·Original Article in English·

Role of nerve growth factor receptor in neuroblastoma angiogenesis

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Abstract: Objective The nerve growth factor receptor (TrkA) gene is an independent marker of the favourable prognosis of neuroblastoma (NB). High expression of TrkA can not only induce differentiation and inhitit proliferation of NB cells but can also be involved in the regulation of tumor angiogenesis. This study aims to investigate the role of the TrkA gene in human NB angiogenesis. **Methods** The NB SY5Y cells were named SY5Y-TrkA cells and SY5Y-Vec cells after being transfected with TrkA gene and PBPSTR1 empty vector respectively, and the NB SY5Y cells without transfection were named the SY5Y cells. Fifteen nude mice were assigned into a Control group, an Empty-Vec group an Experiment group (n = 5 each). The SY5Y cells of the Control group, the SY5Y-Vec cells of the Empty-Vec group and the SY5Y-TrkA cells of the Experiment group were inoculated. The mice were then sacrificed 50 days after inoculation. The tumor volume was measured; the microvessel density (MVD) of the tumor was evaluated and the expression of vascular endothelial growth factor (VEGF) was determined by RT-PCR and immunohistochemistry. **Results** The tumor volume in the Experiment group (0.39 ± 0.02 cm^3) was significantly smaller than that of the Control group (1.74 ± 0.49 cm^3) and also was smaller than the Empty-Vec group (1.80 ± 0.75 cm^3) ($P < 0.01$). The expressions of VEGF mRNA and VEGF protein in the Experiment group were significantly lower than those of both the Control and the Empty-Vec groups ($P < 0.01$). The MVD in the Experiment group (4.08 ± 4.72 %) was also significantly lower than that of the Control (27.21 ± 14.58 %) and Empty-Vec groups (27.76 ± 14.15 %) ($P < 0.01$). **Conclusions** Angiogenesis and tumor growth of human NB can be effectively inhibited by the TrkA gene. This experiment provides a theoretical basis for the treatment of NB with anti-angiogenesis gene therapy.

[Chin J Contemp Pediatr, 2004, 6(2): 93 - 97]

Key words: Nerve growth factor receptor; Angiogenesis; Neuroblastoma; Nude mice

神经生长因子受体对神经母细胞瘤血管生成作用的研究

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[**摘　要**] **目的**　神经生长因子受体(TrkA)基因是神经母细胞瘤(NB)良好预后的标识之一,其高表达不仅可以抑制 NB 增殖和诱导良性分化,对 NB 的血管生长可能有一定的影响。该实验探讨 TrkA 基因对 NB 血管生成的作用。**方法**　15 只裸鼠随机分为对照组、空载体组和实验组(每组 5 只)。利用脂质体转染法将 TrkA 基因和 pBPSTR1 空载体转入 NB SY5Y 细胞,分别命名为 SY5Y-Trk A 及 SY5Y-Vec 细胞,未转染细胞为 SY5Y 细胞。将 SY5Y,SY5Y-Vec,SY5Y-TrkA 细胞分别接种在对照组、空载体组和实验组裸鼠皮下,接种 50 d 后处死动物,切除肿瘤,测量肿瘤体积;用 RT-PCR 及免疫组织化学技术检测肿瘤内血管内皮生长因子(VEGF)的表达;计算微血管密度(MVD)。**结果**　SY5Y-TrkA 细胞 TrkA 表达明显高于 SY5Y-Vec 及 SY5Y 细胞组($P < 0.01$);实验组肿瘤终体积小于对照组及空载体组(0.39 ± 0.02 cm^3 vs 1.74 ± 0.49 cm^3);(0.39 ± 0.02 cm^3 vs 1.80 ± 0.75 cm^3),差异有显著性(均 $P < 0.01$);实验组 VEGF mRNA 表达低于对照组及空载体组(0.16 ± 0.09 vs 1.45 ± 0.77);(0.16 ± 0.09 vs 1.35 ± 0.71),差异有显著性(均 $P < 0.01$);实验组 VEGF 蛋白表达低于对照组及空载体组(2.00 ± 0.60 vs 5.67

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±0.49) ; (2.00 ±0.60 vs 5.33 ±0.78) ,差异有显著性(均 $P < 0.01$) ;实验组 MVD 明显低于对照组和空载体组 (4.08 ±4.72 % vs 27.21 ±14.58 %) ; (4.08 ±4.72 % vs 27.76 ±14.15 %) ,差异有显著性(均 $P < 0.01$) 。**结论** TrkA 基因能有效抑制 NB 的血管生成和肿瘤生长,为应用基因抗血管治疗神经母细胞瘤提供了理论依据。

[**中国当代儿科杂志**,**2004** , **6**(**2**) : **93 - 97**]

