RESEARCH ARTICLE

Expression of RECK and MMPs in Hepatoblastoma and Neuroblastoma and Comparative Analysis on the Tumor Metastasis

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Abstract

Objective: To explore the expression of RECK and relevant matrix metalloproteinases (MMPs) in hepatoblastoma (HB) and neuroblastoma (NB) and their clinical significance in the tumor metastasis. Materials and Methods: Forty-five wax-stone samples of HB and 43 wax-stone samples of NB removed by surgical resection and confirmed by pathology in Linyi Yishui Central Hospital were selected. According to presence and absence of metastasis, both NB and HB samples were divided into metastatic group and non-metastatic group, namely NB metastatic group (n=28), NB non-metastatic group (n=15), HB metastatic group (n=15) and HB non-metastatic group (n=30). The expression of RECK, membrane type-1 matrix metalloproteinase (MT1-MMP) in HB tissue and RECK, MMP-14 in NB tissue was detected using immunohistochemical method, and the correlation between RECK and MT1-MMP, MMP-14 was analyzed. Results: The metastatic rate of NB was dramatically higher than that of HB, with statistical significance (*P*=0.003). The positive rate of RECK expression in NB group (30.2%) was slightly lower than in HB group (40.0%), but no significant difference was presented (*P*=0.338). The positive rate of MMPs expression in NB metastatic group was evidently higher than in HB metastatic group (*P*=0.024). The results of Spearman correlation analysis revealed that the expression of RECK in HB and NB tissues had a significantly-negative correlation with MT1-MMP and MMP-14, respectively (r=-0.499, *P*=0.012; r=-0.636, *P*=0.000). Conclusions: In HB and NB tissues, RECK is expressed lowly, while relevant MMPs highly, and RECK inhibits the tumor invasion and metastasis through negative regulation of relevant MMPs.

Keywords: Hepatoblastoma - neuroblastoma - RECK - matrix metalloproteinase - metastasis

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Introduction

Both hepatoblastoma (HB) and neuroblastoma (NB) are commonly-encountered malignant tumors in children. HB originates from pluripotent stem cells in undifferentiated embryonic tissue which can differentiate into biliary epithelial cells and liver cells, approximately accounting for two-thirds primary malignant tumors in the liver. Its occurrence is closely related to proliferation and abnormal differentiation of liver cells (Khaderi et al., 2014; Kremer et al., 2014). Deriving from original neural crest, NB pertains to an embryonic tumor in sympathetic nervous system and the children at the age of 1~5 tend to be attacked. Metastasis can occur at an early stage of NB, and malignant degree is rather high. The recurrence rate of NB is still high in a progressive period after comprehensive treatment, such as radiotherapy, chemotherapy and surgery, and the long-term survival rate is only 20%~40% (Yáñez et al., 2015). The study has confirmed that distant metastasis tends to appear in NB than HB, especially bone marrow metastasis (Morandi et al., 2015).

As a new-type inhibitor of matrix metalloproteinases (MMPs), RECK gene can block the tumor invasion and metastasis via negative regulation of MMP-2, MMP-9, MMP-14 and membrane type-1 matrix metalloproteinase (MT1-MMP) (Wang et al., 2014; Yang et al., 2014). RECK is hardly ever or lowly expressed in a variety of tumor tissues, but expressed in all normal tissues. A lot of studies displayed that the expression of RECK in a lot of tumor tissues including the liver cancer, gastric cancer and colorectal cancer went down dramatically, but went up in normal tissues adjacent to cancer, suggesting that the level of RECK expression is associated with patient's prognosis and the tumor with low expression of RECK has stronger invasiveness (Furumoto et al., 2001; Cho et al., 2007; Xie et al., 2014). MMPs play pivotal roles in the degradation of basilar membrane, and their over-expression is related to the tumor malignant degrees. In this study, the expression of RECK and MMPs-related proteins (MT1-MMP and MMP-14) in HB and NB tissues was detected, and their correlation with invasion and metastasis of HB and NB was also analyzed in order to provide theoretical evidences for prevention and treatment

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of malignant tumors in children.

