Prognostic Significance of TIMP-1 in Non-small Cell Lung Cancer

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Abstract. *Tissue inhibitor of metalloproteinases 1 (TIMP1) regulates not only extracellular matrix catabolism but the major effect in tumor tissue is promotion of cell growth and anti-apoptotic activity. The aim of our study was to evaluate plasma TIMP1 levels and tissue TIMP1 mRNA expression as prognostic markers in NSCLC patients. Patients and Methods: We studied a group of 108 patients with NSCLC who had undergone lung surgery. Estimation of TIMP1 mRNA was performed by quantitative polymerase chain reaction (qPCR) and estimation of plasma TIMP1 protein using enzyme-linked immunosorbent assay (ELISA). Results: There was shorter disease-free interval (DFI) for NSCLC patients at stage II, with a higher expression of TIMP1 mRNA in tumor tissue (p=0.0246). We recorded a relationship between tumor tissue TIMP1 mRNA expression and DFI in squamous cell carcinoma (SCC) (p=0.0117). Shorter overall survival was found in patients at stages IIIa+IIIb+IV, with a higher expression of TIMP1 mRNA (p=0.0389). We found differences in plasma TIMP1 levels between patients with SCC and those with adenocarcinoma (p=0.0491). Conclusion: A higher tissue level of TIMP1 mRNA is related to an adverse prognosis of patients. However, our results did not show any relation of TIMP1 protein in blood plasma to prognosis.*

Lung cancer is very commonly diagnosed cancer and its incidence in the European Union is 52.5/100 000 per year, the mortality 48.7/100 000 per year (1). Non-small cell lung cancer (NSCLC) accounts for 80% of all cases (2).

Although there are tumor markers routinely used for solid tumors, some of them having become gold standards (3), there is still an effort being made to search for new ones. Tissue inhibitor of metalloproteinases 1 (TIMP1) has been studied intensively over the last ten years and in colorectal cancer it seems to be promising as a prognostic marker in clinical use (4). A Danish-Australian endoscopy study group on colorectal cancer detection is trying to use TIMP1 as a detection marker (5).

There are many published results on the prognostic significance of *TIMP1* mRNA expression in tumor tissue and plasma TIMP1 protein levels in colorectal, breast and endometrial cancer (4, 6-11). However, results for the significance of TIMP1 for outcome in NSCLC are still contradictory (12-15). Therefore we decided to evaluate the use of TIMP1 as a prognostic marker in NSCLC.

TIMP1 belongs to a family of proteins that are natural inhibitors of the matrix metalloproteinases (MMPs), the enzymes involved in extracellular matrix maintenance and remodeling. The TIMP family consists of four members (TIMP1, TIMP2, TIMP3, TIMP4). *TIMP1* differs from the other members of the family in having a short exon 1 that is transcribed but not translated. The function of exon 1 appears to be related to the control of the specificity of tissue expression and may contain tissue-specific repressor elements (16).

TIMP1 regulation of cell processes is very complex and depends on the cell environment, often involving contradictory mechanisms which complicate the understanding of the separate steps. In general, TIMP1 effects can be classified as MMP-dependent (processes realized through interaction with MMPs) and MMP-independent (direct influence on cell growth, apoptosis and angiogenesis) (17).

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Table I. *The distribution of NSCLC patients according to TNM, stage, and histology.*

Group			T				N		M		Stage				Histology		
			1	2	3	4	0	≥1	0	1	I	II	III	IV	Adeno	SCC	Other
Men	64	n	14	39	8	3	43	21	64	0	36	13	15	0	20	41	3
Women	24	n	9	13	1	1	16	8	22	2	15	3	4	2	14	7	3
All	88	n	23	52	9	4	59	29	86	2	51	16	19	2	34	48	6
	100	%	26	59	10	5	67	33	98	2	58	18	22	2	39	54	7

Adeno: Adenocarcinoma; SCC: squamous cell carcinoma.

