Association of TIMP3 expression with vessel density, macrophage infiltration and prognosis in human malignant melanoma

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KEYWORDS
TIMP3; Melanoma; Angiogenesis; Macrophage infiltration; Promoter methylation

Abstract Aims: Several anti-tumour properties have been ascribed to the tissue inhibitor of matrix metalloproteinases-3 (TIMP3) gene, including inhibition of neovascularisation in tumour xenografts. Reduced protein expression has been linked to promoter hypermethylation and allelic loss of heterozygosity in various human malignancies. In melanoma-positive lymph nodes from patients, we evaluated the association between TIMP3 expression, vessel density, macrophage infiltration and potential correlations with disease-free survival (DFS) and overall survival (OS).

Patients and methods: TIMP3 expression was analysed by immunohistochemistry (IHC) in melanoma lymph node biopsies of stage III melanoma patients (n = 43). Blood vessel density and macrophage infiltration were quantitatively assessed and correlation with TIMP3 expression was investigated. Methylation status of the gene promoter was determined using methylation-specific polymerase chain reaction (MSP). Protein expression and promoter methylation status were investigated for associations with DFS and OS.
Results: Reduced expression of TIMP3, as determined by IHC, was observed in 74% of the cases (32 in 43). A significant inverse correlation was observed between TIMP3 expression and vessel density (p = 0.031). Correlation between TIMP3 expression and macrophage infiltration was not statistically significant (p = 0.369). MSP analysis revealed methylation of the gene promoter in 18% (7 in 38) of the analysed cases. No differences in OS and DFS were observed between cases with high and low TIMP3 expression. Gene promoter methylation was significantly associated with both poor 5-year DFS (p = 0.024) and OS (p = 0.034).

Conclusions: Our data indicate that TIMP3 is a dominant negative regulator of angiogenesis in cutaneous melanoma and gene silencing by promoter methylation is associated with poor outcome.

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1. Introduction

Extracellular matrix remodelling is crucial to neovascularation initiation and development and is orchestrated by an interplay between matrix metalloproteinases (MMPs) and their endogenous inhibitors, such as tissue inhibitor of matrix metalloproteinases (TIMPs) [1]. The TIMP3 gene located at 22q12.3 codes for a 24-kDa glycoprotein with broad inhibitory effects. TIMP3 has been described as a tumour suppressor in a number of human malignancies, including breast [2], colorectal [3] and prostate cancers [4], with decreased expression correlating with poor prognosis and outcome. Reduced TIMP3 expression has been attributed to aberrant promoter hypermethylation [5–8] and allelic loss of heterozygosity [9,10] in several tumour types. Concurrent with its role as a tumour suppressor, TIMP3 has been documented to exert anti-tumour effects via both MMP-dependent and -independent pathways. Overexpression of TIMP3 has been reported to induce apoptosis [11,12] and inhibit tumour growth [13–15] and metastasis [16] in several tumour cell lines. Additionally, TIMP3 is a dominant negative regulator of angiogenesis, and has been shown to block the binding of vascular endothelial growth factor (VEGF) to its receptor [17] and suppress neovascularisation in several tumour xenografts [18,19].

Human malignant melanoma is an aggressive disease, with the highest increase in incidence in the western world of all malignancies, and accounts for the majority of skin cancer-related deaths [20,21]. Melanomas are highly vascular tumours and the angiogenic element is crucial for disease progression and subsequent metastatic dissemination [22,23]. We have previously shown that TIMP3 inhibits directionally persistent endothelial cell migration and impairs angiogenesis and macrophage infiltration in melanomas in a xenograft model [24]. However, the clinical association between TIMP3 expression and angiogenesis in melanoma is not known. In the present study, we evaluated TIMP3 expression and correlation to mean vessel density and macrophage infiltration in a cohort of melanoma-positive lymph node biopsies from stage III melanoma patients using immunohistochemistry (IHC). We also assessed the methylation status of the TIMP3 gene promoter CpG island using methylation-specific polymerase chain reaction (MSP) analysis. Finally, the association of protein expression and gene promoter methylation to clinicopathological variables were analysed.

2. Materials and methods

2.1. Patient samples

Forty-three cases of stage III melanoma lymph node biopsies were obtained from the pathology archives of the Erasmus Medical Center, with the approval of the research ethics committee. The tumour specimens used in this study were obtained between 2008 and 2009 and verified by pathological diagnosis. Tumour staging was based on the American Joint Committee on Cancer TNM staging system [25]. All patient data for the present study were collected from medical records according to local Institutional Review Committee guidelines and national legislation.

