Expression of Metallothionein-III in Patients with Non-small Cell Lung Cancer

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Abstract. *Background: Currently, there is little knowledge concerning expression of metallothionein-III (MT-III), also known as growth-inhibitory factor, in non-small cell lung cancer (NSCLC). Materials and Methods: In this study, we evaluated MT-III expression in 184 patients using immunohistochemistry and in 61 cases using real-time polymerase chain reaction. Results: MT-III mRNA expression was significantly higher in NSCLC as compared to non-malignant lung tissues (NMLT; p<0.0086). MT-III expression was noted in the cytoplasm and nucleus of cancer cells. Significantly lower nuclear MT-III (p<0.0001) expression and significantly higher cytoplasmic MT-III (p=0.0068) expression was noted in the pneumocytes of NMLT, as compared to NSCLC. Nuclear MT-III expression was significantly higher in G1 cases as compared to G2 (p=0.0308) and G3 (p=0.0194) cases. Low cytoplasmic MT-III expression was associated with larger primary tumour size (p=0.0378). Lower MT-III mRNA and cytoplasmic MT-III expression was associated with poor patient outcome (p=0.0410 and p=0.0347, respectively). Conclusion: MT-III expression may have an impact on the pathogenesis of NSCLC.*

Lung cancer has the highest mortality rate among all types of cancer. Despite advances in diagnosis and treatment, the prognosis remains unfavorable, with fewer than 16% of patients surviving more than five years (1).

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Metallothioneins (MTs) were first isolated mote than 50 years ago (2). MTs are intracellular, low molecular weight proteins (6-7 kDa), characterized by a high cysteine content, allowing them to bind heavy metal ions *e.g.* zinc, copper, cadmium, lead and mercury (3). In regard to their structure and function, four basic groups of MT proteins are currently known: MT-I, MT-II, MT-III, MT-IV (3, 4). Members of the MT-I/II family, depending on cell type, are responsible for zinc and copper homeostasis and exert antioxidant properties by acting as scavengers of reactive oxygen species (5, 6). MT-I/II are broadly expressed in eukaryotic cells, in normal as well as in neoplastic cells. In some malignancies, overexpression of MT-I/II has been associated with poor patient outcome (7-15). In neoplastic cells, MT-I/II also exhibit anti-apoptotic activity and augment cancer cell proliferation (4, 12, 16, 17). MT-IV has been found to be expressed in stratified squamous epithelium (18).

MT-III, also known as growth-inhibitory factor, was first identified in tissues of the nervous system (19-22). MT-III is characterized by having an additional seven amino acids and differs in its properties from the MT-I/II isoforms (19). Like other MTs, MT-III comprises of α- and β-domains. The β-domain of the MT-I/II family binds up to six metal ions without leaving any free cysteine residues, whereas that of MT-III binds up to four metal ions leaving two free cysteine residues which may participate in interactions with thiol and disulfide groups of other proteins in the cell's nucleus (23). Aside from the nervous system, MT-III expression was found in normal human kidney and some cancer types, including those of the urinary bladder, prostate, esophagus, stomach and breast (24-30). In gastric and esophageal cancer, its expression was down-regulated due to promoter hypermethylation (28, 29). In breast cancer, lack of MT-III expression was rare, but has been associated with good prognosis (30). Similarly to MT-I/II, higher MT-III

Table I. *Patients' and tumour characteristics*.

	All patients (n=184)		SQC (n=82)		AC (n=87)		LCC (n=15)		Real-time PCR group (n=61)*	
Mean age, years (range)	63.04±8.61 (39-87)		64.29±8.61 (39-87)		61.95±8.85 (39-80)		62.53±6.24 (54-76)		64.77±7.31 (49-81)	
Parameter	N	%	N	%	N	%	N	%	N	%
Gender										
Male	136	73.9	59	72.0	67	77.0	10	66.7	40	65.5
Female	48	26.1	23	28.0	20	23.0	5	33.3	21	34.5
Tumour size										
pT1	48	26.0	22	26.8	21	24.1	5	33.3	19	31.1
pT2	99	53.9	43	52.4	47	54.0	9	60.0	33	54.1
pT3	19	9.8	12	14.7	6	6.9	0	0	7	11.6
pT4	19	10.2	5	6.1	13	15.0	1	7.7	2	3.2
Lymph node status										
pN0	95	51.6	48	58.5	37	45.1	10	66.7	41	67.2
pN1-pN3	89	48.4	34	41.5	50	54.9	5	33.3	20	32.8
pTNM										
1A	36	19.5	17	20.7	15	17.2	4	26.7	16	26.3
1B	45	24.5	22	26.8	19	21.9	4	26.7	19	31.1
2A	17	9.2	7	8.6	8	9.2	2	13.3	3	4.9
2B	15	8.2	9	11.0	6	6.9	0	0	6	9.8
3A	50	27.2	22	26.8	23	27.6	4	26.7	15	24.6
3B	14	7.6	3	3.7	10	11.5	1	0.6	0	0
4	7	3.8	2	2.4	5	5.7	0	0	2	3.3
Grade										
G1	10	5.9	5	6.1	5	5.7	-	-	2	3.7
G2	124	73.4	60	73.2	64	73.6	-	-	45	81.8
G3	35	20.7	17	20.7	18	20.7	-	-	8	14.5

SQC, Squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma. *The group comprises 23 AC, 32 SQC and 6 LCC.

expression was associated with cancer cell resistance to cytostatic drugs (31-33).