The MMP-dependent effects of TIMP not only include the regulation of extracellular matrix catabolism (remodelation of tissue) but also MMP-dependent TIMP regulation of cell signaling. Recent studies have begun to identify signaling pathways involved in the MMP-dependent growth-promoting effects of TIMP1. MMPs are implicated in cell growth through the activation of mitogenic factors. Another function of MMPs is the shedding of a variety of receptors, which is a mechanism of communication between cells and their enviroment. The inhibition of the shedding by TIMPs is also an important regulatory effect (17-19).

TIMP functions independent of MMPs include promotion of cell growth and antiapoptotic activity, but a growth inhibitory effect was also described. TIMP1 and TIMP2 promote cell division (20, 21) and growth stimulation by TIMPs is independent of their inhibition of MMP activity (21). The effects of TIMPs on cell growth may be mediated by their direct binding to cell surface receptors. Several distinct signaling pathways have been implicated in TIMP growth-promoting activity, including the mitogen-activated protein kinase (MAPK) and adenosine 3',5'-monophosphate (cAMP)-protein kinase A (PKA) pathways (22, 23). The growth-promoting activities of TIMP1 and TIMP2 may require the activation of Ras, albeit through distinct pathways suggesting independent cell receptor mechanisms (24).

TIMP1 protein was recently identified to be a tetraspanin-interacting cell surface protein. Tetraspanins consist of four transmembrane domains and are known to interact with integrins, which may regulate apoptosis through caspase-independent mechanisms. TIMP1 is able to inhibit apoptosis through binding to a CD63 receptor, a tetraspanin superfamily member. Subsequently, it was observed that TIMP1 enhances expression of survival and differentiation cytokines, such as interleukin-10 (IL-10), which also contribute to the anti-apoptotic effect (25). These findings are consistent with the apparent growth-stimulatory activity of TIMP1. Furthermore, there is evidence that TIMP1 activates the focal adhesion kinase (FAK)-phosphoinositol-3 kinase (PI3K) pathway to protect cells from intrinsic and extrinsic cell death (26).

In addition to the numerous reports describing growth-stimulating activity of TIMP1 and TIMP2, there are reports documenting growth-inhibitory activity for these TIMP family members. Again, this suggests that the specific effects of TIMPs on the fate of cells depend on the cell context and specific model system under study (27). A recent report shows that TIMP1 induces cell cycle arrest in G_1 in association with down regulation of cyclin D1, up-regulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, and hypophosphorylation of the retinoblastoma (Rb) protein (28).

Despite the contradictory effects, on cell growth and proliferation, it has been shown that expression of TIMP1 is higher in tumor tissue in comparison with corresponding normal tissue in many tumor types (29-31), including NSCLC (15, 32).

The aim of our study was to evaluate plasma TIMP1 levels (and tissue *TIMP1* mRNA expression) as a prognostic marker. We assessed the *TIMP1* mRNA levels in tumor tissue of NSCLC and preoperative plasma levels of TIMP1 protein in patients treated for NSCLC and correlated these levels with disease-free interval (DFI) and overall survival (OS).

Patients and Methods

Patients. We studied a group of 108 patients with NSCLC (median age 62.3 years, range 42.9-74.8 years, at the time of surgery) who had undergone lung surgery at the Department of Surgery, University Hospital Pilsen, from 2005-2007. We recieved informed consent from all research participants. The study was approved by the local Ethical Committee. Tissue samples for *TIMP1* mRNA expression estimation were available from 88 patients and the distribution according to histology and stage is shown in Table I. Plasma samples for TIMP1 protein level measurement were available from 90 patients. Postoperative adjuvant chemotherapy was indicated according to the current Guidelines of ASCO (American Society of Clinical Oncology) 2005-2007. Twenty-three patients with stage Ia and Ib did not undergo adjuvant chemotherapy. The others recieved chemotherapy based on platinum derivates (carboplatin, cisplatin) in different therapeutic schemes. No radiotherapy was applied. The median follow up was 33.7 months. For the staging verification, Kaplan-Meier DFI curves were generated for each stage (Figure 1). The exclusion criteria for