2.2. Immunohistochemistry

Five-micrometer serial tissue sections were cut from the formalin-fixed, paraffin-embedded (FFPE) melanoma blocks using a Microm HM325 microtome (Thermo Fisher Scientific, Waltham, USA) and mounted on Objectglas Superfrost Plus slides (VWR). One tissue section was used for haematoxylin and eosin (H&E) staining and tumour areas were demarcated. Serial tissue sections were immunohistochemically stained for TIMP3 (rabbit polyclonal, Abcam ab2169, 1:100), CD31 (mouse monoclonal, Abcam, clone JC/7A, ab9498, 1:50) and CD68K (mouse monoclonal; DAKO, clone KP1, M0814, 1:1600) using the Ventana Benchmark Ultra Stainer (Ventana Medical Systems, Tucson, AZ, USA). The staining procedure included pretreatment with CC1 (cell conditioner 1, pH 8) for
64 min followed by primary antibody incubations at 36 °C for 32 min. Stainings for TIMP3, CD31 and CD68K were visualised using the Ventana ultraView Universal Alkaline Phosphatase Red Detection Kit (760-501). For all antibodies, the Ventana Amplification Kit (760-080) was used. Nuclei were counterstained with haematoxylin. Human placenta tissue was used as a positive control for TIMP3 staining.

2.3. TIMP3 staining evaluation

Evaluation of TIMP3 immunohistochemical staining was performed by two experienced pathologists (S.K. and V.N.H.) who were blinded towards patient characteristics, patient outcome, and results of promoter methylation status. TIMP3 staining was graded semi-quantitatively as previously described [26], with modifications. Staining intensity was graded as 1 (no staining), 2 (weak stain), 3 (clear stain), or 4 (strong stain). To account for intratumoral heterogeneity, the percentage of cells exhibiting the various intensities was recorded. The intensity and abundance (expressed as a fraction) scores were multiplied to obtain a total immunoreactivity score which ranged from a maximum total score of 4 (intensity score 4, abundance 100%) to a minimum total score of 1 (intensity score 1, abundance 100%). For correlation and survival analyses, samples with an immunoreactivity score of >2.4 were classified as high and ≤2.4 were classified as low. Differences in scoring were evaluated by Kappa test and discrepant scores were resolved by consensus.

2.4. CD31 and CD68K density evaluation

To quantitatively determine vessel density and macrophage infiltration, whole-slide scanning was performed on serial sections immunohistochemically stained for CD31 and CD68K. Stained slides were scanned using the Hamamatsu NDP slide scanner (Hamamatsu Nanozoomer 2.0HT) and digitally converted into virtual slides [27]. Depending on the size of the tumour tissue, 6–12 tumour areas were randomly annotated at ×20 magnification using the NDP View analysis platform. Images of annotated areas were exported as .jpg and analysed with a self-written plugin for the Image J software to obtain percentages of blood vessel density and macrophage infiltration.

2.5. Methylation-specific polymerase chain reaction

Two to three 10-μm FFPE tissue sections were macrodissected with reference to the H&E-stained sections to include areas with >80% tumour cells. Genomic DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen Benelux B.V., Venlo, NL) and quantified using the Qubit® 2.0 fluorometer (Life Technologies, CA, USA). Five hundred nanograms of DNA was bisulphite modified using the EpiTect Bisulfite Kit (Qiagen). Fifty nanograms of modified DNA was amplified by polymerase chain reaction (PCR) in 24 μL reactions, using AmpliTaq Gold® DNA Polymerase (Life Technologies) and 0.25 μM of each primer per reaction. Primer sequences for TIMP3 MSP have been described before [5]. Primers specific for unmethylated DNA were 5'-TTTTGTTTTGTTATTTTTGTTTTTTGTTTT-3' (sense) and 5'-CCCCAAAAACCCCCACCTCA-3' (antisense), yielding a 122-bp product. Primers specific for methylated DNA were 5'-CGTTTCGTTATTTTTGTTTTCGGTTC-3' (sense) and 5'-CGGAAAAACCACGCCCTCG-3' (antisense), yielding a 116-bp product. The PCR reactions were performed with an annealing temperature of 59 °C. Ten microliters of each PCR sample was resolved by electrophoresis on 2% agarose gels. Epitect Control DNA Set (Qiagen) was used as controls for MSP.