To date, as far as we are aware, no information concerning the role of MT-III in non-small cell lung cancer (NSCLC) exists. Therefore, in this study we investigated the expression of MT-III using immunohistochemistry and real-time polymerase chain reaction (PCR) in a series of patients with NSCLC and we correlated its expression levels with recognized clinicopathological parameters, including the expression of the Ki-67 antigen.

Materials and Methods

Patients and tissue samples. The studies were performed on archival material embedded in paraffin blocks from 184 patients with NSCLC operated on in 1997-2011 at the Lower Silesian Centre of Pulmonary Diseases in Wroclaw before treatment initiation. In the case of 61 patients, additional tumour material was deep-frozen in liquid nitrogen and stored at –80˚C.

Immunohistochemistry (IHC) was performed for the whole patient cohort, comprising of 87 adenocarcinomas (AC), 82 squamous cell carcinomas (SQC) and 15 large cell carcinomas (LCC). *MT-III* mRNA expression level was additionally examined in 61 cases (23 AC, 32 SQC, six LCC) and in 12 samples of non-malignant lung tissue, (NMLT; normal and inflammatory lung tissue from patients with no neoplastic history). All the samples used for IHC and real-time PCR studies were characterized by a cancer cell content exceeding 70% of the whole obtained tissue. Clinical and pathological data of the examined patients are presented in Table I. The whole study group was followed-up for 31.1±38.8 (range=1-147) months. In this period, 96 (52.2%) of the patients died of their disease. In cases where real-time PCR examinations were performed, the patients were followed up for 11.6±8.8 (range=1-29) months and 17 (27.4%) of the patients died. This study was approved by the Commission of Bioethics at Wroclaw Medical University.

Immunhistochemistry. Resected tissues were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. The paraffin sections, stained with hematoxylin/eosin (HE), were used to verify the diagnosis and degree of tumour malignancy grade.

The IHC reactions were performed as follows. Paraffin blocks were cut into 4-μm-thick paraffin sections and fixed on microscopic slides (SuperFrost+; Menzel Glässer, Braunschweig, Germany). De-paraffinization and antigen retrieval were then performed using

