EGFR-Regulated EMT is a Driver of Vasculogenic Mimicry in Nasopharyngeal Carcinoma

Yue Yuan¹; Yunfan Luo²; Huiru Feng¹; Rui Deng¹; Bijun Liang¹; Haoran Huang¹; Xiaofei Yuan¹; Zilu Chen¹; Bolong Yu¹*; Xiong Liu¹*; Xiaohong Peng¹*

¹Department of Otolaryngology, Head and Neck Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China.
²Department of Otolaryngology, Head and Neck Surgery, Second People’s Hospital, Shenzhen, China.

Abstract

Background: Vascular mimicry is emerging as a potential target for anti-tumorigenesis. It involves the formation of microvascular channels composed of tumor cells. However, the mechanism of how tumor cells build into microvascular channels is not clear. The existence of a relationship between EMT and VM has been reported in the literature but the exact regulatory mechanism is unclear. Whether EMT regulates VM formation and its specific mechanism need to be further verified in NPC.

Materials and methods: We detected the relationship between EMT indicators and VM by immunohistochemical experiments. Also, the relationship between EMT indexes and VM indexes and clinical staging was analyzed. Cellular assays and immunoprotein blotting assays were used to detect EMT and VM changes in cells after addition of EGFR inhibitors. VM and EMT indices were examined after EGFR-targeted drug treatment in a subcutaneous tumorigenesis assay in nude mice.

Conclusion: EGFR-regulated EMT is a driver of vasculogenic mimicry in Nasopharyngeal Carcinoma.

Keywords: Nasopharyngeal carcinoma; EGFR; Vasculogenic mimicry EMT.

Introduction

Vasculogenic mimicry (VM), the phenomenon by which tumor cells mimic ECs and form vascular channels themselves in the first reported in 1999 [1-3]. It refers to the plasticity of invasive cancer cells to form new vascular networks, thus contributing to the perfusion of rapidly growing tumors, delivery of fluid from leaking vessels, and/or connection with constitutional endothelial layer vessels [4]. Over the next 20 years, VM has been reported in a variety of malignancies, including melanoma, glioblastoma, osteosarcoma, and hepatocellular carcinoma, as well as breast, lung, gastric, colorectal, and prostate cancers. In patients with malignancies such as breast, colorectal, prostate, liver, lung, ovarian, gastric, and bladder cancers, VM is associated with high tumor grade, invasion, metastasis, and poor prognosis [5]. VM is considered an important factor in the poor anti-tumor angiogenesis effect gradually becoming a hot research topic. However, the mechanism of how tumor cells build into microvascular channels is not clear.

Some studies have shown that a hypoxic and acidic microenvironment that strongly favours VM in xenografts [5,6]. Tumor...
activated platelets also possible induce vascular mimicry in mesenchymal stem cells and aid metastasis [7]. A study showed that microbial metabolite deoxycholic acid promotes vasculogenic mimicry formation in intestinal carcinogenesis [8]. All these factors are involved in the formation of VM. Hypoxic and acidic microenvironment is closely associated with epithelial mesenchymal transition (EMT) [9-11]. EMT is a process in which epithelial cells, under the action of some factors, lose their cell polarity, lose their tight intercellular connections and adhesion connections, and gain the ability of infiltration and migration, becoming mesenchymal cells with morphology and characteristics [12]. Whether EMT is a VM driver raises concerns. We speculate that EMT enables tumor cells to acquire tentacles and invasive ability, which may be one of the driving factors for their formation of VM. A small amount of literature mentions the association of EMT with angiogenic mimic formation in some tumors, such as gastric cancer [13] and melanoma [14]. However, the exact relationship between EMT and VM still needs to be further clarified.

Nasopharyngeal carcinoma (NPC) is a malignant tumor of nasopharynx with high affection in Southeast Asia [15]. In previous studies, we have shown that Foxq1 significantly promotes Vasculogenic mimicry (VM) formation, tumor growth, and metastasis and is effectively inhibited by EGFR inhibitors [16]. In the text we suggest that EGFR promotes the formation of VM. Whether EMT is an EGFR-regulated VM is worth further investigation.

