

Original Article

Expression and clinical significance of microvessel density and its association with TWIST in nasopharyngeal carcinoma

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Abstract: Nasopharyngeal carcinoma (NPC) is characterized by a high frequency of nodal and distant metastasis at diagnosis. Microvessel density (MVD) is an indicator for angiogenesis that has been shown to correlate with metastasis of cancers. Evidence regarding the roles of MVD in NPC has rarely been reported. Thus, we aimed to investigate the state of angiogenesis in NPC. CD34 and TWIST were detected in sixty-five NPC specimens by immunohistochemistry, respectively. Then, MVD and its association with clinical features as well as TWIST expression have been assessed. As a result, MVD was closely related to cancer progression as reflected by distant metastasis ($P < 0.05$), and nevertheless, the data failed to show its association with lymph node metastasis and other clinicopathological features ($P > 0.05$). Interestingly, a marked correlation between TWIST positive expression with increased MVD was observed. The results suggested that MVD might play important roles in the development of NPC and TWIST might promote cancer progression by facilitating angiogenesis. Further studies are needed to confirm the results.

Keywords: Nasopharyngeal carcinoma, TWIST, expression, immunohistochemistry, microvessel density

Introduction

Nasopharyngeal cancer (NPC) is a type of fast-growing tumor originates from nasopharyngeal region and its incidence is rare in many areas of the world but common in Southeast Asia and North Africa [1]. NPC is characterized by a high frequency of nodal and distant metastasis at diagnosis [2]. Nevertheless, the mechanisms of metastasis are not fully understood.

A growing body of literature has shown that tumor angiogenesis is closely associated with metastasis of malignant carcinomas [3]. The newly established vessels can support the proliferation of tumor cells by supplying essential substance and gas exchange. Besides, the vessels act as a direct portal that facilitates cell metastasis [4]. Tumor angiogenesis not only act as an early event of tumorigenesis, but also play important roles in the advanced phases of cancers [5]. The degree of angiogenesis is

usually determined by microvessel density (MVD) in tissue specimens, which is a valuable indicator of the development and progression of cancers [6]. Reports showed that MVD might be an important prognostic factor for predicting the aggressiveness of gastric carcinomas [7]. Moreover, MVD has a correlation with high tumor grade and vascular invasion in patients with colon cancer [8]. Thus, we hypothesized that MVD might play important roles in the development of NPC.

Many factors, such as VEGF, bFGF and PDGF have been suggested to play a role in the formation of microvessels [9]. However, other factors might also make important contributions to the process of vascularization because it includes complicated steps. Recently, epithelial-mesenchymal transition (EMT), a key event of embryogenesis that has been shown to be correlated closely with development and progression of tumors, has attracted much attention [10].

Table 1. Patient characteristics

Characteristic	No. of patients
Age (year)	
< 45	40
≥ 45	25
Gender	
Male	39
Female	26
Clinical stage	
I	8
II	20
III	13
IV	24
T stage	
T1	19
T2	11
T3	21
T4	14
N stage	
N0	22
N1	17
N2	14
N3	12
Distant metastasis	
M0	55
M1	10

Aberrant activation of EMT in epithelial cells usually has been implicated in genesis of cancers. During this process, TWIST, a basic helix-loop-helix transcription factor, has been thought to act as an inducer and play critical roles in a variety of carcinomas. Recent reports showed that TWIST is a useful predictor of unfavorable prognosis for ovarian cancer [11] and renal cell carcinoma [12]. Moreover, over-expression of TWIST might be associated with lymph node metastasis for thyroid cancer [13] and gastric cancer [14]. In addition, TWIST renders the invasiveness to cancers by promoting angiogenesis [15].

To the best our knowledge, the significance of MVD and its association with TWIST in NPC has not virtually been reported in the open literature. The aim of the present study was to determine the status and significance of angiogenesis in NPC. Thus, manual counting of MVD was used to evaluate the state of angiogenesis in NPC tissue specimens. Moreover, the relation-

ships of MVD with clinical features as well as TWIST expression were assessed.

