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EphB4 promotes the proliferation, invasion, and angiogenesis of human colorectal cancer

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ABSTRACT

Objective: Eph/Ephrin signalling plays an important role in tumorigenesis, neovascularization, and vasculogenesis. However, studies concerning the role of EphB4 in colorectal cancer (CRC) show inconsistent results, and the function of EphB4 in the formation of CRC-related blood vessels is not fully understood. The aim of this study is to investigate the EphB4 expression in CRC and the role of EphB4 in tumour angiogenesis.
Materials and methods: EphB4 and EphrinB2 expressions were detected in 200 CRC samples and 50 paired colorectal mucosae by immunohistochemistry. Xenograft animal models were established by stable knockdown and stable overexpression of EphB4, and control cell lines were used to investigate the role of EphB4 in CRC. Microvessels were stained with anti-CD34, and microvessel density (MVD) was assessed.
Results: EphB4 protein was more highly expressed in CRC tissues compared with adjacent normal mucosae ($P < 0.05$), while EphrinB2 levels were unchanged. Modulation of EphB4 levels in colon cancer cell line SW480 resulted in significant effects on tumour growth and invasion *in vivo*, with stable overexpression of EphB4 associated with faster growth and invasion. Furthermore, microvessel density values in xenograft tumours were significantly correlated with EphB4 ($P < 0.05$).
Conclusion: EphB4 acts as a tumour promoter associated with proliferation, invasion, and angiogenesis, and may be used as a potential CRC therapeutic target.

1. Introduction

The Eph (erythropoietin-producing human hepatoma) family is the largest among transmembrane receptor tyrosine kinases (RTKs). Fifteen Eph RTKs are classified into two subclasses based on their structural features and binding affinities to various ephrin ligands (Lemke, 1997). The Eph receptors and their membrane-anchored ephrin ligands regulate tumorigenesis and angiogenesis (Heroult et al., 2006). Among the EphB receptors, increased expression of EphB4 has been reported in various tumour types, including colon (Kumar et al., 2009), prostate (Li et al., 2015), breast (Brantley-Sieders et al., 2011), oesophageal (Hu et al., 2014), pancreatic (Bai et al., 2014), lung (Ferguson et al., 2013), and mesothelioma (Liu et al., 2013), but with low or no expression in most normal tissues. As for CRC tissue, EphB4 was shown to be expressed in all 102 CRC specimens analysed, but was absent in normal colon mucosae, and its expression level was correlated with higher tumour grade and stage. Additionally, EphB4 inhibition decreased tumour growth and metastasis (Kumar et al., 2009). Another study analysing expression of all Eph and ephrin genes in 153 clinical specimens and CRC cell lines showed that EphA1, EphA2, EphB2, and EphB4 were all significantly overexpressed in CRC (Herath et al., 2012). Other studies showed that EphB4 inactivation resulted in higher proliferation of both intestinal tumours and normal epithelium (Dopeso et al., 2009). These opposing conclusions illustrate why the functional role of EphB4 in colorectal tumours remains undefined.

EphB4 and EphrinB2 play critical roles in the development of the vascular system and contribute to vascular function (Pitulescu and Adams, 2010). Research concerning the angiogenic role of EphB4 mainly focuses on embryonic and retinal vasculogenesis (Katsuta et al., 2013; Pitulescu et al., 2010; Xue et al., 2014). The role of EphB4 signalling in CRC has only recently been elucidated, and there are limited studies focusing on the relationship between microvessel density in human CRC and EphB4 expression. In a preliminary study, we stably transfected EphB4 overexpression and knockdown lentiviral vectors into CRC cell line SW480, and *in vitro* experiments showed that EphB4 overexpression promoted cell proliferation and stimulated migration and invasion, while EphB4 knockdown resulted in reduced cell viability, migration, and invasion. Here, we evaluated the expression of EphB4 and EphrinB2 using tissue microarrays from 200 colorectal tumours by immunohistochemistry. Furthermore, we performed *in vivo* experiments using xenograft models to investigate the role of EphB4 in CRC proliferation, invasion, and angiogenesis.

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2. Material and methods

2.1. Patients and clinical samples

Paired tissue samples of CRC and adjacent normal mucosae were obtained from patients who had undergone a colectomy due to CRC from January 2012 to December 2014, at the Suzhou Hospital affiliated with Nanjing Medical University. None of these patients received radiotherapy or chemotherapy before surgery. Data including clinicopathological parameters were collected from the medical records and pathological reports. The procedures were approved by the Ethics Committee of Human Experimentation.

2.2. Xenograft experiments

For xenograft tumour *in vivo* growth analysis, 5-week-old female Balb/C athymic mice (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were maintained under controlled conditions in individually ventilated cages and grouped randomly (six mice per group). The nude mice were inoculated by subcutaneous injection with 1×10^7 cells transfected with vectors enabling stable EphB4 overexpression (SW480/EphB4), stable EphB4 knockdown (SW480/shEphB4), or cells transfected with an empty vector (SW480/control). Tumours were measured by calliper twice a week. Tumour volume (mm^3) was calculated with the formula $V = 0.5ab^2$, where a and b are the largest and smallest lengths of the xenografted tumour. The mice were killed after six weeks, and tumours were harvested. Tumour samples were fixed with 10% formalin and embedded in paraffin. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals and related ethical regulations.