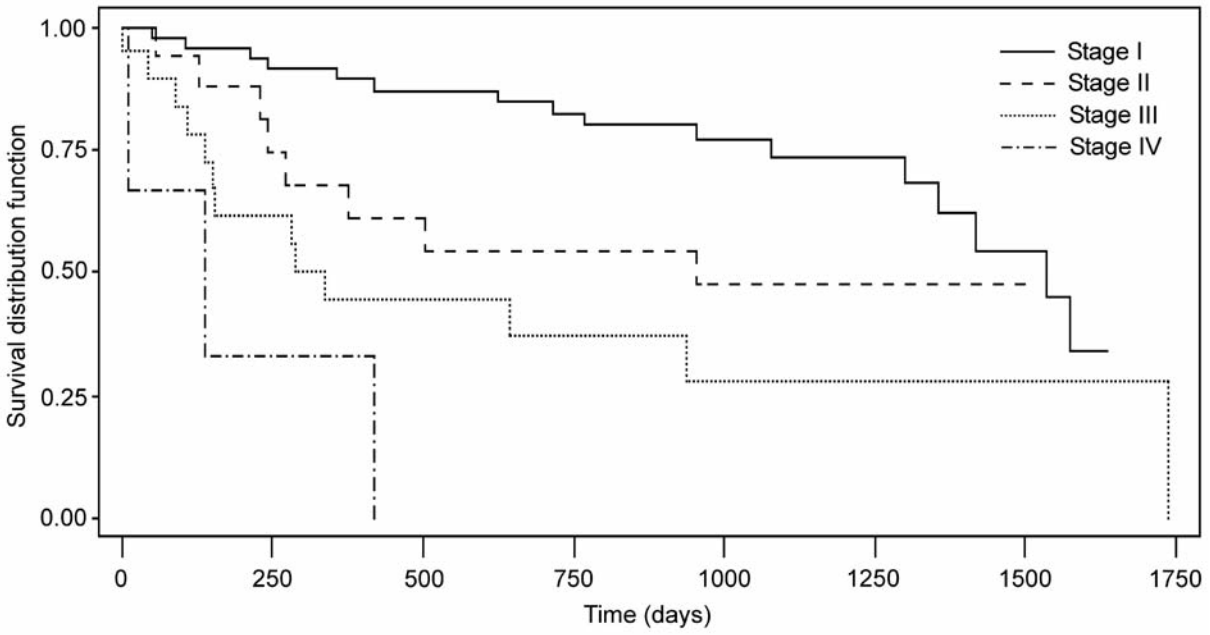

Figure 1. *Relation of stage of disease to Table II. The (DFI) in NSCLC patients. For the staging verification, Kaplan-Meier DFI curves were generated.*

entering the study were age over 75 years, other malignancy, high cardiopulmonary risk (*e.g.* chronical obstructive lung disease, condition after myocardial infarction).

Tissue samples. Eighty-eight paired (tumor and control) lung tissue samples were taken directly from the tumor tissue and from adjacent, histologically cancer-free lung tissue (normal lung tissue) from the same patient during surgery. These resected tissue samples were immediately frozen at −70˚C and stored at this temperature until use. All the samples were histologically verified.

Blood plasma samples. Blood plasma samples were collected preoperatively, one day before surgery. The peripheral blood of 90 patients was drawn using Vacuette® tubes (Greiner Bio-One, Austria) with EDTA as an anticoagulant. Plasma was separated by centrifugation at 1300 ×g and all specimens were immediately aliquoted and frozen. Samples were stored at −70˚C. No more than one freeze–thaw cycle was allowed before an analysis.

Quantitative estimation of mRNA using reverse-transcription (RT) real-time PCR. Total RNA was isolated from 100 mg of 88 pairs of tumor and control lung tissue using the FastRNAPro Green Kit (Q-BIOgene, Irvine, CA, USA). RT was performed from 3 μg of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and oligo d(T)21 as a primer. The sequence of primers used for *TIMP1* mRNA quantification was as follows: forward primer 5'-AGACCTACACTGTTGGCTGTGAG-3'; reverse primer 5'-GACTGGAAGCCCTTTTCAGAG-3' synthesized by GeneriBiotech (Hradec Kralove, Czech Republic). All samples were also assessed for the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The real-time PCR procedure and the sequence of *GAPDH* primers were described in our previous publication (29). The results are presented as both absolute and normalized values. Normalized values were calculated as the ratio of the number of copies of *TIMP1* to that of the housekeeping gene *GAPDH*.