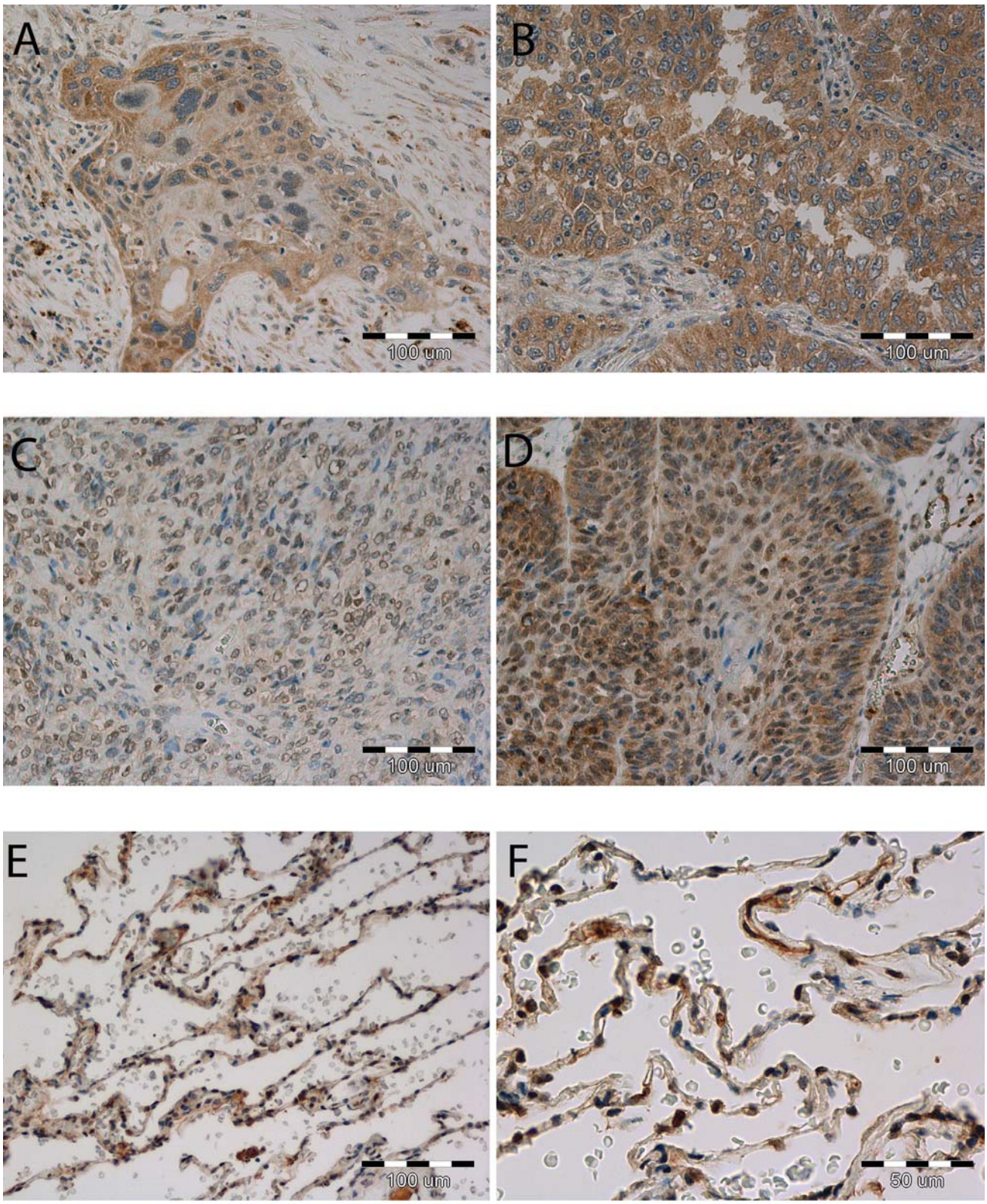

Figure 1. *Metallothionein-III (MT-III) immunohistochemical expression in non-small cell lung cancer (NSCLC) presenting low (A) and high (B) cytoplasmic MT-III expression. In a minority of the cases, weak (C) and strong (D) nuclear MT-III expression in NSCLC was also noted. In non-malignant lung tissue (NMLT) mainly nuclear MT-III expression could be noted (E, F).*

Table II. *Associations between cytoplasmic and nuclear metallothionein-III immunohistochemical expression and selected clinicopathological parameters for the group of 184 patients with non-small cell lung cancer.*

Parameter	No. (%)	Cytoplasmic		*p*-Value	Nuclear		*p*-Value
		IRS 0-4	IRS 6-12		Score 0	Score 1-4	
Age							
≤60	70 (38.0)	29 (41.4)	41 (68.6)	0.5320	55 (78.6)	15 (21.4)	0.8500
>60	114 (62.0)	41 (36.0)	73 (64.0)		92 (80.7)	22 (19.3)	
Gender							
Male	136 (73.9)	51 (37.5)	85 (62.5)	0.1802	112 (82.4)	24 (18.6)	0.2080
Female	48 (26.1)	19 (39.6)	29 (60.4)		35 (72.9)	13 (18.1)	
Tumour size							
pT1	48 (26.1)	12 (25.0)	36 (75.0)	**0.0378**	39 (81.3)	9 (18.7)	0.8376
pT2-pT4	136 (73.9)	58 (42.6)	78 (57.4)		108 (79.4)	28 (20.6)	
Lymph node status							
pN0	95 (51.6)	33 (34.7)	62 (65.3)	0.3648	78 (82.1)	17 (17.9)	0.4665
pN1-pN3	89 (48.4)	37 (41.6)	52 (58.4)		69 (77.5)	20 (22.5)	
Ki-67 index							
≤25%	76 (41.3)	30 (39.4)	46 (60.6)	0.7596	60 (78.9)	16 (21.1)	0.8526
>25%	108 (68.7)	40 (37.0)	68 (63.0)		87 (80.6)	21 (19.4)	

Antigen Retrieval Solution (pH 9.0, 97˚C, 20 min; DakoCytomation, Glostrup, Denmark). Subsequently, the sections were washed in Tris-buffered saline buffer and incubated with primary antibodies in a Dako Autostainer Link48 for 20 min at room temperature. MT-III expression was studied using rabbit polyclonal antibody raised against GGEAAEAEAEKC peptide (Invitrogen, Carlsbad, CA, USA) and Ki-67 expression by using MIB-1 antibody (DakoCytomation). The sections were visualized with EnVison FLEX (DakoCytomation) reagents. All slides were counterstained with haematoxylin (DakoCytomation) and all the reactions were conducted with negative controls. Cases in which tumour tissues did not express Ki-67 were subjected to repeated staining with positive control (another tumour tissue positive for Ki-67 expression) in order to exclude a reaction error.

Evaluation of IHC reactions. IHC reactions were analyzed under a BX-41 light microscope (Olympus, Tokyo, Japan). Expression of MT-III in the cytoplasm was evaluated using a 12-point semi-quantitative immunoreactive score (IRS), according to Remmele and Stegner (34), which was used in our previous work for MT-I/II assessment in NSCLC (12). The scale is based on the percentage of tumour cells showing positive reaction (0 points: absence of cells with positive reaction, 1 point: 1-10% cells, 2 points: 11-50%, 3 points: 51-80%, 4 points: over 80% cells with positive reaction), as well as the intensity of the reaction (0: no reaction, 1: low-intensity reaction, 2: moderate-intensity reaction, 3: intense reaction). Nuclear expression of MT-III and Ki-67 antigen expression were evaluated in whole tumour sections using a semi-quantitative five-grade scale based on the proportion of cells with reaction product: (0 points: absence of reaction, 1: 1-10% cells with positive reaction, 2: 11-25%, 3: 26-50%, 4 points: more than 50% cells with positive reaction).