[**关 键 词**] 神经生长因子受体;血管生成;神经母细胞瘤;裸鼠

[**中图分类号**] R73 [**文献标识码**] A [**文章编号**] 1008 - 8830(2004)02 - 0093 - 05

Neuroblastoma (NB) is a malignant embryogenic tumor derived from primitive neuroblast. It is one of the common malignant solid tumors in children , with the characteristics of abundant vessels , rapid growth and early metastasis. Recent research has shown that growth and metastasis of malignant tumors are largely dependent on angiogenesis. NB has become a key target for anti-angiogenic therapy of tumors because of its high vascularity. At present , most anti-angiogenic drugs and reagents for the treatment of NB are in the process of vitro experiments or animal experiments. Also , obvious toxic side-effects have been found. It has been reported that the TrkA gene , a nerve growth factor (NGF) receptor , was associated with the differentiation and proliferation of NB cells and might be involved in the regulation of angiogenesis[1]. So far , no reports about the role of TrkA in NB angiogenesis have been found in China. This study aims to evaluate the role of TrkA in NB angiogenesis and the feasibility of the TrkA gene in the treatmemt of human NB.

Materials and methods

Reagents

The Lipofectin kit was provided by Roche Biochemical Company (Germany). The TrkA-cDNA primer and β-actin primer were synthesized by Gibco Company (USA). The vascular endothelial growth factor (VEGF) primer and the RT-PCR kit were provided by Takara Company. The VEGF immunohistochemistry kit was provided by Boster Biotechnology Company of Wuhan. The SH-SY5Y NB cell line , vector (pBPSTR1 , with puromycin-resistant gene) and full-length TrkA cDNA plasmid that connected with the vector (pBPSTR1-TrkAcDNA) were a generous gift from Professor Carol Thiele (National Institute of Health , USA).

Establishment and identification of SY5Y-TrkA cell lines

SH-SY5Y (SY5Y) NB cells were cultured in the routine way. The pBPSTR1-TrkA cDNA and pBPSTR1 empty vector were transfected into the packaging cell line according to the directions of the Lipofectin kit and the method provided by Joseph Sambrook[2]. The tranfected cells were treated with 0.5 μg/ ml of puromycin for a week. The surviving , drug-resistant cells were subcloned by limited dilution to single-cell clonal lines. The cells transfected with pBPSTR1-TrkA were named the SY5Y-TrkA cell line and the cells transfected with pBPSTR1 were named the SY5Y-Vec cell line. The cells without transfection were SY5Y cell line. The expression of TrkA , which was represented by the ratio of the TrkA product density to the β-actin product density (PCR ratio) , was detected by RT-PCR[2]. The predicted product of TrkA was 523 bp[3]. The predicted product of β-actin was 700 bp.

Inoculation of NB cells and grouping

Fifteen 4-week-old female Balbc nude mice from the Animal Department of China Medical University were randomly assigned into three groups (n = 5 each) : an Experiment group , an Empty-Vec group and a Control group. The mice in the Experiment group were inoculated with 600 μl PBS SY5Y-TrkA cells suspension solutions in the right front axillary flank subcutaneously , with the inoculation density of 2.5×10^7/ ml[4]. The mice in the Empty-Vec group and the Control group were inoculated with SY5Y-Vec cells and SY5Y cells respectively , with the same dosage , inoculation site and inoculation time as the Experiment group. The growth of tumors were observed and the tumor volumes were measured 21 , 35 and 50 days after inoculation. The mice were sacrificed 50 days after inoculation and the tumors were resected. Some of the tumor specimens were made in-

to 5 μm paraffin sections and the other were snap-frozen in liquid nitrogen.

Detection of VEGFmRNA expression

After the frozen tumor specimens had been quickly thawed, pieces with less connective tissues were taken out. Pieces of tissue with a volume of 0.5 mm ×0.5 mm ×0.5 mm were then cut in the sterile condition. The total cellular RNA was extracted and then the VEGF mRNA expression was detected by RT-PCR. The VEGF mRNA expression was represented by the PCR ratio. The predicted product of VEGF was 535 bp[5].

Detection of VEGF protein expression

The processes were performed according to the directions of the VEGF immunohistochemistry kit. The cells with brown yellow granules in the plasm were defined as positive. The results were evaluated according to the total score of staining intensity and the percentage of positive cells: (a) staining intensity scoring: 0 = negative; 1 = weak positive; 2 = positive; 3 = strong positive; (b) percentage of positive cells scoring: 0 = 0 %; 1 = 25 %; 2 = < 26 % - 50 %; 3 = > 50 %.

Calculation of microvessel density (MVD)

The tumor tissues in the paraffin sections were stained with HE. The percentage of the vessel area in per unit field of vision area was calculated using a UIC/Olympus digital image analysis computer system. The observed visions were selected from the outer area, central area and inner area of the tumor tissure sections.