Quantitative estimation of TIMP1 protein using ELISA. Plasma TIMP1 levels were measured by ELISA technology using commercial kits: Quantikine Human kits (R&D Systems, USA).

Statistical analysis. Statistical analysis was performed using SAS 8.02 software (SAS Institute Inc., Cary, NC, USA). The statistical results of comparing groups were calculated using a Wilcoxon two-sample test and Kruskal-Wallis test. For evaluation of prognostic significance (DFI, OS) Cox regression hazard model was used. In statistically significant results of the Cox model, 'optimal cut off' expression values for the examined markers were found. For the cut-off with the strongest *p*-value, Kaplan-Meier DFI curves were generated. *P*-values were considered statistically significant at the 0.05 level.

Results

We found statistically significant differences in *TIMP1* mRNA expression in NSCLC tissue samples between stages I, II, III and IV (p=0.0446). The highest expression was recorded during stage II (Figure 2).

We recorded a statistically significant relation between *TIMP1* mRNA NSCLC tumor tissue expression and DFI at stage II. There was shorter DFI in patients with a higher expression of *TIMP1* (Cox regression model, p=0.0246) For the cut-off with the strongest *p*-value (p<0.0001), Kaplan-Meier DFI curves were generated (Table II, Figure 3). We did

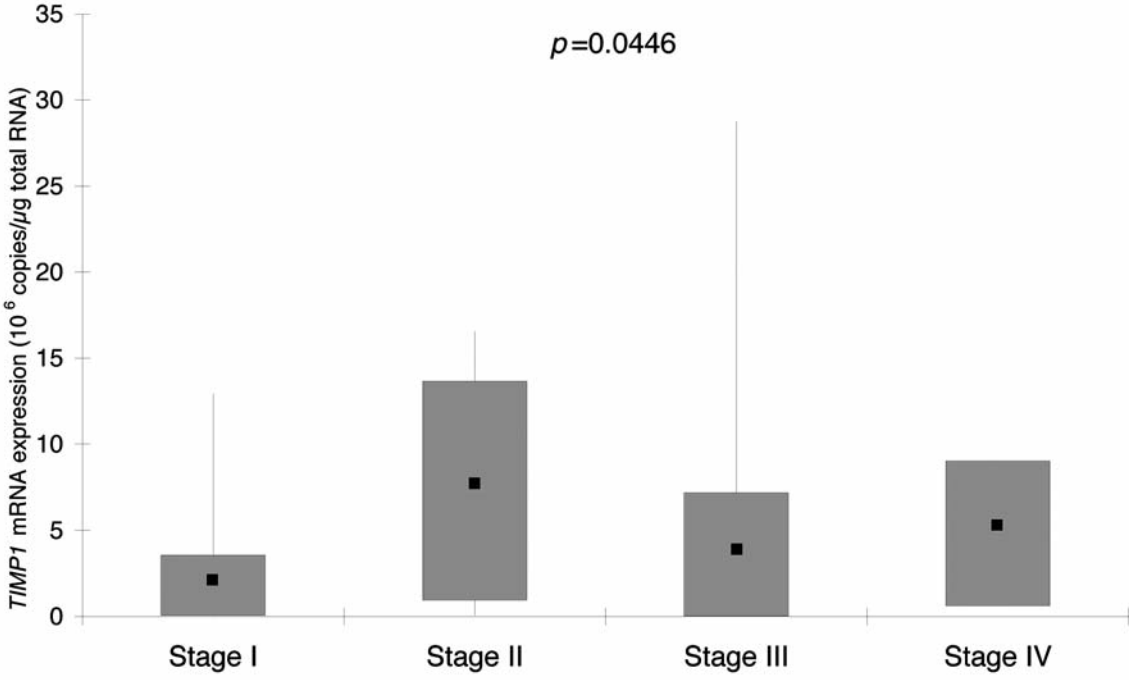

Figure 2. *Differences in tissue inhibitor of metalloproteinases 1 (TIMP1) mRNA expression in NSCLC tissue samples between stages I, II, III and IV (p=0.0446, Kruskal-Wallis test). The values shown in the figure are minimum (5%) and maximum (95%) (line), lower and upper quartile (rectangle) and median (small square).*

not find any connection between *TIMP1* mRNA expression and DFI in the whole patient group or in subgroups of patients with disease stage I or stages IIIa + IIIb + IV.