Materials and Methods

Sample source

Forty-five wax-stone samples of HB and 43 wax-stone samples of NB removed by surgical resection and confirmed by pathology in Linyi Yishui Central Hospital from May 2008 to May 2014 were selected, in which the males and females were 52 and 26 cases, respectively. They were 8 months~6 years old, averagely (3.5±0.8) years old. Based on international neuroblastomas staging system (INSS), there were 7 cases in phase I (non-metastasis), 8 in phase II (non-metastasis), 17 in phase III (in which 11 children suffered from bilateral lymph node metastasis and 6 from the tumor surpassing the midline) and 11 in phase IV (in which 6 suffered from bone marrow metastasis, 3 from distant lymph node metastasis and 2 from distant organ metastasis) among 43 children with NB. According to the malignant staging system of liver in children made by American Children Oncology Group, there were 12 cases in phase I, 15 in phase II, 11 in phase III (in which 8 children encountered regional lymph node metastasis) and 7 in phase IV (distant lymph node metastasis occurred in 7 children) among 45 children with HB. The enrolled samples in this study were all approved by Ethics Committee of Linyi Yishui Central Hospital, and the children or their relatives signed informed consent forms.

Reagents

Concentrated rabbit anti-human RECK mAb was purchased from American Santa Cruz Corporation. Both MT1-MMP and MMP-14 rabbit anti-human mAbs were provided by Wuhan Boster Biological Products Co., Ltd. Universal two-step detection kit (PV-6000) and DAB chromogenic kit were purchased from Beijing Zhongshan Golden Bridge Bio-tech Co., Ltd.

Methods

Grouping: According to presence and absence of metastasis, both NB and HB samples were divided into metastatic group and non-metastatic group, namely NB metastatic group (n=28), NB non-metastatic group (n=15), HB metastatic group (n=15) and HB non-metastatic group (n=30).

Immunohistochemical method: All samples were embedded by paraffin, cut into slices and stained by hematoxylin-eosin (HE) to confirm the pathological types and differentiated degrees of tissue. The expression of RECK, MT1-MMP in HB tissue and RECK, MMP-14 in NB tissue was detected using immunohistochemical method. The working concentrations of primary antibodies (RECK, MMP-14 and MT1-MMP) were respectively 1: 100, 1: 100 and 1: 200. The concrete operation methods were as follows: a. cut the wax-stone tissue into slices with the thickness of 3 μm and dewax routinely; b. swash with running water first, and then swash with distilled water twice, 3 min per time after hydration by gradient alcohol; c. swash with distilled water twice again, 3 min per time after incubation 10 min with 3% hydrogen peroxide at room temperature; d. carry out microwave repair for 30 min according to requirements for primary antibodies, and swash with phosphate buffer solution (PBS) twice, 2 min per time; e. After precondition, dropwise add primary antibodies, put them in a humidified box for 1 h at 37°C, and then swash with PBS three times, 3 min per time; f. dropwise add universal IgG antibody and incubate 20 min in a water bath at 37°C, swash with PBS three times again, 2 min per time; g. use DAB for coloration, hematoxylin for counterstaining, xylene for transparency and neural resin for sealing sections. All the operations were carried out strictly according to the kit instructions. Known positive sections of HB and NB tissues were regarded as positive controls and PBS as negative controls instead of primary antibodies.

Judging criteria

The positive expression of RECK, MT1-MMP and MMP-14 proteins was presence of claybank substances like granules in tumor cytoplasma. Two clinical pathologists observed the sections in a double-blind method, randomly selected 5~10 high-power visual fields (×400) in each section and calculated ≥200 cells in every visual field. The staining grading was performed based on staining degrees of tumor cells and percentage of positive cells. Scoring criteria for staining degrees: colorless (0 point), light yellow (1 point), claybank (2 points), sepia (3 points). Scoring criteria for the percentage of positive cells: the percentage of positive cells <30% (1 point), ranging between 30%~70% (2 points) and >70% (3 points). The product of two sorts of scores above was as the final score, namely 0~1 point being negative (-), 2~3 points being weakly positive (+) and ≥4 points being positive (++). In this study, the weakly-positive and positive expression was classified into the positive categorization.