**Materials and methods**

**Clinical samples**

60 cases of nasopharyngeal carcinoma tissue samples were collected from the Southern Hospital of Southern Medical University. The research subjects selected nasopharyngeal carcinoma patients with pathologically confirmed nasopharyngeal carcinoma from 2007 to 2019. Detailed pathological, clinical data and survival time of all NPC patients were collected through outpatient and telephone follow-up. Each patient signed an informed consent form. The TNM grading is based on the definition of the UICC American Joint Committee on Cancer Staging Criteria, 7th edition. The employment of these tissue samples was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University.

**Immunohistochemical and CD31-PAS dual staining**

The tissue was fixed with formalin, embedded in paraffin, and sliced at a thickness of 4 mm. After collection, the tissue was fixed with 4% paraformaldehyde at 4°C overnight. Antigen blocking was performed using 10% goat serum (AR0009, Boster, China). Anti-e-cadherin ((24E10) Rabbit mAb #3195, CST), anti-cd31 ((PE-CAM-1) (D8V9E) XP Rabbit mAb #77699, CST), anti-Ve-cadherin ((D21H3) XP Rabbit mAb #5741, CST), anti-cd31 ((PE-CAM-1) (D8V9E) XP Rabbit mAb #77699, CST), and anti-cd31 ((PE-CAM-1) (D8V9E) XP Rabbit mAb #77699, CST) were incubated overnight at 4°C. DAB system (ZLI-9017, Zsbio, China) was used to visualize the antigen. The number of positive cells was obtained from 5 randomly selected fields and 400x magnification.

**Immunohistochemical score**

Immunohistochemical scoring criteria: comprehensive score = staining intensity × positive area. Staining intensity score: strong positive (3 points), positive (2 points), weak positive (1 point), negative (0 points). The proportion of positive (including strong positive) regions: 100%-76% (4 points), 75%-51% (3 points), 50%-25% (2 points), 0-25% (1 point). The above scores were averaged by two pathologists independently.

**Cell culture**

All nasopharyngeal carcinoma cells were obtained from the Cancer Research Center of Southern Medical University, and were cultured in RPMI-1640 medium (Thermo Fisher Scientific Corporation PM15101) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Corporation 10270-106), 100 U/ml penicillinin (15140-122, Thermo Fisher Scientific, USA), 100 mg/ml streptomycin (15140-122, Thermo Fisher Scientific, USA), and humidified in 5% CO2. The environment was maintained at 37°C.

**Three-dimensional culture**

24-well plates coated with 100 μL Matrigel (354230, BD Biosciences, USA) reduced growth factor for each well, incubated at 37°C for 1 h, Take 500 μL medium containing 10% FBS (1×105 cells), spread it on the gel surface, and incubate at 37°C for 24 h. Each group provides three holes. The cells were then photographed under an inverted microscope (IX71, OLYMPUS, Japan). ImageJ calculates the average number of tubular structures.

**RNA isolation, reverse transcription, and quantitative Real-time PCR**

Total RNA was extracted from samples using RNA iso Plus (R401-01, Vazyme, China) and reverse transcribed using HiSctp III RT SuperMix for Quantitative Real-time PCR (+gDNA wiper) (R323-01, Vazyme, China) as cDNA. Quantitative reverse transcription PCR (qRT-PCR) was performed on ABI Quantaas5 system using ChamQ SYBR qRT-PCR Master Mix (Low ROX master mix) (Q331-02, Vazyme, China). GAPDH served as an mRNA endogenous control. All samples were normalized to an internal control and relative expression levels were calculated by using relative quantification.

**Western blot**

The proteins extracted from samples were assayed using lysis buffer (P0013B, Beyotime, China) containing protease inhibitor cocktail (HY-K0010, MCE, USA) using radioimmunoprecipitation. Proteins were solubilized in SDS loading buffer (FD0016, Fdbio, China), and the lysates were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (IPVH00010, Millipore, USA). Anti-E-Cadherin (24E10, CST, USA), Vimentin (D21H3, CST, USA), VE-Cadherin (D87F2, CST, USA), N-Cadherin (D4R1H, CST, USA), β-Catenin (D10A8, CST, USA) or GAPDH (D16H11, CST, USA) polyclonal antibodies were incubated at 4°C overnight at a dilution of 1:1000, and then incubated with species-specific enzyme-labeled secondary antibodies (1:5000 dilution) for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (WBKLS0100, Millipore, USA).
Statistical analyses

Statistical analysis was performed using SPSS 25.0 software. All data are from at least three independent experiments. Unless otherwise stated, data are presented as SEM means. A p-value < 0.05 was considered statistically significant.