We also found a shorter OS in patients at stages IIIa + IIIb + IV associated with a higher expression of *TIMP1* mRNA (Cox regression model, p=0.0389). We did not record any relation between *TIMP1* mRNA expression and OS in the whole patient group or in the subgroups of patients with stage I and stage II disease.

The data were also evaluated in relation to histological subtypes of NSCLC. We did not find differences in *TIMP1* mRNA expression between tissue samples of adenocarcinoma and squamous cell carcinoma (SCC).

We did find there to be a statistically significant relation between tumor tissue *TIMP1* mRNA expression and DFI in SCC (Cox regression model, p=0.0117). For the cut-off with the strongest p-value (p<0.0001), Kaplan-Meier DFI curves were generated (Table II, Figure 4).

One of the purposes our study was to evaluate plasma TIMP1 levels for clinical use. We found statistically significant differences in plasma TIMP1 levels between SCC and adenocarcinoma NSCLC patients (p=0.0491). Differences in TIMP1 between stage I, II, II and IV NSCLC patients were statistically insignificant (p=0.0849) (Figure 5). We did not record any relationship between plasma TIMP1 levels and prognosis (DFI, OS) in any studied group.

No statistically significant correlation between *TIMP1* mRNA expression and plasma TIMP1 levels was recorded.

Discussion

The purpose of our study was the evaluation of plasma TIMP1 levels (and tissue *TIMP1* mRNA expression) as a prognostic marker with possible clinical use. We found differences in *TIMP1* mRNA expression in NSCLC tissue samples between stages I, II, III and IV. The highest level of expression was observed at stage II. Only at this stage was a relation between NSCLC tumor tissue expression of *TIMP1* mRNA and DFI recorded. Simi *et al*. described a correlation of *TIMP1* mRNA expression with the disease stage (15). On the contrary, Aljada *et al*. and Iniesta *et al*. recorded no association of the level of TIMP1 with stage (33, 34) but they were only dealing with TIMP1 protein in tissue.

Furthermore, we found a relation between *TIMP1* mRNA expression in NSCLC tumor tissue and OS at stages IIIa + IIIb + IV. It is not surprising that we recorded a relation between *TIMP1* expression and DFI at stage II but none for OS because in cases of recurrence at stage II, there are possibilities for the treatment of this unfavourable course of the disease.

Gouyer *et al*. and Fong *et al*. (12, 35) described higher *TIMP1* mRNA levels as beeing associated with adverse prognosis. According to Gouyer *et al*. (12) overexpression of