Statistical data analysis

SPSS 15.0 statistical software was used to analyze the data. The enumeration data were compared with chi-square test and expressed by the percentage. The indexes in each group were compared using x^2 test of the four-fold table. Spearman correlation analysis was applied for the relationship between RECK and MMPs-related proteins. All statistical tests were made in a two-sided way. $P<0.05$ was considered to be statistically significant.

Results

Comparison on the metastasis of HB and NB

Metastasis appeared in 15 out of 45 children with HB, and the metastatic rate was 33.3% (15/45). Metastasis occurred in 28 out of 43 children with NB, and the metastatic rate came up to 65.1% (28/43). The metastatic rate of NB was higher than that of HB dramatically, with statistical significance ($\chi^2=8.889$, $P=0.003$).

Immunohistochemical staining of RECK and relevant MMPs in HB and NB

The positive signals of RECK and MT1-MMP proteins in HB tissue were located in tumor cytoplasma,

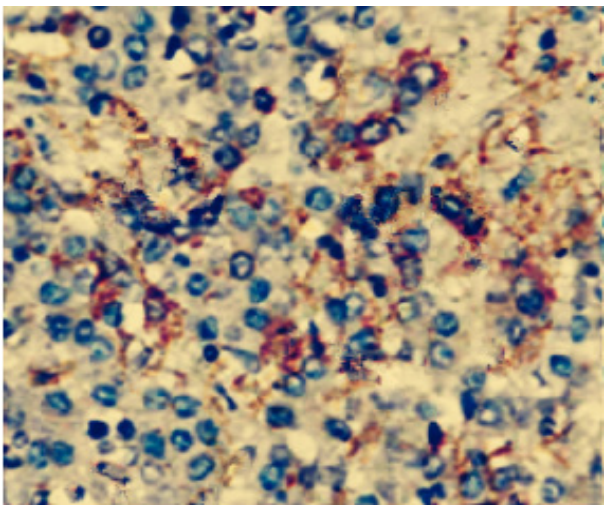

Figure 1. Positive Expression of RECK in HB Tissue (×400)

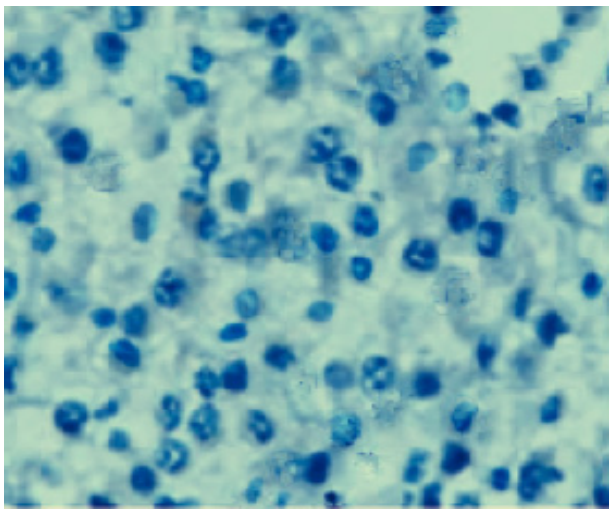

Figure 2. Positive Expression of MT1-MMP in HB Tissue (×400)

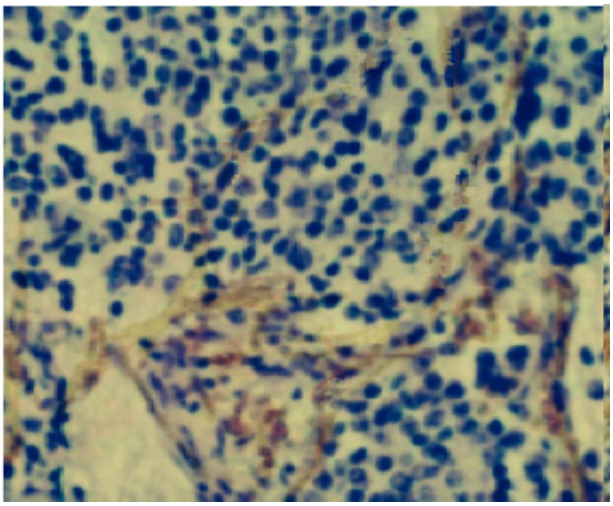

Figure 3. Positive Expression of RECK in NB Tissue (×400)