Materials and methods

Patients and tissue samples

A total of 65 paraffin-embedded NPC samples from NPC patients who were histologically and clinically diagnosed from the Affiliated Hospital of Guiyang Medical College, Southwest Hospital and Xinqiao Hospital in China between 2001 and 2010. All of the patients received no radiotherapy or chemotherapy before operation. Patients were informed of the investigational nature of the research and each provided written informed consent prior to recruitment. The stage and the histology of cases were classified according to the 2010 American Joint Committee on Cancer (AJCC, 7th edition). The characteristics of the NPC patients are listed in **Table 1**. All patients were treated with standard curative radiotherapy with or without relevant chemotherapy.

Immunohistochemical staining

Protein expressions in NPC tissues were tested by using the two-step PV method of immunohistochemistry (IHC). Samples were fixed with 10% neutral formaldehyde solution. The staining was performed on 4 µm sections from formalin-fixed and paraffin-embedded tissue. The slides were deparaffinized, rehydrated and treated with 3% hydrogen peroxidized for 20 m to inhibit endogenous peroxidase. The sections were rinsed with distilled water and saturated in phosphate buffered saline (PBS) for 5 min and then were incubated with a 1:50 dilution of the primary antibodies (Rabbit anti-TWIST polyclonal antibody, Abcam; Mouse anti-CD34 monoclonal antibody, Abcam) overnight at 4°C. Then the reaction was performed using the PV-9000 Polymer Detection System (Zhongshan Biotech, Beijing, China). The staining was visualized using DAB solution and counterstained with hematoxylin. IHC staining was conducted according the manufacture's instructions.

Evaluation of IHC staining

The IHC staining results for TWIST were identified by integrated scoring. Four fields of view, each at 400× magnification were randomly sampled from the upper, lower, left, right and

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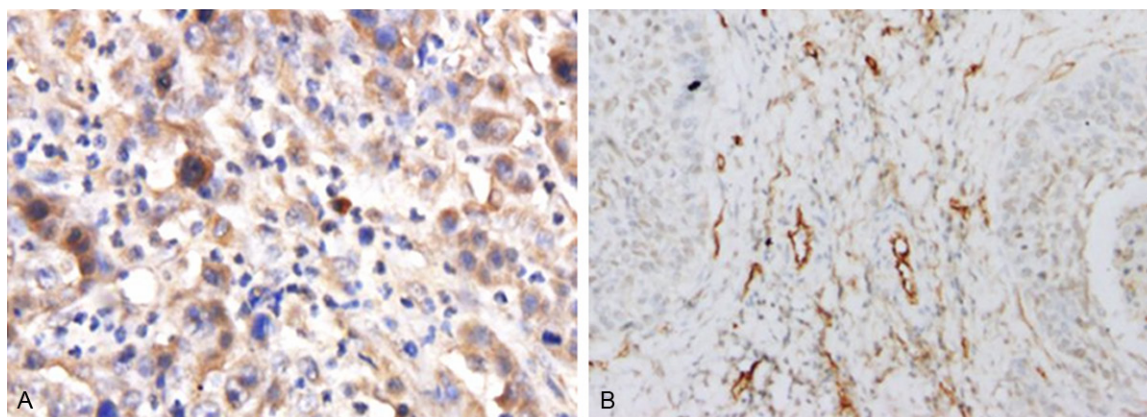


Figure 1. Expression of TWIST protein ($\times 400$) and CD34 antigen ($\times 200$) in NPC tissues assessed by IHC, respectively. A: TWIST; B: CD34.