RNA extraction, cDNA synthesis and real-time PCR. Total RNA was extracted from 61 fresh-frozen NSCLC samples and 12 control NMLT samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the procedure of the manufacturer. Briefly, genomic DNA was removed by DNase (Qiagen) digestion. Total RNA sample quality was evaluated utilizing agarose gels and staining with ethidium bromide. *18S* and *28S* bands were visualized under UV light. Concentration and quality of the isolated RNA was measured in a NanoDrop1000 instrument (Life Technologies, Carslbad, CA, USA). Reverse transcription was performed using SuperScript III (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

MT-III mRNA expression level was evaluated by real-time PCR, performed in 7900HT Fast Real-Time PCR System using the TaqMan® Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA). The results of mRNA expression were normalized to the mRNA expression of β-actin. MT-III Hs00359394_g1 and β-actin Hs99999903_m1 (Applied Biosystem) primers and probes from TaqMan® were used. All of the reactions were performed in triplicates. Polymerase was activated at 50˚C for 2 min. Initial denaturation was performed at 94˚C for 10 min followed by 40 cycles of denaturation at 94˚C for 15 s, and an annealing step and synthesis at 60˚C for 1 min. Relative expression of the studied MT-III was calculated using the ΔΔCt method. The average Ct of all NMLT samples for both MT-III and β-actin was calculated and the average ΔCt (Ct_{MT} - $Ct_{\beta\text{-actin}}$) was determined. The average ΔCt of NMLT was used as $\Delta Ct_{calibrator}$ in all the calculations, as shown below. In this way, the relative expression (RQ) of the MT-III isoform was calculated for each studied sample of NMLT and NSCLC according to the following formula:

$\Delta Ct_{sample}=Ct_{MT} - Ct_{\beta\text{-actin}}$
$\Delta\Delta Ct=\Delta Ct_{sample} - \Delta Ct_{calibrator}$
$2^{-\Delta\Delta Ct}=RQ$

Statistical analysis. For statistical analysis, the Prism 5.0 (GraphPad, La Jolla, CA, USA) statistical software was used. Mann-Whitney test was used to analyse the expression level of cytoplasmic MT-III, and nuclear MT-III and MT-III mRNA in NSCLC in comparison to NMLT, which served as the control. Correlations between clinicopathological

parameters and MT-III expression were analysed using Mann-Whitney *U*-test, Fisher's exact and Spearman's correlation test. Kaplan-Meier method and the log-rank test was used to analyse patient survival. For each variable, the hazard ratio (HR) and 95% confidence interval (95% CI) were estimated. In all the analyses, results were considered statistically significant when $p<0.05$.

Results

Relationship between MT-III IHC expression and patients' clinicopathological data. MT-III expression was noted in the cytoplasm and nuclei of NSCLC cells (Figure 1A-D), as well as in pneumocytes of NMLT (Figure 1E-F). Cytoplasmic MT-III expression was observed in 178 (96.7%) out of the 184 examined cases, whereas nuclear MT-III expression was noted only in 37 (20.1%) cases. Based on statistical analysis estimation, the cases which showed cytoplasmic MT-III expression less than or equal to IRS score 4 were regarded as having 'low' expression, whereas cases with scores of six and higher were regarded as 'high' in further analyses. For nuclear MT-III, cases scored as zero were regarded as negative and those scoring one or more were regarded as positive.

We have noted a significantly lower nuclear MT-III expression in NSCLC as compared to its expression in NMLT ($p<0.0001$; Figure 2A). However, the opposite relationship was noted for cytoplasmic MT-III expression, which was significantly higher in NSCLC in comparison to NMLT ($p=0.0086$; Figure 2B). No correlation was observed between cytoplasmic and nuclear MT-III expression in NSCLC (Spearman correlation test).

No differences were noted in cytoplasmic MT-III expression regarding the grade of malignancy of all the analyzed tumours (IRS G1=6.2±3.36; G2=5.68±2.57; G3=5.83±2.80). Similar results were obtained when SQC and AC types were analyzed separately. Cytoplasmic MT-III expression did not differ between the histological NSCLC types. Statistical analysis revealed that, lower cMT-III expression was associated with larger primary tumour size ($p=0.0378$) considering all the analysed tumours (Table II). A similar association was noted for the AC subtype ($p=0.0403$), but not for SQC and LCC subtypes, although a trend for both these types was observed ($p=0.0787$ and $p=0.0769$, respectively). No relationship was noted between the cytoplasmic MT-III expression and the presence of lymph node metastases in the studied patient cohort or histological subtypes of NSCLC.