2.6. Statistical analysis

Statistics were performed with Statistical Package for the Social Sciences version 22.0 (SPSS Inc., Chicago, IL). Disease-free survival (DFS) was defined as the number of months from the date of surgery to the date of first recurrence (locally or distant), to death without relapse or to last follow-up. Overall survival (OS) was defined as the number of months from the date of surgery to death of any cause as registered by the social security death index or to last follow-up. Univariate analysis of the clinicopathologic characteristics was performed using χ² test for nominal variables, Kendall tau for ordinal variables and Mann–Whitney U test for non-parametric continuous variables. Survival curves were plotted using the Kaplan–Meier method, and comparison of survival times was performed with the log-rank test. All tests were two sided, and p < 0.05 was considered statistically significant.

3. Results

3.1. Patient population

Baseline characteristics of the patient population are provided in Table 1. All 43 patients had stage III disease at the time of surgery. Surgery consisted of a sentinel node biopsy, a diagnostic lymph node biopsy, or a therapeutic lymph node dissection. Only histologically confirmed metastatic lymph node samples were used for this study.

Median age was 54 years (interquartile range [IQR] 46–66 years), and median follow-up was 29 months (IQR 11–63 months). Median Breslow thickness was
3.00 mm (IQR 1.80–7.00 mm). Most primary melanomas were situated on the lower extremity (n = 19, 44%), 14 melanomas (33%) were situated at the trunk, 8 melanomas (19%) were situated in the head and neck area and there was 1 melanoma (2%) of unknown primary origin.

3.2. Immunohistochemical analysis of TIMP3 protein expression

TIMP3 was graded semiquantitatively and staining intensity was scored on a scale of 1–4 as illustrated in Fig. 1a. TIMP3 protein was expressed heterogeneously between and within the tumour samples (Fig. 1b) and expression was predominantly cytoplasmic and membranous. The median TIMP3 score was 2.0 (IQR 1.9–2.5). Based on the total immunoreactivity scoring cut-off criteria of 2.4, 11 cases (26%) were classified as high and 32 cases (74%) were classified as low.

3.3. Correlation between TIMP3 expression, vessel density and macrophage infiltration

Vessel density and presence of infiltrating macrophages were visualised by staining with CD31 (Fig. 2a) and CD68K (Fig. 2b) antibodies, respectively. The median CD31 score was 2.5 (IQR 1.4–3.6) and CD68K score was 2.8 (IQR 1.7–4.9). Tumours with a high total TIMP3 immunoreactivity score had a lower vessel density (median 1.5, IQR 1.2–2.2) as compared to cases with a low TIMP3 score (median 2.7, IQR 1.7–4.0). CD68K abundance did not differ between TIMP3 high (median 2.8, IQR 1.7–3.4) and TIMP3 low (median 2.9, IQR 1.7–5.3) samples. Associations between these parameters were evaluated using Spearman correlation analysis (Fig. 2c, d, e and Table 2). We observed a significant inverse correlation between TIMP3 expression and vessel density (correlation coefficient −0.330, p = 0.031). A positive correlation was observed between vessel density and macrophage infiltration (correlation coefficient 0.438, p = 0.003). However, no significant association was observed between TIMP3 expression and macrophage infiltration (correlation coefficient −0.140, p = 0.369).

3.4. TIMP3 promoter methylation analysis

The frequency of TIMP3 promoter methylation in the tumour samples was detected by MSP. Adequate DNA was available for 38 cases and the methylation status of TIMP3 promoter was evaluated (Fig. 3 a, b). TIMP3 promoter methylation was detected in 18% (n = 7 in 38) of the cases.