Table 2. Relationship between MVD and clinicopathological factors

Variables	No. of cases	Microvessel counts	P value
Gender			
Male	39	44.15 \pm 8.77	> 0.05
Female	26	43.62 \pm 7.17	
Age (years)			
< 45	40	44.30 \pm 8.21	> 0.05
\geq 45	25	43.36 \pm 8.09	
Clinical stage			
I + II	28	44.04 \pm 8.14	> 0.05
III + IV	37	43.87 \pm 7.98	
T stage			
T1 + T2	30	43.73 \pm 8.31	> 0.05
T3 + T4	35	44.11 \pm 8.05	
N stage			
N0 + N1	39	43.87 \pm 8.67	> 0.05
N2 + N3	26	44.04 \pm 7.36	
Lymph nodal metastasis			
Negative	22	42.90 \pm 8.38	> 0.05
Positive	43	44.67 \pm 7.90	
Distant metastasis status			
M0	55	42.86 \pm 7.84	< 0.05
M1	10	49.90 \pm 7.25	

central part of the slice. The results were evaluated and scored independently by two pathologists without knowledge of the clinical parameters of the cases. The expression of TWIST was classified into several groups according to the percentage of positively staining cells: Grade 0 (negative), \leq 5%; Grade 1, 5%-25%; Grade 2, 25%-50%; Grade 3, 50%-75%; and Grade 4, \geq 75%. The staining intensity was categorized as

follows: Grade 0, negative; Grade 1, weak; Grade 2, moderate; and Grade 3, strong. The proportion and intensity scores were then multiplied to gain a total score: score 0-1, negative (-); score \geq 2, positive (+).

Counting method for MVD

With the CD34 staining, the MVD was calculated according to the method raised by Wendner et al. [16]. In brief, each section was observed under low-powered fields (LPF) at $\times 100$ magnification to find its high points of MVD (hot spots). Then, the microvessels were counted under $\times 200$ magnification. A single endothelial cell or a cluster of endothelial cells that were positively stained in dark brown and could be obviously separated from adjacent cancer cells or connective tissues were recognized as one microvessel. For each slide, five random selected fields at $\times 200$ magnification were counted. Then, the mean MVD were calculated. Likewise, two pathologists who had no knowledge of the patients' information observed the staining results of IHC.

Statistical analysis

For continuous variables, data were expressed as mean value \pm SD. Differences between groups were analyzed with a *t*-test. χ^2 test was used to differentiate the rates of different groups. These analyses were performed by utilizing Microsoft excel program (Version: 2003) and SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL). A *P* value of less than 0.05 was considered statistically significant.

Table 3. Relationship between MVD and TWIST expression

TWIST	No. of cases	Microvessel counts	P value
Positive	46	45.20 ± 7.68	< 0.05
Negative	19	40.90 ± 8.53	

patients with distant metastasis have a higher MVD than that in patients without distant metastasis. TWIST positive expression in NPC was associated with increased MVD.

Results

Microvessel staining and expression of TWIST protein assessed by IHC

The samples were tested for TWIST and CD34 expression by IHC with the specific antibodies. As a result, MVD was higher in highly proliferation sections than that in areas of high cancer cell density. The microvessels differed in size and shape. Incontinuity of endothelial cells could be observed in some regions. Positive staining of TWIST was observed in 70.8% (46/65) of NPC samples. Specific staining was found in the nuclei and cytoplasm of the cells (**Figure 1**).

Relationship between clinicopathologic features and MVD

The association of MVD with clinicopathologic parameters was presented in **Table 2**. No associations could be observed between MVD and age, gender, T and clinical stage as well as lymph node metastasis, respectively. Nevertheless, there was a marked association of MVD with distant metastasis status. The MVD in patients with distant metastasis (49.90 ± 7.25) was significantly higher than that in patients without distant metastasis (42.86 ± 7.84) ($P < 0.05$).

Association of TWIST protein expression with MVD

The association between TWIST expression and MVD was evaluated. As shown in **Table 3**, the MVD in TWIST positive patients were significantly higher than that in TWIST negative patients ($P < 0.05$), indicating that TWIST positive expression might have an association with increased MVD.