The highest nuclear MT-III expression was noted in the SQC subtype (0.37±0.71), followed by LCC (0.20±0.41) and AC (0.16±0.43) subtypes. Nuclear MT-III expression differed significantly between the SQC and AC subtypes ($p=0.0283$; Figure 3A). Nuclear MT-III was differentially expressed regarding the grade of malignancy of the tumours ($p=0.0496$; Kruskal-Wallis test; Figure 3B). Significantly higher nuclear MT-III expression was found using the Mann-Whitney test in G1 cases (0.60±0.69), as compared to G2 (0.26±0.61; $p=0.0308$) and G3 (0.17±0.45; $p=0.0194$) cases. In the SQC subtype, nuclear MT-III expression was significantly higher in the G1 (0.80±0.84) than in the G3 (0.12±0.33) cases ($p=0.0243$; Figure 3C). No associations between nuclear MT-III expression and primary tumour size and presence of lymph node metastases were noted in regard to the entire study group, nor in particular NSCLC subtypes-alone (Fisher exact test; data not shown).

Cytoplasmic and nuclear MT-III expression showed no relationship with patient age, gender and the expression of the Ki-67 antigen regarding the whole analyzed cohort (Table III), nor for NSCLC subtypes (data not shown).

Table III. *Metallothionein-III mRNA expression in non-small cell lung cancer (NSCLC) and its histological subtypes for the group of 61 patients. Means±SD (median) are given as relative mRNA expression (RQ). Differences between MT-III mRNA expression in non-malignant lung tissue in comparison to NSCLC and its histological types were estimated using the Mann-Whitney test. Significant p-values are given in bold.*

Tissue	*MT-III* mRNA RQ	*p*-Value
	Mean±SD (median)	
NMLT	1.841±2.084 (0.90)	
NSCLC	22.96±49.9 (3.91)	**0.0086**
SQC	19.31±46.93 (3.87)	**0.0039**
AC	23.54±44.14 (3.161)	0.1061
LCC	40.20±84.66 (7.01)	0.0678

SQC, Squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma.

MT-III mRNA expression in NSCLC and its impact on patients' clinicopathological parameters. Real-time PCR showed *MT-III* mRNA expression to be significantly up-regulated in the 61 examined cases of NSCLC as compared to NMLT ($p<0.0001$; Table III). No statistical differences were observed between the expression of *MT-III* mRNA in the SQC, AC and LCC subtypes, and *MT-III* mRNA expression was only significantly up-regulated in SQC compared to NMLT ($p=0.0039$; Table III). *MT-III* mRNA expression did not correlate with the cytoplasmic or nuclear MT-III expression (Spearman correlation test). No significant associations were noted between *MT-III* mRNA expression and patient age, gender, tumour grade of malignancy, primary tumour size, presence of lymph node metastases or the expression of Ki-67 antigen.

Prognostic value of MT-III IHC and mRNA expression in NSCLC. Univariate analysis of the group of 184 patients revealed that only presence of lymph node metastases ($p<0.0001$) and of AC histological subtype ($p=0.024$) were associated with poor overall survival (OS). Of note, patients

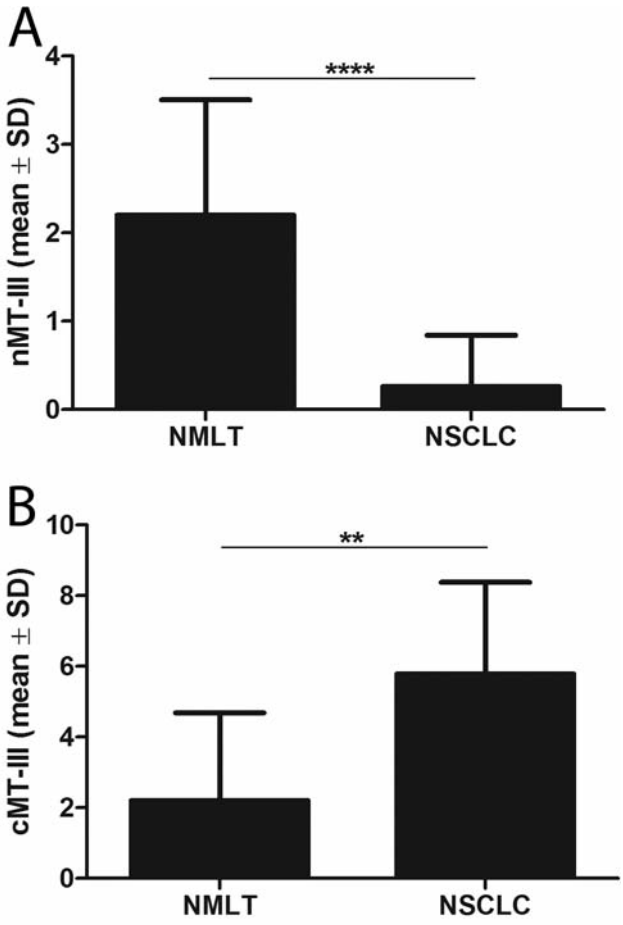

Figure 2. *Nuclear (n) (A) and cytoplasmic (c) metallothionein-III (MT-III) (B) immunohistochemical expression in non-malignant lung tissue (NMLT) in comparison to its expression in non-small cell lung cancer (NSCLC). The differences were tested utilizing the Mann-Whitney test. **p<0.005; ****p<0.0001.*