Table 1
Baseline characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All (n = 43)</th>
<th>TIMP3 high (n = 11)</th>
<th>TIMP3 low (n = 32)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 (50)</td>
<td>4 (36)</td>
<td>18 (56)</td>
<td>0.255</td>
</tr>
<tr>
<td>Female</td>
<td>21 (50)</td>
<td>7 (64)</td>
<td>14 (44)</td>
<td></td>
</tr>
<tr>
<td>Breslow thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (≤1 mm)</td>
<td>1 (2)</td>
<td>0</td>
<td>1 (3)</td>
<td>0.244</td>
</tr>
<tr>
<td>T2 (1.1–2 mm)</td>
<td>14 (33)</td>
<td>3 (27)</td>
<td>11 (34)</td>
<td></td>
</tr>
<tr>
<td>T3 (2.1–4 mm)</td>
<td>12 (28)</td>
<td>2 (18)</td>
<td>10 (31)</td>
<td></td>
</tr>
<tr>
<td>T4 (&gt;4 mm)</td>
<td>11 (26)</td>
<td>4 (36)</td>
<td>7 (22)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>5 (11)</td>
<td>2 (18)</td>
<td>3 (9)</td>
<td></td>
</tr>
<tr>
<td>Ulceration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>13 (30)</td>
<td>3 (27)</td>
<td>10 (31)</td>
<td>0.531</td>
</tr>
<tr>
<td>Absent</td>
<td>16 (37)</td>
<td>5 (46)</td>
<td>11 (34)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>14 (33)</td>
<td>3 (27)</td>
<td>11 (34)</td>
<td></td>
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<tr>
<td>Tumour burden</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopic</td>
<td>5 (12)</td>
<td>1 (9)</td>
<td>4 (13)</td>
<td>0.761</td>
</tr>
<tr>
<td>Macroscopic</td>
<td>38 (88)</td>
<td>10 (91)</td>
<td>28 (87)</td>
<td></td>
</tr>
<tr>
<td>Number of excised lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (median, IQR)</td>
<td>12 (5–17)</td>
<td>9 (2–16)</td>
<td>14 (5–18)</td>
<td>0.288</td>
</tr>
<tr>
<td>Positive (median, IQR)</td>
<td>2 (1–5)</td>
<td>2 (1–5)</td>
<td>2 (1–5)</td>
<td>0.501</td>
</tr>
<tr>
<td>Number of positive lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16 (37)</td>
<td>5 (46)</td>
<td>11 (34)</td>
<td>0.410</td>
</tr>
<tr>
<td>2–3</td>
<td>11 (26)</td>
<td>3 (27)</td>
<td>8 (25)</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>16 (37)</td>
<td>3 (27)</td>
<td>13 (41)</td>
<td></td>
</tr>
<tr>
<td>LNR (median, IQR)</td>
<td>0.33 (0.14–0.67)</td>
<td>0.38 (0.10–1.00)</td>
<td>0.33 (0.14–0.65)</td>
<td>0.555</td>
</tr>
<tr>
<td>Extranodal growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29 (67)</td>
<td>9 (82)</td>
<td>20 (63)</td>
<td>0.238</td>
</tr>
<tr>
<td>Yes</td>
<td>14 (33)</td>
<td>2 (18)</td>
<td>12 (37)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as n (%) unless otherwise specified.
Abbreviations: IQR, interquartile range; LNR, lymph node ratio; TIMP3, tissue inhibitor of matrix metalloproteinases-3.
*Significant at p < 0.05 level, calculated using χ² test, Mann–Whitney U test.
3.5. Clinical correlations

Lymph node positive melanoma (stage III) is extremely heterogeneous and 5-year survival rates can vary depending on the three most important prognostic factors for stage III melanoma which are (i) microscopic versus macroscopic (palpable) nodal involvement, (ii) the number of involved nodes (one versus two to three

Fig. 1. Immunohistochemical staining and scoring of tissue inhibitor of matrix metalloproteinases-3 (TIMP3) expression. (a) Melanoma lymph node biopsies were immunohistochemically stained with polyclonal TIMP3 antibody. Staining intensity was graded semiquantitatively on a scale of 1−4, where score 4 represents a strong stain, score 3 represents a clear stain, score 2 represents a weak stain and score 1 represents no staining. Representative images of the intensity scores are shown. Magnification ×40. (b) Overview (×1.25) and magnification (×20) of a tumour showing heterogeneous expression of TIMP3. L, low TIMP3 expression and H, high TIMP3 expression. Scale bars, 200 μm.