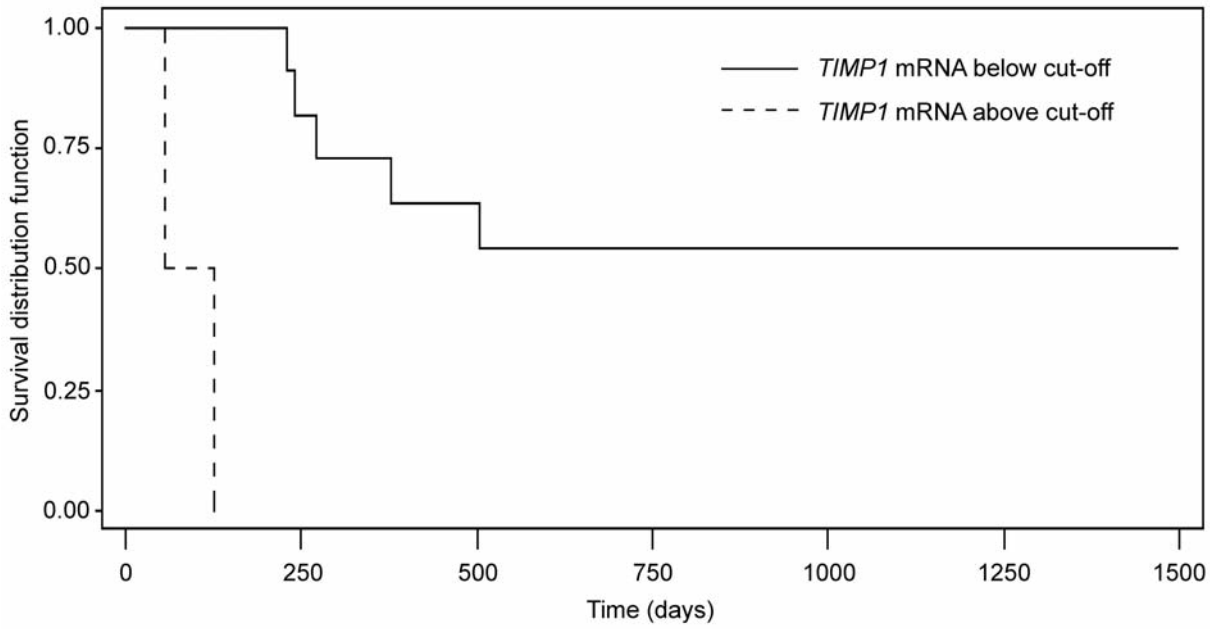

Figure 3. *Relation of tissue inhibitor of metalloproteinases 1 (TIMP1) mRNA expression to disease-free interval (DFI) in stage II NSCLC patients (Kaplan-Meier DFI curve). There is a significant difference in DFI between patients with tissue expression of TIMP1 below and above the cut-off value (p<0.0001).*

Table II. *The distribution of patients in Kaplan-Meier disease-free interval (DFI) curves according to tissue inhibitor of metalloproteinases 1 (TIMP1) mRNA tumor tissue expression with the strongest p-value.*

Group of patients	Patients above cut-off		Optimal cut-off (copy number)	Patients below cut-off		Log-rank *p*-value	Wilcoxon *p*-value
	N	Median DFI (days)		N	Median DFI (days)		
NSCLC stage II	2	91	16290000	14	503	<0.0001	<0.0001
SCC	4	107	15100000	43	1079	<0.0001	<0.0001

SCC: Squamous cell carcinoma; DFI: disease-free interval.

TIMP1 is an independent predictor of worse survival. Aljada *et al*. (33) used immunohistochemistry to demonstrate a longer survival time in patients with low tumor tissue TIMP1 protein expression. But there are also studies that do not support these results. Simi *et al*. (15) did not observe differences in outcome between patients with low and high tissue level of *TIMP1* mRNA (p=0.06). Iniesta *et al*. published quite opposite findings using protein estimation with the ELISA method. Disease-free survival time was higher for patients whose tumors showed increased levels of TIMP1 (34). Despite some inconsistency in results, it seems that higher tissue levels of TIMP1 are related to an adverse prognosis of patients.

The data from histological subtypes of NSCLC was also evaluated. We did not find any differences in *TIMP1* mRNA expression between tissue samples of adenocarcinoma and SCC. However, we found tumor tissue *TIMP1* mRNA expression to be related to DFI in SCC. This result may reflect a difference in the pathogenesis of SCC from adenocarcinoma. Despite the fact that recorded mRNA levels of *TIMP1* were similar in these histological subtypes the effect of TIMP1 could differ.

As discussed below, promising prognostic results of TIMP1 in tumor tissue are not coupled with results obtained for TIMP1 protein levels in peripheral blood plasma. We are fully aware that the route from mRNA in tissue to protein in blood is a multistep process and is far from linear. Nevertheless, we expected that results obtained for plasma TIMP1 protein levels would reflect the relation of tissue TIMP1 to prognosis, as was shown in studies dealing with different cancers (*e.g*. colorectal cancer) (3). Unfortunately, in NSCLC, this was not confirmed. Differences in TIMP1 between stage I, II, II and IV NSCLC patients were observed to be insignificant. We did not record