Discussion

In the present study, we examined the status of MVD in NPC tissues and its association with an EMT inducer, TWIST, by IHC and found that NPC

Whether MVD has a correlation with cancer development remains controversial. Though many studies indicated that increased MVD might reflect the progression of cancers [6], a proportion of studies disagreed with this point. A previous study on prostate cancer revealed that MVD was not a useful prognostic indicator and had no relation with tumor grade, pathologic stage, and clinical outcome [17]. A recent study on colon cancer suggested that MVD failed to provide predictive value of advanced or aggressiveness of the disease [18]. Thus, the roles of MVD might differ in various malignancies.

In the present study, MVD might only have an association with distant metastasis but not other clinical features such as lymph node metastasis. The reasons for this phenomenon might be that lymph node metastasis mainly results from lymphovascular invasion, while distant metastasis results from blood vessel invasion [19]. Thus, vascular invasion, but not lymphatic invasion, has been suggested to be an indicator of high biological aggressiveness and a strong prognostic factor for cancers [20, 21]. This might help explain why increased MVD in NPC has an association with distant metastasis in the present study. However, though the difference was insignificant, we observed that MVD in lymph node positive group (44.67 ± 7.90) was higher than that in lymph node negative group (42.90 ± 8.38). This result implied a tendency that MVD might also have a relation with lymph node metastasis. The insignificance may be due to chance because the sample size in this study was small. Therefore, future studies using large sample sizes were needed.

NPC is characterized by silent development and atypical clinical features, such as lymph node and distant metastasis. In the present study, an important inducer, TWIST, has been shown to correlate with increased MVD in NPC, indicating that TWIST might also act as an angiogenic factor for NPC. In our recent research, we found that positive expression of TWIST has a

correlation with both lymph node metastasis and distant metastasis in NPC (data not shown), in accordance with a previous study [22]. Thus, TWIST might accelerate cancer progression by promoting angiogenesis. However, the underlying mechanisms of NPC metastasis are not fully understood. Evidence suggests that TWIST could alter gene expression and promote loss of cell-cell adhesion, leading to a shift in cytoskeletal dynamics and a change from epithelial morphology and physiology to the mesenchymal phenotype [23]. Thus, the cells acquired elevated migratory abilities. Moreover, TWIST can promote angiogenesis by recruiting stromal macrophages [24] and increase microvessel density through up-regulation of MMP-9 expression [25]. Additionally, TWIST can promote the synthesis of a factor, VEGF, that is a well-characterized pro-angiogenic factor [26], and thus, MVD might be increased. Notably, we observed the existence of the discontinuity of the endothelial cells as blood vessel walls. Thus, the growing microvessels with incomplete walls provided a portal for malignant cells and facilitated their distant metastasis. This might help clarify the possible relationship between TWIST and MVD as well as their roles in the distant metastasis of NPC.

IHC staining targeting specific markers on microvessels can reflect the extent of angiogenesis, and thus may provide valuable information about tumor progression for clinical cancer therapy. Three antibodies targeting markers on endothelial cells were commonly used for research, including anti-factor VIII, anti-CD31 and anti-CD34. Though these antibodies are often used to label the endothelial cells, a large variability in the results was presented. Among them, using CD34 has generated more intense staining than using the other two antibodies. Meanwhile, the density of stained microvessels was greater with anti-CD34 compared to anti-CD31 and anti-factor VIII-related antigen [27]. Thus, we used CD34 antibodies in our study because it might produce stable and reliable results.

Several limitations might be included in this study. First, the present study evaluated the roles of MVD in NPC. Nevertheless, the molecular mechanisms could not be assessed due to the shortage of IHC. Second, this study only involved patients with relatively detailed information rather than patients with incomplete

information. Therefore, any selection bias might exist. Third, the sample size in the present study is limited. Thus, future studies with large sample sizes in vivo and in vitro using diverse bio-techniques are needed to explore the underlying mechanisms and increase power to get a more confidential result.

In summary, the results of the present study showed that increased MVD might be associated with distant metastasis of NPC. TWIST might act as an angiogenic factor in NPC development. MVD measurement using IHC of CD34 is a useful tool that could evaluate the progression of cancerigenesis and the efficacy of anti-angiogenic treatment.

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Disclosure of conflict of interest

None.

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