Statistical analysis

All data were expressed as $\bar{x} \pm s$ and t test was used to analyze the differences.

Results

TrkAmRNA expression of NB cells

The PCR ratios in the SY5Y cell line and the SY5Y-Vec cell line were 0.43 ±0.07 and 0.44 ±0.09 respectively. In the SY5Y-TrkA cell line, there was a bright band at 523 bp segment and the PCR ratio was 2.69 ±0.22. The TrkAmRNA expression of the SY5Y-TrkA cell line was significantly higher than that of the SY5Y cell line and was also significantly higher than that of SY5Y-Vec cell line (both $P <$ 0.01) (Figure 1).

Vivo tumor growth in nude mice

The tumor occurred 12 days after inoculation in the Control and Empty-Vec groups. While in the Experiment group, the tumor occurred 28 days after inoculation. The average tumor volumes 21, 35, and 50 days after inoculation in the Control group were 0.13 ± 0.04 cm^3, 0.48 ± 0.11 cm^3, 1.74 ± 0.49 cm^3, and those in the Empty-Vec group were 0.14 ± 0.05 cm^3, 0.50 ± 0.24 cm^3, 1.80 ± 0.75 cm^3. Those in the Experiment group were 0 cm^3, 0.10 ±0.02 cm^3, 0.39 ±0.02 cm^3. The tumor volume in the Experiment group was significantly smaller than those of the Control and the Empty-Vec groups ($P < 0.01$). There was no statistical difference between the Control and the Empty-Vec groups.

VEGF mRNA expression of tumor tissues

The PCR ratios in the Control, Empty-Vec and Experiment groups were 1.45 ±0.77, 1.35 ±0.71 and 0.16 ±0.09 respectively. The results showed that the VEGF mRNA expression of tumor tissues in the Experiment group were significantly lower than those of the Control and Empty-Vec groups (both $P < 0.01$) (Figure 2).

VEGF protein expression of tumor tissues

The scores of immunohistochemistry staining in the Control, Empty-Vec and Experiment groups were 5.67 ±0.49, 5.33 ±0.78, and 2.00 ±0.60 respectively. The results showed the expression of VEGF protein in the Experiment group was significantly lower than those of the Control and Empty-Vec groups (both $P < 0.01$). There was no statistical difference between the Control and Empty-Vec group. See Figures 3, 4 and 5.

MVD of tumor tissuses

The MVD in the Experiment group was significantly lower than those of the Control group and the Empty-Vec group respectively (4.08 ±4.72 % vs 27.21 ±14.58 %; 4.08 ±4.72 % vs 27.76 ± 14.15 %) (both $P < 0.01$). There was no statistical difference between the Control group and the Empty-Vec group (Figures 6, 7 and 8).

Discussion

Onset of NB is a result of some neural crest-de-

rived neuroblasts malignant proliferation. The normal regulation of these cells growth is disturbed because of gene variation in embryonic developmental stage[6]. The Trk oncogene family, which encodes tyrosine protein kinase receptors, and their ligands-nerve nutritions (NTs) may be involved in the incidence and development of NB to various extents. It has been shown that the high expression of TrkA can induce benign differentiation in NB cells and that the TrkA is a marker of favourable prognosis of NB. Mutiple defects in the TrkA-mediated signaling pathway were one of the important mechanisms of tumor formation and progression[7]. Recent studies have shown that the activated Trk-NGF complexes transmitted a neurotrophic signal from nerve terminals to cell bodies, resulting in a series of alterations in the biochemistry, gene regulation and morphological differentiation in NB cells, and then turned the NB cells into ganglinoblast phenotype. The high expression of TrkA not only might affect the differentiation and proliferation of NB cells but might also be involved in the regulation of tumor angiogenesis[1,8,9].

Angiogenesis is essential for tumor growth and metastasis[10]. VEGF is a highly specific mitosin on vascular endothelial cells and may intensely stimulate vascular endothelial cells to perform mitosis, proliferate, migrate and establish new vessels. As an autocrine and paracrine growth factor, VEGF can interact with the other angiogenic factors to contribute the angiogenesis of tumors[11]. The high expression of the VEGF gene was in accordance with N-myc amplification, remote metastasis and poor outcome of NB. However, the mechanism of regulating expression of angiogenic factors in tumor cells is still not well explained. Recent research has indicated that tumor angiogenesis is controlled by alterations of oncogene expression and tumor suppressor gene expression. The TrkA gene might be a suppressor gene in NB. In this study, the NB cell line with high expression of TrkA was established. Compared with the Empty-Vec and Control groups, the time of tumor appearance was later, tumor growth was slower, tumor volume was smaller, and the VEGFmRNA expression, the VEGF protein expression and the MVD were lower in the Experiment group. It is suggested that the oncogenic ability of the SY5Y-TrkA cells with high TrkA gene expression was greatly decreased. The mechanism might be that the TrkA gene could reduce VEGF expression and then inhibit NB angiogenesis. The results in this study were obtained without exogenous NGF, which was low in the SY5Y cells, and this suggested that the low autophosphorylation of TrkA at low level of endogenous NGF might be sufficient to cause inhibition of NB angiogenesis[3]. Or the reduction of TrkA on VEGF expression might be realized through the non-TrkA-NGF pathway.