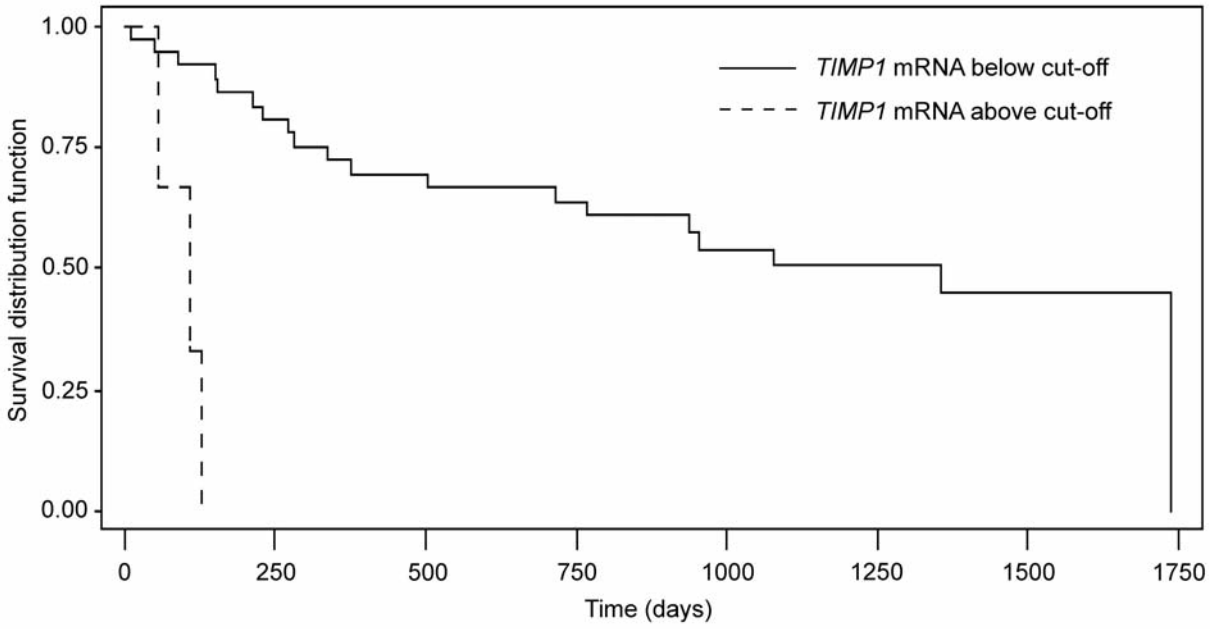

Figure 4. *Relation of tissue inhibitor of metalloproteinases 1 (TIMP1) mRNA expression to disease-free interval (DFI) in squamous cell carcinoma patients (Kaplan-Meier DFI curve). There is significant difference in DFI between patients with tissue expression of TIMP1 below and above cut off value (p<0.0001).*