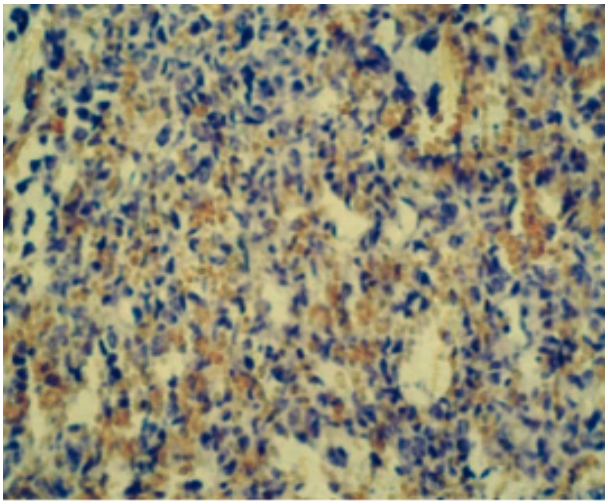

Figure 4. Positive Expression of MMP-14 in NB Tissue (×400)

Table 1. Expression of RECK in Each Group of HB and NB [n (%)]

Groups	n	RECK		χ^2	*P*
		Negative	Positive		
HB non-metastatic group	30	17 (56.7)	13 (43.3)	0.046	0.831
NB non-metastatic group	15	9 (60.0)	6 (40.0)		
HB metastatic group	15	10 (66.7)	5 (33.3)	0.337	0.561
NB metastatic group	28	21 (75.0)	7 (25.0)		

Table 2. Expression of Relevant MMPs in Each Group of HB and NB [n (%)]

Groups	n	Relevant MMPs		χ^2	*P*
		Negative	Positive		
HB non-metastatic group	30	10 (33.3)	20 (66.7)	0.194	0.660
NB non-metastatic group	15	6 (40.0)	9 (60.0)		
HB metastatic group	15	6 (40.0)	9 (60.0)	5.062	0.024
NB metastatic group	28	3 (10.7)	25 (89.3)		

manifesting different sizes of claybank substances like granules (Figure 1, 2). The positive signals of RECK and MMP-14 proteins in NB tissue were also positioned in tumor cytoplasma, showing claybank substances like granules (Figure 3, 4).

Expression of RECK in each group of HB and NB

The positive rates of RECK expression in HB and NB non-metastatic groups as well as their metastatic groups were 43.3% and 40.0%, 33.3% and 25.0%, respectively. No significant difference was presented by comparison to them (P=0.831, P=0.561). The positive rate of RECK expression in NB group (metastatic group + non-metastatic group) (30.2%) was slightly lower than in HB group (metastatic group + non-metastatic group) (40.0%), but there was no statistical significance (χ^2=0.919, P=0.338) (Table 1).

Expression of relevant MMPs in each group of HB and NB

The positive rates of MMPs expression in HB and NB non-metastatic groups were 66.7% and 60.0%, and there was no statistical significance (P=0.660). The positive rate of MMPs expression in NB metastatic group was evidently higher than in HB metastatic group, with statistical significance (χ^2=5.062, P=0.024). No significant difference was presented by comparison to the positive

rates of MMP expression between HB and NB groups (metastatic group + non-metastatic group) (χ^2=2.313, *P*=0.128) (Table 2).

Correlation between RECK and relevant MMPs in HB and NB tissues

The results of Spearman correlation analysis revealed that the expression of RECK in HB and NB tissues had a significantly-negative correlation with MT1-MMP and MMP-14, respectively (r=-0.499, *P*=0.012; r=-0.636, *P*=0.000).