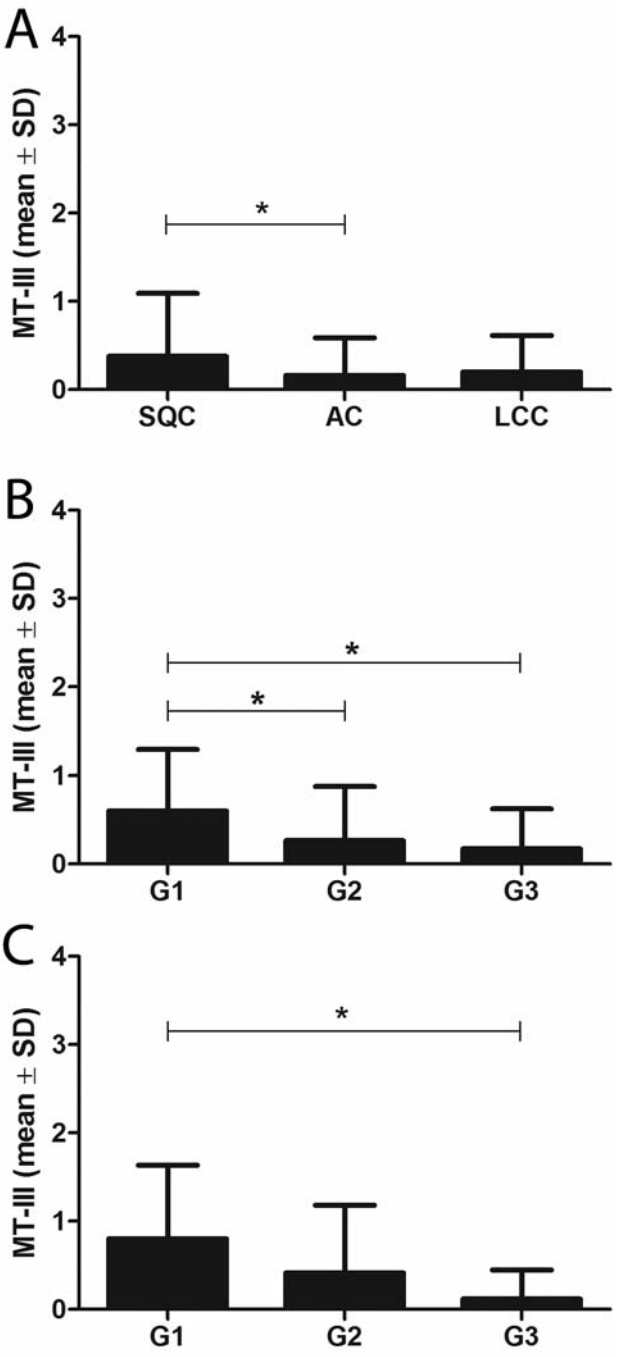

Figure 3. *Differential expression nuclear metallothionein-III (MT-III) expression in non-small cell lung cancer (NSCLC) subtypes (A). Nuclear MT-III expression regarding the grade of malignancy of the analysed cases of squamous cell carcinoma and adenocarcinoma subtypes (B) and in squamous cell carcinoma subtypes only (C). *p<0.05.*

with pT2-pT4 tumours tended to have a shorter OS, but this trend did not reach the threshold of significance (p=0.0509). Patients' age, gender, cytoplasmic and nuclear MT-III, and Ki-67 antigen expression had no impact on patient OS (Table IV).

For the statistical analysis purposes, patients were dichotomized regarding the median *MT-III* mRNA expression. Cases characterized with *MT-III* mRNA RQ>3.91 were classified as 'up-regulated', whereas those with RQ≤3.91 were classified as 'down-regulated'. Down-regulated *MT-III* mRNA expression was associated with poor OS (p=0.0347). Similar observations were noted for patients with low IHC cytoplasmic MT-III expression in this group (p=0.0189). The impact of cytoplasmic and nuclear *MT-III* and *MT-III* mRNA expression on OS is shown in Figure 4. Similarly to the group of 184 patients, the presence of lymph node metastases predicted a poor patient outcome (p=0.0045; Table IV). Patients age, gender, nMT-III and Ki-67 antigen expression had no impact on OS for the group of 61 patients (Table IV).