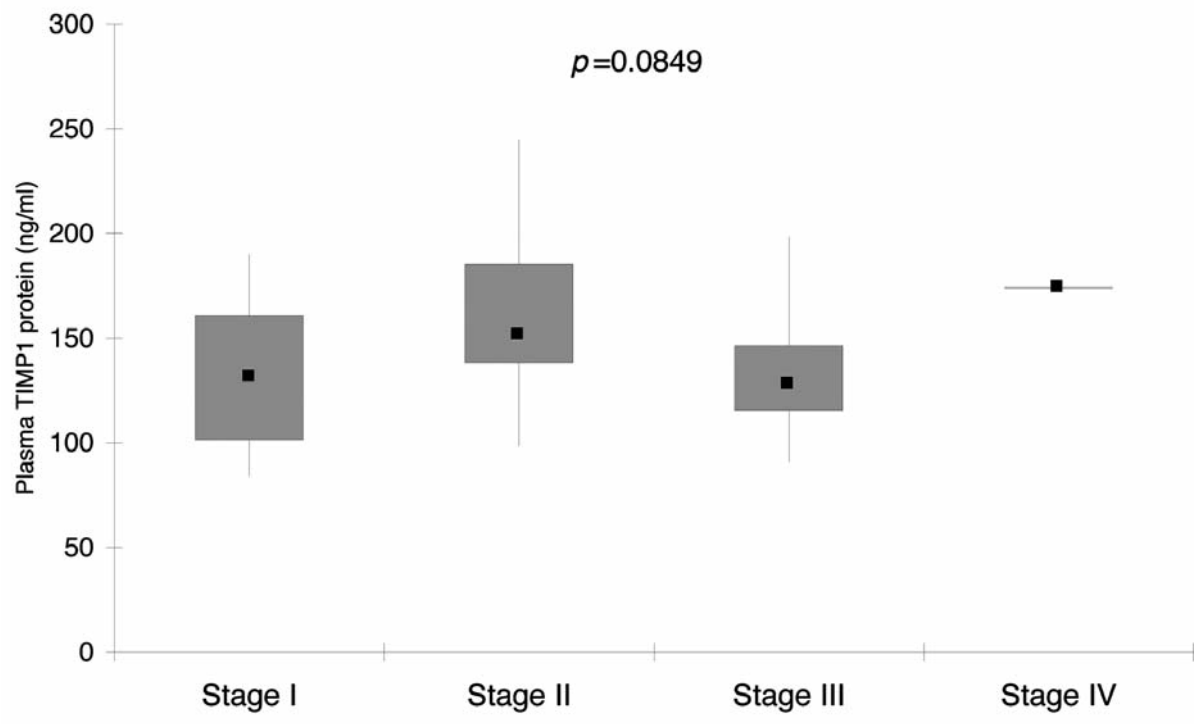

Figure 5. *Differences in plasma level of tissue inhibitor of metalloproteinases 1 (TIMP1) protein in NSCLC tissue samples between stages I, II, III and IV (p=0.0849, Kruskal-Wallis test). The values shown in the figure are minimum (5%) and maximum (95%) (line), lower and upper quartile (rectangle) and median (small square).*

any relationship between plasma TIMP1 levels and prognosis (DFI, OS) in any studied group. In addition, no statistically significant correlation between *TIMP1* mRNA expression and plasma TIMP1 levels was recorded either. In 2000, Ylisirnió *et al*. (36) published a study showing that a high TIMP1 level in blood is an indicator of poor prognosis, especially in SCC and in NSCLC patients with stage III disease: 66% and 70% respectively of the patients with low serum TIMPl levels survived for more than one year, while only 25% and 20%, respectively, of patients with high serum levels for TIMP1 protein survived after the same period of time. In 2004, Suemitsu *et al*. (37) stated that patients with high TIMP1 values had significantly shorter disease-free survival (p=0.0479). So far we have only found these two works (Ylisirnió *et al*. and Suemitsu *et al*.) investigating plasma TIMP1 levels in NSCLC patients. In contrast, there have been many studies investigating tumor tissue TIMP1 expression in relation to DFI and OS. It is possible that negative results concerning plasma TIMP1 levels were not published.

There are many factors which influence plasma levels of tumor markers, including renal excretion, degradation of molecules, and other factors reflected in the biological half-life. However, there are many tumor markers valuable in clinical use (3). Even solid cancer is not an isolated disease and there is at least a reaction of adjacent tissue in advanced stages affecting the whole organism. In addition, these processes depend on the type and subtype of cancer. Among consequences of such processes, we might include our finding of differences in plasma TIMP1 levels between SCC and adenocarcinoma NSCLC patients while no difference was observed in tumor tissue measurements of *TIMP1* mRNA.

Despite the promising positive results and also the light shed on the functions and molecular pathways of TIMP1, the value of assessments of TIMP1 for the prediction of survival in NSCLC patients is still uncertain for routine clinical use. The reason for this could lie in the multiple and often contradictory effects of TIMP1, as was discussed above.

On the basis of our results, the plasma level of TIMP1 protein in NSCLC patients as a predictor of survival does not seem to be significant.

Conclusion

We found there to be a relationship between *TIMP1* mRNA expression in NSCLC tumor tissue and prognosis. A higher tissue level of TIMP1 is related to an adverse prognosis of patients. However, our results did not show any relation of TIMP1 protein in blood plasma of patients with NSCLC to prognosis.

Conflict of Interest Statement

The Authors report no conflict of interests.

Acknowledgements

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