2.3. Immunohistochemistry

For immunohistochemical analysis, 4-μm sections were cut from paraffin blocks and boiled in 10 mM sodium citrate buffer following deparaffinization and dehydration. The following antibodies were used: anti-EphB4 (1:200, BS2018; Bioworld Technology, St. Louis Park, MN, USA), anti-EphrinB2 (1:100, sc15397; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD34 (1:150, MEC14.7; Biolegend, San Diego, *CA*, USA), and anti-E-cadherin antibody (1:100, Dako; Agilent Technologies, Santa Clara, CA, USA). Phosphate-buffered saline solution [0.01 M (pH 7.2)] was used instead of antibody as a negative control. The sections were stained with haematoxylin-eosin (HE) and observed under a light microscope and photographed.

2.4. Staining evaluation

The results were independently judged by two expert pathologists who were blinded to the clinicopathologic information. EphB4 was expressed on the cell membrane and in the cytoplasm. The estimated fractions of cells with EphB4 expression were denoted as 0 (0% to 1%), 1 (1% to 10%), 2 (11% to 50%), and 3 (>50%). The intensity of EphB4 staining was scored as follows: 0 = negative, 1 = weak, 2 = moderate, 3 = intense. A combined score was constructed by multiplying the fraction and intensity of staining. E-cadherin involved membranous staining, while EphrinB2 was expressed in the cytoplasm. The standards used for evaluating E-cadherin and EphrinB2 were the same as that used for EphB4.

2.5. Evaluation of tumour MVD

MVD determination was assessed by two pathologists in a double-blind format. The immunohistochemical staining of CD34 was used for MVD counting, as previously reported (Foote et al., 2005). First, sections stained for CD34 were scanned at a low magnification (100×), and all areas with a high density of highlighted microvessels ("hot spots") were identified. In the second step, five hot spots were selected randomly and observed, and the number of MVD values counted by scanning at a total magnification of 200×. Any positively stained endothelial cells or clusters that were clearly separated from adjacent microvessels, tumour cells, or other connective tissue were considered as separate countable vessels. The mean score of the five areas was then calculated as the level of MVD for each animal. Each assessment was repeated in triplicate (Preusser et al., 2006).

2.6. Statistical analysis

Data were analysed using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). The statistical significance of differences between groups was evaluated by two-tailed Pearson's χ^2 test. A $P < 0.05$ was considered statistically significant. The statistical methods and statistical significance for each test are indicated.

3. Results

3.1. EphB4 is overexpressed in CRC

To investigate the protein expression of EphB4 in clinical CRC samples, we assessed EphB4 protein expression in a clinical cohort including 200 cases of primary CRC tumour tissues and 50 cases of matched adjacent non-tumour tissues by immunohistochemistry. EphB4 protein was strongly expressed in the cellular membrane of CRC cells (Fig. 1A), while minimal to no EphB4 expression was detected in adjacent normal colon mucosa cells (Fig. 1B). The positive expression ratio of EphB4 in primary CRC specimens (167/200; 83.5%) was notably higher than that observed in adjacent normal samples (4/50; 8.0%; $P < 0.05$). EphrinB2 was expressed in the cytoplasm, and the level of EphrinB2-positive staining in CRC tissues was the same as that observed in normal mucosae. There was no obvious correlation between the expression level of EphB4 and EphrinB2. Furthermore, we analysed the correlation of clinicopathological features with EphB4 protein expression in CRC lesions (Table 1). Our data demonstrated that high EphB4 protein expression was significantly associated with invasion depth, lymph node metastasis, distant metastasis, and TNM stage ($P < 0.05$). No significant associations were found between EphB4 protein expression and other clinicopathological characteristics, such as sex, age, tumour diameter, or tumour differentiation ($P > 0.05$).

3.2. EphB4 promotes the growth of established xenograft tumours in vivo

Our previous work demonstrated that EphB4 promoted the proliferation, migration, and invasion of CRC cell line SW480 (manuscript in press). To test whether EphB4 could promote SW480 cell proliferation *in vivo*, we established subcutaneous xenograft models using the stable-transfection cell lines SW480/control, SW480/EphB4, and SW480/shEphB4. Tumours were measured two times per week. Then, the mice were killed after six weeks, visceral organs were inspected, and all tumours were harvested. Since day 28, significant differences of average tumour volume in the three groups of xenograft nude mice could be detected. When grown as xenografts in nude mice, EphB4 overexpression led to significantly faster tumour growth (Fig. 2A). The average tumour weight of the SW480/EphB4 tumours was significantly larger than that observed in control tumours ($P < 0.05$) (Fig. 2B). In contrast, SW480/shEphB4 tumours grew more slowly, and exhibited the lowest average tumour weight. Immunochemistry results showed no difference in EphrinB2 expression among the three groups (Fig. 2C–E), though the EphB4 expression levels were distinct (Fig. 2F–H). These observations indicated that EphB4 may promote CRC cell viability and proliferation *in vivo*.