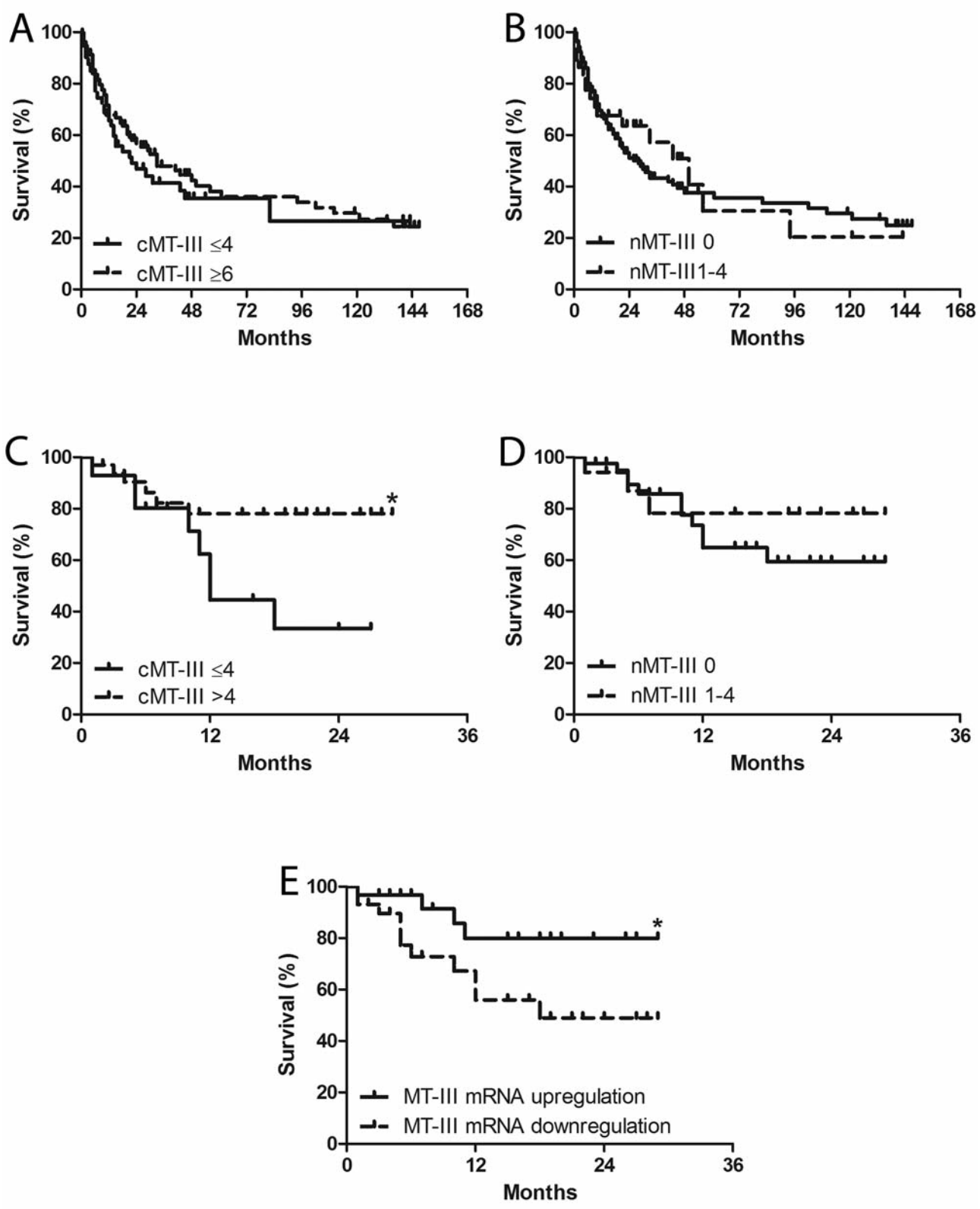

Figure 4. *Kaplan-Meier survival curves for the whole analysed patient cohort regarding cytoplasmic (c) (A) and nuclear (n) metallothionein (MT-III) (B) expression. Patient survival was also analysed for the real-time PCR group regarding cytoplasmic (C) and nuclear MT-III (D) and MT-III mRNA (E) expression. *p<0.05.*

Table IV. *Univariate overall survival analysis of patients with non-small cell lung cancer by immunohistochemistry (IHC) and real-time polymerase chain reaction (PCR). Significant p-values are given in bold.*

Clinicopathological parameter	184 patients (IHC)				61 patients (real-time PCR)			
	Deaths/Cases	HR	95% CI	*p*-Value	Deaths/Cases	HR	95% CI	*p*-Value
IHC cMT-III								
IRS 0-4	58/116	0.9665	0.6372-1.466	0.8725	10/28	3.622	1.237-10.610	**0.0410**
IRS 6-12	38/67				5/32			
IHC nMT-III								
Score 0	79/146	1.093	0.6523-1.831	0.7362	12/43	1.597	0.5178-4.926	0.4153
Score 1-4	17/37				3/17			
MT-III mRNA								
RQ≤3.91	NA	NA	NA	NA	11/29	3.029	1.083-8.474	**0.0347**
RQ>3.91					4/31			
Age, years								
≤60	37/70	1.219	0.8101-1.834	0.1684	6/21	0.8320	0.2850-2.429	0.7365
>60	59/113				9/39			
Gender								
Male	77/135	0.7228	0.4554-1.147	0.4954	10/39	0.9173	0.3034-2.773	0.8785
Female	19/48				5/21			
Histology								
AC	54/87	1.782	1.179-2.692	**0.0240**	5/23	1.065	0.3518-3.221	0.9119
SQC, LCC	41/54				27/37			
Tumour size								
pT1	20/48	1.568	0.9982-2.464	0.0509	2/19	2.424	0.8051-7.297	0.1152
pT2-pT4	76/135				13/41			
Lymph node status								
pN0	30/94	2.752	1.834-4.147	**<0.0001**	6/40	5.334	1.682-16.91	**0.0045**
pN1-pN3	66/89				9/20			
Grade of malignancy*								
G1, G2	69/132	1.574	0.9016-2.747	0.3613	11/46	2.990	0.3747-23.85	0.3013
G3	21/35				2/8			
Ki-67 index								
≤25%	44/75	1.062	0.7048-1.601	0.7732	3/15	1.206	0.3578-4.062	0.7629
>25%	52/108				12/45			