Fig. 2. Analysis of blood vessel density and macrophage infiltration. Quantitative analysis of blood vessel density and macrophage infiltration was performed using the Nanozoomer digital pathology platform and Image J as specified in the Materials and methods section. (a) Representative annotated image of CD31 staining and quantification analysis. (b) Representative annotated image of CD68K staining and quantification analysis. Scale bars, 200 μm. Correlation analysis of (c) tissue inhibitor of matrix metalloproteinases-3 (TIMP3) expression and vessel density, (d) TIMP3 expression and macrophage infiltration, and (e) vessel density and macrophage infiltration.
versus greater than or equal to four positive nodes), and (iii) absence versus presence of ulceration of the primary tumour [25,28]. For microscopic disease only, the absence versus presence of ulceration of the primary tumour [25,28]. For microscopic disease only, the absence versus presence of ulceration of the primary tumour, the correlation analysis was performed on DNA isolated from the tumour samples to evaluate promoter methylation status of TIMP3 and polymerase chain reaction products were resolved on 2% agarose gels. M, EZ Load 100-bp Molecular Ruler; u, unmethylation amplification fragment (116 bp). Bisulfite converted unmethylated (C1) and methylated (C2) human control DNA were used as controls for MSP.

Fig. 3. Methylation-specific polymerase chain reaction (MSP). (a) Overview images (×2.5) of tumours with low and high tissue inhibitor of matrix metalloproteinases-3 (TIMP3) expression. (b) MSP was performed on DNA isolated from the tumour samples to evaluate promoter methylation status of TIMP3 and polymerase chain reaction products were resolved on 2% agarose gels. M, EZ Load 100-bp Molecular Ruler; u, unmethylation amplification fragment (122 bp); and m, methylation amplification fragment (116 bp). Bisulfite converted unmethylated (C1) and methylated (C2) human control DNA were used as controls for MSP.

### Table 2
Correlation analysis.

<table>
<thead>
<tr>
<th>TIMP3 score</th>
<th>CD31 (%)</th>
<th>CD68K (%)</th>
<th>TIMP3 score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient</td>
<td>-0.330*</td>
<td>-0.140</td>
<td>1.000</td>
</tr>
<tr>
<td>p Value</td>
<td>0.031</td>
<td>0.369</td>
<td></td>
</tr>
<tr>
<td>TIMP3 (%)</td>
<td>1.000</td>
<td>0.438*</td>
<td>-0.330*</td>
</tr>
<tr>
<td>p Value</td>
<td>0.003</td>
<td>0.0003</td>
<td>0.369</td>
</tr>
<tr>
<td>CD68K (%)</td>
<td>0.438*</td>
<td>1.000</td>
<td>-0.140</td>
</tr>
<tr>
<td>p Value</td>
<td>0.330*</td>
<td>0.140</td>
<td></td>
</tr>
</tbody>
</table>

* Correlation is significant at *p* < 0.05 level (two tailed), Statistical test: nonparametric, Spearman rho test.

4. Discussion

Malignant melanomas arise from the neoplastic transformation of melanocytes, and disease progression and metastatic dissemination rely on the angiogenic cascade. Several lines of evidence indicate the importance of angiogenesis in melanoma development, including increased expression of proangiogenic modulators [32] and correlation of angiogenesis to aggressiveness and poor clinical outcome [33]. In addition to tumour-secreted angiogenic factors, the tumour stromal environment facilitates tumour growth by functioning as a reservoir of angiogenic ligands. In particular, inflammatory cells such as macrophages have been shown to contribute to tumour angiogenesis and have been linked to disease progression and poor prognosis in several tumour types [34], including melanomas [35–37]. Despite the fundamental role of angiogenesis in melanoma pathophysiology, strategies to combat this attribute have remained relatively unsuccessful with marginal clinical benefits (reviewed in [23,38]). Thus, the further identification of relevant molecular targets mediating this vital cascade is essential for the development of successful therapies. This may be of particular importance to prevent progression of micrometastatic residual disease in the adjuvant setting, as would be the case for stage III patients after lymph node dissection.

TIMP3, a member of the TIMP family of endogenous MMP inhibitors, is known to exert tumour-suppressive functions in several human malignancies. These effects are mediated via both MMP-dependent and -independent mechanisms and include inhibition of tumour growth, angiogenesis and invasion. We extended our observations of the inhibitory effect of TIMP3 on the angiogenic cascade in melanoma xenografts [24] to a cohort of lymph node biopsies from stage III melanoma patients. We first evaluated TIMP3 protein expression in our dataset using IHC. Intratumoural heterogeneity in TIMP3 expression was observed in the biopsies and this was taken into account for the scoring system. Based on our cut-off criteria, the majority of the lymph node biopsies scored low (74%) on the total immunoreactivity score. These results are in accordance with the reduced expression of TIMP3 reported in other human malignancies with high-grade tumour phenotype [9,26,39] and suggest that the loss of TIMP3 expression is an important event in melanoma progression and pathogenesis.