Discussion

HB in children is an embryonic malignant tumor and may be related to proliferation and abnormal differentiation of liver cells during embryonic development. In prenatal or postnatal period, continuous proliferation of immature embryonic tissue in the liver forms infantile tissue block so as to easily transform into malignant HB (Zhang et al., 2013). With the development of medical technology and further research on biological features of the tumor, both the curative rate and long-term survival rate in children with HB improve dramatically, but the tumor invasion and metastasis are still the major factors that affect the prognosis (Martínez-Criado et al., 2013). As an embryonic tumor in sympathetic nervous system during childhood, NB can occur at any part of the sympathetic nervous system. Bone marrow metastasis or metastasis to general organs can appear in about 60%~70% children with NB at the early stage. Although both HB and NB belong to embryonic malignant tumors, there is still great difference regarding their growth modes, metastatic pathways and incidence as well as biological features. Hence, to investigate the influencing factors and metastatic mechanisms of HB and NB is of great importance for the prevention and treatment of malignant tumors in children.

Local infiltration and distant metastasis are the most important biological features of malignant tumors. Invasiveness and metastatic capability of the tumor cells are closely associated with the capability of proteases to degrade basilemma and extracellular matrix (ECM). The study demonstrated that RECK gene and MMPs play critical roles in the process of tumor invasion and metastasis (Alexius-Lindgren et al., 2014; Verma et al., 2014). MMPs, a serious of proteolytic enzyme families, can remodel the tissue by degrading macromolecular substances in ECM. Their degradation on the endothelial ECM is the precondition of angiogenesis, and the activity goes up conspicuously in various tumors, such as breast cancer and colorectal cancer. As a membrane-fixed glycoprotein that can inhibit angiogenesis and regulate MMPs, RECK can inhibit the tumor invasion and metastasis at the level of transcription by inhibiting the expression of MMPs. It is extensively expressed in normal tissue, and its expression is inhibited in fibroblasts transformed by tumor cells and oncogenes (Clark et al., 2007). Additionally, RECK is also related to angiogenesis intimately. Its decreased expression can effectively block angiogenesis (Jeon et al., 2010). The balance between RECK and MMPs is an important factor to maintain the integrity and stability of ECM under a normal physiological status. When RECK is loss of expression, over-expression of relevant MMPs causes a lot of ECM to degrade and integrity of blood vessels and peripheral tissue decrease, consequently leading to promotion of angiogenesis. When RECK is highly expressed, low expression of relevant MMPs inhibits ECM to degrade so as to inhibit angiogenesis (Kimura et al., 2010; Peters et al., 2010).

The research results in this study displayed that the metastatic rate of NB was dramatically higher than that of HB, and the positive rate of MMPs expression in NB metastatic group was evidently higher than in HB metastatic group, whereas no significant difference was presented between non-metastatic groups, illustrating that MMPs exert a key effect in the process of tumor invasion and metastasis. The positive rate of RECK expression in NB group was slightly lower than in HB group, and that in each metastatic group was also lower than in non-metastatic group, but there was no statistical significance, indicating that the increase of tumor infiltration is not evidently correlated with RECK expression in the children's malignant tumor tissue. The reasons may be related to the cytological features of the malignant tumors in children different from the adult or the nature and number of the selected samples. Relevant MMPs in HB and NB tissues were highly expressed, and the expression went up when the tumor invasion and metastasis appeared, suggesting that when RECK expression decreases, increased expression of relevant MMPs can enhance the tumor invasiveness and metastatic capability. Besides, the results of Spearman correlation analysis showed that the expression of RECK in HB and NB tissues had a significantly-negative correlation with MT1-MMP and MMP-14, indicating that RECK in malignant tissues inhibits the tumor invasion and metastasis through negative regulation of relevant MMPs.

In conclusion, in HB and NB tissues, RECK is expressed lowly, while relevant MMPs highly, and RECK inhibits the tumor invasion and metastasis through negative regulation of relevant MMPs. Both RECK and MMP are likely to be important reference indicators for prognostic assessment in the children's malignant tumors.

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