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Original Research

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

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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.

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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.

1. Introduction

Extracellular matrix remodelling is crucial to neovasculature 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