Several reports indicate that TIMP3 functions as a dominant negative regulator of angiogenesis and these effects are thought to be mediated by the ability to inhibit Vascular endothelial growth factor (VEGF)-Vascular endothelial growth factor receptor 2 (VEGFR2) signalling [17]. We and others have shown that TIMP3 inhibits endothelial cell migration and tube formation, and enforced expression or viral delivery...
reduces neovascularisation and macrophage infiltration in various tumour xenografts [13,14,17–19,24,40]. We analysed blood vessel density in our patient set and observed a significant inverse correlation with TIMP3 expression. The presence of infiltrating macrophages has previously been described to correlate with blood vessel density in melanomas [35,36], and we observed a significant correlation in our cohort. As TIMP3 has been shown to inversely correlate with infiltrating macrophages in xenograft models [18,24], we also explored the possible correlations between these parameters. In our dataset, we did not observe a significant correlation between tumour infiltrating macrophages and TIMP3 expression. Of note, in this study, we used the pan-monocyte/macrophage marker CD68K which does not discriminate between pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages. It might be of interest to evaluate potential associations between infiltrating macrophage subsets and TIMP3 expression.

As loss of expression of TIMP3 has been attributed to gene promoter hypermethylation in several tumour types, we evaluated the methylation status of the TIMP3 promoter in our dataset. Our results show that methylation-associated silencing of TIMP3 is a low-frequency event in melanomas (7 in 38, 18%). These results are in accordance with a previous study where promoter methylation gene profiling analysis of melanoma cells

Fig. 4. Tissue inhibitor of matrix metalloproteinases-3 (TIMP3) expression and promoter methylation in the prognosis of malignant melanoma. Kaplan–Meier curves of overall survival and disease-free survival of patients with tumours expressing (a, b) high or low TIMP3 and (c, d) methylated or unmethylated gene promoter.

Nr at risk 0 12 24 36 48 60
TIMP low 32 22 15 11 9 7
TIMP high 11 9 8 6 4 4

P = 0.02

Nr at risk 0 12 24 36 48 60
TIMP low 32 14 9 8 8 6
TIMP high 10* 6 6 3 3 3

* One patient with missing date of first recurrence

Nr at risk 0 12 24 36 48 60
Meth - 31 22 17 14 11 9
Meth + 7 5 3 0 0 0

P = 0.024

Nr at risk 0 12 24 36 48 60
Meth - 30* 14 11 9 9 7
Meth + 7 2 0 0 0 0

* One patient with missing date of first recurrence
and tumours revealed TIMP3 promoter methylation to be a low-frequency event [41]. As promoter methylation alone does not explain the low levels of TIMP3 protein expression, alternate mechanisms of loss of expression have been suggested, such as the post-transcriptional regulation by microRNAs (miRNAs). A recent study by Martin del Campo et al. [42] reports reduced TIMP3 expression upon overexpression of miRNA-21 in melanoma cell lines, suggesting an alternate mode of TIMP3 expression regulation.

Finally, we explored the clinical relevance of reduced TIMP3 expression in melanomas. TIMP3 expression did not show any association with the analysed clinicopathological variables for stage III melanoma. We did not observe differences in 5-year DFS or OS between high- and low-expression samples. Interestingly, when classified based on promoter methylation status, we found that patients with methylated TIMP3 promoter experienced significantly shorter 5-year DFS and OS compared to patients without methylation. Although these results suggest that promoter methylation status of TIMP3 could have value as a prognostic marker in cutaneous melanomas, it is important to bear in mind that the total number of patients at risk in the current study is very low. Thus, larger studies need to be conducted to ascertain TIMP3 promoter methylation as a truly significant prognostic factor in melanoma.

Collectively, our results suggest a tumour suppressor role of TIMP3 in melanoma pathogenesis. Reduced TIMP3 expression was observed in the majority of stage III melanoma cases studied and protein expression inversely correlated with microvesSEL density. Although promoter methylation was detected in only 18% of the samples analysed, this epigenetic regulation seemed to impact outcome. Our results suggest that promoter methylation may not be the only mechanism responsible for reduced protein expression and thus other modes of gene inactivation, such as post-transcriptional regulation by microRNAs, need to be investigated.

Conflict of interest statement

None declared.

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