). In LCC, we detected a strong correlation between the higher expression of S100A2 and lowered expression of LATS2 ($r_s = -1.000$, $p < 0.001$).

For the statistical evaluation of the relationship between the differentially expressed genes and the stage of the tumour in the NSCLC patients, we used Spearman's Rho test statistic. A significant positive correlation was found between higher hTERT expression and a later tumour stage ($p < 0.05$, $r_s = 0.42$). All the other tested genes (MDM2, COX-2, LATS2 and S100A2) showed no correlation between the gene expression and the stage of the tumour ($p > 0.05$).

DISCUSSION

The expression of the hTERT, COX-2, S100A2, MDM2 and LATS2 genes has already been researched for different types of tumours, but the relationships between those genes were never established for NSCLC. Most expression analysis is still done at the protein level via imunohistochemical assays, so

discrepancies in the results for the researched genes can be a consequence not just of different NSCLC types or different pathways of tumourigenesis, but also of the different techniques used for gene expression analysis and the different interpretations of the results.

We proved that COX-2 and hTERT are already over-expressed at the mRNA level and that higher expression levels are related to NSCLC tumours, as already stated [17, 18, 27, 28]. We also confirmed that COX-2 gene expression was significantly higher in ADC, as was already known [12-15]. For the hTERT gene, we discovered that the expression was higher in ADC than in SCC (Fig. 1), which is not in accordance with previously published results [7]. The relationship between the higher expression levels of the hTERT and COX-2 genes was already detected with IHC and the RT-PCR method [27], and we confirmed that this relationship exists by comparing the mRNA levels, meaning the deregulation occurs at the transcription level. Lu *et al.* [18] published the same observation when they tested six molecular markers on a larger patient group. There is still no precise explanation for the correlation, although it has been shown that different COX-2 inhibitors inhibit tumour growth and telomerase activity in mice [41, 42].

The interesting result that MDM2 gene expression is lowered in NSCLC put that gene into a different perspective in lung tumourigenesis. Although most publications with IHC as the main methodology state that the MDM2 gene is over-expressed, contrary results showing that MDM2 can be lowered also exist. MDM2 is suppressed by FUS1, which could provide the basis for new strategies in cancer treatment [43]. p53 over-expression induces over-expression of MDM2, which, in turn, results in rapid degradation of the p53 protein [44]. It is also necessary to evaluate the level of properly functional p53 in those tumours, because if p53 cannot induce MDM2, that could explain the under expression of the MDM2 gene. MDM2 can be down-regulated by small interfering RNA, and is involved in the inhibition of CDK2 activity via p21 [24]. Although this lowered expression was not significant, relationships between the lowered expression of MDM2 and differences in the expression of other researched genes (LATS2, S100A2, hTERT) for specific types of NSCLC were significant. Thus, there is a possibility of using even not-significant markers, combined with others, to detect differences between NSCLC tumours.

Down-regulation of the LATS2 gene was noted in all the studied NSCLC, and was specific regardless of the NSCLC type. We showed that the LATS2 gene is down-regulated in tumours, as has already been detected in breast and testis cancers, leukaemias and astrocytomas [45-49]. An analysis of the expression of the two genes (LATS2 and MDM2) showed a positive correlation between the down-regulation of LATS2 and MDM2 (Spearman's Rho correlation, $p < 0.05$), which may indicate the importance of LATS2 in the checkpoint pathway LATS2-Mdm2-p53, as has recently been identified [34]. However, this study cannot establish whether the finding is relevant for lung tumourigenesis.

The S100A2 gene is significantly differently expressed in ADC versus SCC, an observation that had already been detected with IHC and RT-PCR in 2004 by Smith *et al.* [38]. Many discrepancies in the results concerning S100A2 expression have arisen: it was stated that the gene is down-regulated in NSCLC, while further research showed that its expression levels depend on the stage of the NSCLC, and that in the main, S100A2 is over-expressed in lung cancer tumours. We discovered that its expression levels are much lower in ADC and much higher in SCC, and that the expression level of the S100A2 gene in our study cohort is not dependant on staging (Spearman's Rho correlation, $p > 0.05$) as previously stated [39, 40]. This may indicate that the S100A2 gene acts differently in ADC than in SCC tumourigenesis, and that the pathways including S100A2 are different in different NSCLC types. It has been published that the S100A2 gene is down-regulated in lung ADC cell lines, but frequently observed in primary lung ADC cells [50], so there is more proof that the expression of S100A2 is precisely regulated with different mechanisms, and it may be that the deregulation of this gene's expression is specific to the pathological characteristics of different tumours.

The study is limited by the numbers of specimens included in the study and by its retrospective nature. It is difficult to speculate on potential biases affecting the results. Tobacco consumption level was previously related to an abnormal expression of hTERT and COX-2 genes in NSCLC patients [51-53], so it could also be related with the differences in the expression of the chosen genes in this study. However, the group of non-smokers was too small for any meaningful statistical estimation (less than 10%). We did not confirm the previously published relationship between MDM2 expression and the gender of the patients [54], although we did discover higher expression levels of COX-2 and hTERT for female patients. The higher expression of those two genes may be a consequence of the different pathways of tumourigenesis and different triggers for the expression of COX-2 and hTERT, or due to potential gender-specific differences. We proved the relationship between the later stage of the tumour of the patients and the higher hTERT gene expression (Spearman's Rho, $p < 0.05$). The reason for the lack of significance between the other differentially expressed genes and the tumour stage grouping could be the number of specimens used in this study. Another reason for the lack of significance could be differences in gene expression according to the different NSCLC types. We did not test the relationships between the tumour stage and gene expression in the separate NSCLC types, because the statistics would be uninformative due to the small sample size for each stage. There is also a possibility that the tumour stage is not related to differentially expressed genes, or that the relationship between gene expression and stage (which is a very informative variable) is not strong enough to show statistical significance with a limited sample size.

Our results should be evaluated on larger separate population cohorts of NSCLC patients, and functional studies performed to evaluate the real relationships between the tested markers. It would be interesting to inspect these markers in

relation to the clinical and histological signs of NSCLC, but a much bigger sample would be needed for accuracy of interpretation. Nevertheless, we have shown that relationships exist at the mRNA level, and are different for the different NSCLC types. There is a further possibility that the markers and the relationships between them can eventually provide a molecular staging system and improve prognostic and therapeutic strategies for different types of NSCLC.

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