cMT-III, Cytoplasmic metallothionein-III expression; nMT-III, nuclear metallothionein-III expression; RQ, relative expression; IRS, immunoreactive score; AC, adenocarcinoma; SQC, squamous cell carcinoma; LCC, large cell carcinoma; HR, hazard-ratio; CI, confidence interval; NA, not analyzed. *LCC subtype was not included in the analysis.

Discussion

MT-III was firstly found to be expressed in tissues of the nervous system (19, 20). Recent studies have identified MT-III as being expressed in breast, esophageal, stomach, prostate and urinary bladder cancer, although its exact role in carcinogenesis remains unclear and seems to be tumour type-specific (25-30).

In our study, we have noted an up-regulation of *MT-III* mRNA expression in NSCLC as compared to NMLT, as determined by real-time PCR. Similar findings were noted in urinary bladder, breast and prostate cancer (24, 26, 27), whereas in gastric and esophageal cancer, MT-III expression was down-regulated due to promoter hypermethylation (28, 29). Interestingly, during analysis of IHC expression of MT-III in those tissues, we noted a down-regulation of nuclear MT-III expression which was accompanied by an increase in cytoplasmic MT-III expression. To date, no studies exist which may explain such an expression pattern switch of MT-III. In cancer tissues, MT-III expression has so far been reported to be regulated only by histone modifications or promoter hypermethylation (28-30).

Nuclear MT-III expression was also noted in some of the tested NSCLC samples. Nuclear localization seems to be in accordance with the molecular structure of MT-III, as it differs from the other MT family members by an unique C(6)PCP(9) motif in the β-domain and an acidic hexapaptide insert in the α-domain (35). This renders MT-III highly dynamic and it possesses higher nucleophilicity (36-38). Lack of nuclear MT-III expression was associated with a higher grade of tumour malignancy. Although, nuclear expression of MT-III yielded no prognostic significance and

no associations with patient age, gender and tumour size, presence of lymph node metastases and cancer cell proliferation. These results should be verified in a larger patient cohort to determine the exact role of MT-III in the carcinogenesis of the particular NSCLC subtypes. Of note, the differential nuclear expression of MT-III in NMLT and NSCLC may point to its role in the early steps of pathogenesis of NSCLC.

The statistical analysis revealed that low cytoplasmic MT-III expression was associated with higher primary tumour size, but no other associations with patients clinicopathological data were noted. In *in vitro* studies, MT-III expression suppressed the growth of prostate cancer cells (32). Similar findings were reported by Wei *et al*. who showed differential expression of MT-I/II and MT-III in prostate cancer (39). In their study, MT-I/II was significantly down-regulated, whereas MT-III expression was significantly up-regulated in benign prostate lesions (39). Interestingly, in our study, low cytoplasmic MT-III and low *MT-III* mRNA expression was associated with poor patient outcome only in the 61 patients for whom real-time PCR studies were performed. However, the results concerning cytoplasmic MT-III expression were not confirmed regarding patient survival for the whole study group, although the two groups did not differ significantly concerning patient treatment and management. The results of the IHC analysis performed for the whole patient cohort do not allow MT-III to be defined as a suppressor in the pathogenesis of NSCLC, yet it is clear that MT-III differs from the MT-I/II family in its functional properties (36-38). The study of Wei *et al*. and earlier studies regarding MT-I/II expression in NSCLC seem to confirm our observation, as MT-I/II were shown to be involved in the progression and chemoresistance of this cancer type (4, 12, 39-41). The clinical observations regarding the role of MT-I/II and MT-III expression in carcinogenesis may be justified by their interaction with metal-responsive transcription factor-1, which is highly zinc-dependent and regulates only MT-I/II expression (42).

In summary, we are the first, to our knowledge, to show nuclear MT-III expression in NMLT and NSCLC and, furthermore, that this expression decreases with tumour growing malignancy. This points to its potential role in the early steps of pathogenesis of NSCLC, but these results should be clarified in further studies. Moreover, using real-time PCR, we showed that *MT-III* mRNA expression may be regarded as a potential prognostic marker of NSCLC, but further studies are needed to validate this finding.

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