Results

EMT was significantly positively correlated with VM in nasopharyngeal carcinoma clinical samples

We divided the tissue samples of 60 patients with nasopharyngeal carcinoma into stage I (4 cases), stage II (8 cases), stage III (35 cases), and stage IV (13 cases). The detailed Immunohistochemical staining scores for each group are shown in Table 1. The VM and E-cadherin and Vimentin sections stained are shown in Figure 1A.

The association of VM and E-cadherin and Vimentin with clinical stage and TNM stage was analyzed according to immunohistochemical scores. VM indicators were higher in stage III-IV than in stage I-II nasopharyngeal carcinoma. As the clinical stage of the tumor increased, E-cadherin expression decreased, but vimentin increased. The results suggest that the EMT process is promoted (Figure 1B). Similar results were obtained in the analysis of outcomes in T-stage versus N-stage (Figure 1C,D).

We compare the relationship between EMT and VM. E-cadherin was significantly negatively correlated with VM in nasopharyngeal carcinoma tissues ($r^2=0.5049$, $p<0.001$), while Vimentin was positively correlated with VM ($r^2=0.4116$, $p<0.001$) (Figure 1E,F). This indicates that EMT is significantly positively correlated with VM. The results suggest that EMT may be involved in the VM process.

Table 1: The relationship between VM expression, EMT and NPC clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Immunohistochemical staining score</th>
<th>n</th>
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<tbody>
<tr>
<td></td>
<td>PAS+CD31-</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>Overall stage$^1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>3.917 ± 1.505</td>
<td>6.167 ± 2.167</td>
</tr>
<tr>
<td>III+IV</td>
<td>8.167 ± 1.705</td>
<td>3.104 ± 2.146</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Tumor stage$^2$</td>
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<td></td>
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<tr>
<td>T1+T2</td>
<td>6.269 ± 2.662</td>
<td>4.654 ± 2.637</td>
</tr>
<tr>
<td>T3+T4</td>
<td>8.118 ± 1.805</td>
<td>3.000 ± 2.089</td>
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<tr>
<td>P value</td>
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<td>0.009</td>
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<tr>
<td>Node stage$^3$</td>
<td></td>
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<tr>
<td>N0</td>
<td>5.300 ± 3.591</td>
<td>5.500 ± 3.100</td>
</tr>
<tr>
<td>N1+N2+N3</td>
<td>7.720 ± 1.863</td>
<td>3.360 ± 2.183</td>
</tr>
<tr>
<td>P value</td>
<td>0.003</td>
<td>0.011</td>
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Figure 1: Viability assay in HeLa cell cultures. Arracacia xanthorrhiza Bancr (AXB) cytotoxic effect on HeLa cells was evaluated by MTT assay. Cells were seeded at 1 x 10^4 per well and treated with increased doses of AXB from 10^-11 mg/ml to 10^-1 mg/ml. Experiments were performed in triplicate to evaluate half-maximal inhibitory concentration for AXB.

EMT was positively correlated with VM formation ability in vitro cell experiments

First, to investigate the effect of EGFR inhibitors on the ability of nasopharyngeal carcinoma cells to form VM, we selected two nasopharyngeal carcinoma cell lines: 5-8 F and CNE1. EGFR inhibitors significantly inhibit the tube-forming ability of tumor cells in 3D cell culture. The results suggest that EGFR inhibitors inhibit VM formation (Figure 2A,B). Further observation of the ability of cells to form characteristic tentacles (equivalent to the occurrence of EMT markers). The results showed that after the addition of nimotuzumab, the proportion of “spindle cells” in 5-8 F and CNE1 cells decreased significantly (5-8 F: 66%; CNE1: 60%), indicating that the EMT process was inhibited ($p<0.05$) (Figure 2C,D).

Then, we tested the effectiveness of nimotumab and found that the expression of EGFR did not change significantly in the experimental group (Nimotuzumab +) compared with the control group (Nimotuzumab -), but the content of its phosphorylated product p-EGFR was significantly decreased (Figure 3A).

