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Original contribution

The role of Twist1 in hepatocellular carcinoma angiogenesis: a clinical study☆

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Summary The epithelial-mesenchymal transition regulator Twist1 has been implicated in tumor invasion, metastasis, and vasculogenic mimicry formation of human hepatocellular carcinoma. However, the relationship between Twist1 expression and endothelium-dependent angiogenesis is not clear. In this study, to investigate the role of Twist1 in hepatocellular carcinoma angiogenesis, we measured the microvessel density by CD31 immunohistochemistry stain and explored the microvessel density as an angiogenesis indicator. The microvessel density in paraffin sections from 97 patients was correlated with Twist1 expression up-regulation. Nuclear relocation was also identified based on immunohistochemistry stain, presenting a significant clinical pattern in hepatocellular carcinoma metastasis and prognosis. Twist1 expression, which is both located in the cytoplasm and relocated into the nucleus, was associated with matrix metalloproteinase 9 up-regulation; matrix metalloproteinase 2 did not appear to present these effects in hepatocellular carcinoma. An assessment of microvessel density could provide an estimate of the degree of angiogenic activity in tissues, as well as its association with Twist1 up-regulated expression. To the best of our knowledge, not only is Twist1 related to metastasis by tumor cells, but vasculogenic mimicry is also significantly related to microvessel density; this process is also associated with matrix metalloproteinase 9 up-regulated expression. This work provides a better understanding of the role of Twist1 in hepatocellular carcinoma angiogenesis and metastasis, suggesting that our findings could represent tumor cell epithelial-mesenchymal transition and endothelium-dependent angiogenesis, as can be seen in hepatocellular carcinoma.

1. Introduction

Metastasis is the leading cause of mortality in hepatocellular carcinoma (HCC) patients. HCC invasiveness is a key step to metastasis, which then results in poor prognosis [1,2]. Therefore, HCC invasiveness is of great value for studying the risk factors of molecular events during metastasis. The abilities of cell invasion, migration, and matrix metalloproteinase activation, as well as the relationship between tumor

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cells and angiogenesis, leads to metastasis and poor prognosis in HCC [3,4]. The hypothesis of "seed and soil" reveals that tumor stroma plays an important role in grown tumors, while angiogenesis is considered a key factor in tumor stroma. Thus, progression to metastasis reflects changes in the close relationship between a tumor cell and its host environment [5]. Adequate blood supply is essential for rapidly proliferating tumor cells to obtain oxygen and nutrients. Furthermore, angiogenesis allows tumor cells to access circulation and metastasize to distant sites [6].

Numerous studies have shown that the nature of stromal tissues and tumor cells secreting matrix metalloproteinases (MMPs) that interact with an extracellular matrix contributes to tumor angiogenesis and malignancy at both the early and late stages of tumor progression [7-9]. However, the inducing factor for MMPs in metastasis and angiogenesis is currently not completely understood. Emerging evidence indicates that tumor-associated MMPs can also stimulate processes of epithelial-mesenchymal transition (EMT), which is considered a developmental mechanism during embryogenesis [10,11]. Recent reports show that Twist1, an EMT regulator, is involved in the metastasis of breast cancer, HCC, and other tumors by promoting EMT in vivo and in vitro [12-14]. Our recent study shows that Twist1 can promote vasculogenic mimicry (VM) formation in HCC [15]. We further investigated the relationship between Twist1 expression and endothelial-dependent vessels, which presents a significant clinical pattern in HCC metastasis and prognosis. Twist1 expression, which is both located in the cytoplasm and relocated into the nucleus, was observed to be associated with MMP-9 up-regulation. Meanwhile, MMP-2 did not appear to present these effects in HCC. For endothelial-dependent vessels, we measured microvessel density (MVD) by CD31 immunohistochemistry stain. An assessment of MVD could provide an estimate of the degree of angiogenic activity in the tissue; it is also associated with Twist1 up-regulated expression. To the best of our knowledge, not only does Twist1 relate to metastasis by tumor cells, VM presents a significant relationship with MVD as well; this process is associated with MMP-9 up-regulated expression. The current work provides a better understanding of the role of Twist1 in HCC angiogenesis and metastasis.

2. Materials and methods

2.1. Patient samples

Tissue specimens from 97 patients who underwent hepatectomy for HCC between 2001 and 2005 were obtained with the assistance of the Tumor Tissue Bank of Tianjin Cancer Hospital. Diagnosis of the HCC samples was verified by pathologists. Detailed pathological and clinical data were collected for all samples, including Edmondson tumor grade, metastasis, and survival duration. Paraffin-embedded tumor tissue samples were collected from patients who did not undergo therapy prior to tumor surgical operation. The use of these tissues in this study was approved by the Institutional Research Committee [15].

2.2. Immunohistochemical stain methods

Slides were deparaffinized in xylene. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 50% methanol for 10 minutes at room temperature. Sections were rehydrated in alcohol, washed with phosphate-buffered saline (PBS), and then pretreated with citrate buffer (0.01 mol/L citric acid, pH 6.0) for 20 minutes at 95°C in a microwave oven. After nonspecific binding sites were blocked by exposing them to 10% normal goat serum in PBS for 20 minutes at 37°C, sections were incubated overnight at 4°C using a series of antibodies (refer to sTable 1). Following this incubation, the sections were rinsed with PBS and incubated with biotinylated goat antimouse IgG for 20 minutes at 37°C. The slides were then incubated with 3,3′-diaminobenzidine chromogen for 5 to 10 minutes at room temperature and washed with distilled water. Finally, the sections were slightly counterstained with hematoxylin for 1 minute followed by dehydration and coverslip mounting. PBS was used in place of primary antibodies as a negative control. The staining systems used in this study were PicTure PV6000 (Zhongshan Chemical Co, Beijing, China) and Elivision Plus (Zhongshan Chemical Co, Beijing, China). Twist1, Twist2, E-cadherin, MMP2, and MMP9 levels were quantified according to the method described by Bittner et al [16]. Both the intensity and percentage of positive cells were measured. More than 10 microscopic fields in one section were counted for 100 tumor cells per field, of which 10 were visually evaluated. Cell expression was stratified as follows: 0 (negative) for less than 10% positive cells, 1 (weak) for less than 25% positive cells, 2 (moderate) for less than 50% positive cells, and 3 (strong) for more than 50% positive cells. The sum (staining index) of the stain intensity and positive cell scores was used to determine the final result for each section with 3 or greater as positive sample.

2.3. Microvessel quantification

To identify the hotspots containing the greatest number of stained vessels, vascularity assessment was performed by first scanning the section at a low power (×40) using a light microscope. For manual counts, 5 nonoverlapping fields in each section were considered to have MVD. In each section, 5 areas with the highest vascularization were selected [17].

2.4. Statistical analysis

The methods used for statistical analysis were the Pearson test (for correlation coefficient), rank-sum test, and Kaplan-Meier survival analysis. All the data used in the study were