Further, further validation of the change in EMT index after the addition of nimotuzumab. The experimental results showed that the gene expression and protein expression of E-cadherin in two nasopharyngeal carcinoma cells increased (5-8 F: 52.4%, CNE1: 49.7%), while the gene and protein expression levels of Vimentin and N-cadherin decreased (Vimentin 5-8 F: 53.3%, CNE1: 20%), while the gene expression level of β-catenin was almost unchanged (see Discussion section for details) (Additional Figure 1). The above results suggest the ability of EGFR inhibitors to inhibit epithelial mesenchymal transition in nasopharyngeal carcinoma.

To verify whether the inhibition of EMT and VM by Nimotuzumab is related to drug concentration, we designed a Nimotuzumab drug concentration gradient experiment. The experimental
results showed that with the increase of nimotuzumab concentration, the protein content and gene expression level of E-cadherin in the two cell lines gradually increased, while the protein content and gene expression level of Ve-cadherin gradually decreased (Figure 3D-H).

In animal experiments, nimotuzumab can inhibit EMT process and VM formation

In our previous study, in order to investigate the relationship between vasculogenic mimicry and Foxq1 and EGFR, we performed subcutaneous tumorigenesis experiments [16]. Brief description of the experiment: 5-8 F cells were injected subcutaneously in nude mice. The tail vein of the experimental group was injected with Nitrozumab and the tail vein of the control group was injected with saline after tumor formation. We re-sliced and stained the tumor specimens utilizing previous animal experiments from our experimental group.

First, PAS/CD31 double staining was performed to detect the formation of vasculogenic mimicry in tumor tissues. We found that the tumor tissues of mice injected with nimotuzumab in the tail vein were less prone to vasculogenic mimicry than those of mice injected with normal saline (Figure 4A-B). Next, we examined EMT-related indicators (E-cadherin, Vimentin) and VM related indicators (VE-cadherin) in these animal tissues. We found that e-cadherin expression was decreased and Vimentin and VE-cadherin expression was increased in nimotuzumab treated tumor tissues compared with saline treated mouse tumor tissues. Nimotuzumab inhibited the EMT process and VM suppression was also observed (Figure 4C-F).
In this article, we verified the relationship between angiogenic mimicry and EMT from tissue samples, cellular experiments and animal experiments. The results suggest that EMT may act as a bridge mediating the EGFR pathway and promote angiogenic mimicry in nasopharyngeal carcinoma.

Vasculogenic mimicry (VM) is a vascular-like structure which can mimic the embryonic vascular network pattern to nourish the tumour tissue [17]. As a unique perfusion way, VM is correlated with tumour progression, invasion, metastasis and lower 5-year survival rate. Notably, Epithelial-Mesenchymal Transition (EMT) regulators and EMT-related transcription factors are highly up-regulated in VM-forming tumour cells, which demonstrated that EMT may play a crucial role in VM formation [18]. Therefore, the up-regulation of EMT-associated adhesion molecules and other factors can also make a contribution in VM-forming process [19].

Our study found a correlation between EMT and vasculogenic mimicry in nasopharyngeal carcinoma. When we inhibited the formation of vasculogenic mimicry vessels in nasopharyngeal carcinoma by using EGFR inhibitors, we detected that the EMT process was inhibited. Our results found a strong correlation between EMT and VM. It is highly likely that EMT is a bridge for VM formation. However, experiments are still needed to confirm their direct relationship. We also added that the EGFR signaling pathway can promote the formation of EMT and VM.

During the experiment, we discovered an interesting phenomenon. We began to extract the total protein of the cells to detect the expression of β-catenin. There was no difference in the expression level of the CTNNB1 gene, which regulates the expression of β-catenin. Therefore, we hypothesized that during EMT, the total amount of β-catenin did not change significantly, but its intracellular distribution changed, from cytoplasm to nucleus. The mechanism of β-catenin regulation of EMT and VM remains to be further studied.

Conclusions

EGFR-regulated EMT is a driver of vasculogenic mimicry in Nasopharyngeal Carcinoma.

Declarations

Ethics approval: All clinical studies were approved by the Ethics Committee of Southern Medical University. All animal experiments were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Conflicts of interest: The authors declare no competing interests.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Informed consent: Informed written consent was obtained from each patient.

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