It was found that the high expression of the TrkA gene in the Experiment group could not fully block VEGF expression and angiogenesis. It is suggested that the regulation of the VEGF expression and angiogenesis is a complex procedure, and that there might be other indirect mechanisms which down-regulated VEGF and affected the angiogenesis and invasion of NB besides TrkA. Angiogenesis of tumors might be regulated by multiple angiogenic factors in coordination rather than a single angiogenic factor[10].

In summary, it is helpful in controlling angiogenesis of NB to learn about the regulation mechanism of angiogenesis in NB. The research of the role of TrkA gene on angiogenesis of NB provides a new approach to the treatment of NB.

(Figures are on the back cover)

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·病例报告·

小儿红斑性肢痛症 1 例

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[中图分类号] R758.61 [文献标识码] E

患儿,女,3 岁,因双足疼痛 2 d 于 2003 年 3 月 9 日入院。患儿于入院前 2 d 无明显诱因出现双足剧烈疼痛,啼哭不止。睡觉时因双足放入被中疼痛加重,迫使双足暴露在被外;抬高双足或冷敷疼痛减轻。病程中无发热,无其他关节肿痛。查体:发育正常,心肺未见异常,四肢关节无红肿、变形,足背血管搏动正常。双足第四趾及其相连的足背约 1 cm × 2 cm的皮肤发红,稍肿胀,略高出皮肤表面,局部皮肤温度稍高,但无压痛。实验室检查:血、尿常规、抗"O"、血沉、类风湿因子、C 反应蛋白、血尿酸、足部 X 线摄片均正常。入院诊断:类风湿性关节炎。给予抗炎、解热镇痛药效果不佳。依据病史及症状特征诊断为红斑性肢痛症,改用抗过敏、冷敷、抬高双足等处理,住院 5 d,局部皮肤红肿渐消退,疼痛明显减轻出院。出院 4 d 电话随访,已痊愈。

讨论:红斑性肢痛症是一种原发性血管疾病,在 1872 年首先由 Mitchell 报道。本症病因未明,可能与植物神经功能紊乱有关。Drench 等[1]提出诊断原发性红斑性肢痛症的 6 条标准:①局部血管扩张和充血并发局部皮肤温度升高和灼痛;②两侧改变;③运动和加热能使症状加重;④冷敷、休息和抬高患肢会使症状减轻;⑤无原发或并发疾病存在;⑥对药物治疗有抗药性。本例符合上述诊断标准。治疗目前尚无特效药物。

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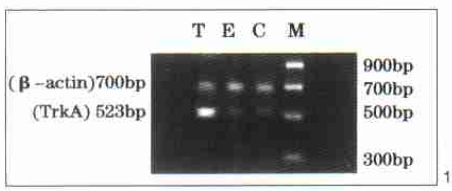
Role of nerve growth factor receptor in neuroblastoma angiogenesis

(These figures refer to the paper on page 93)



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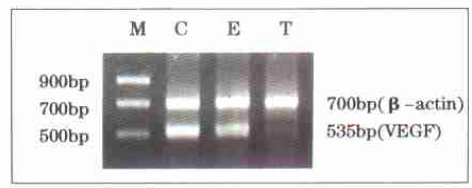

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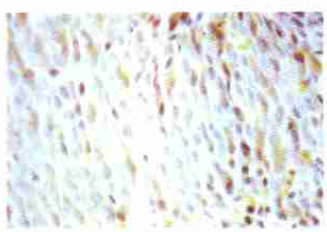
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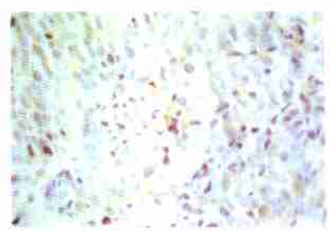
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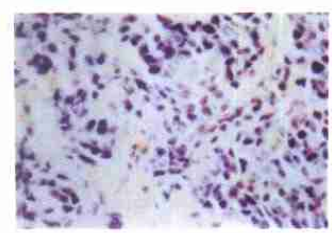
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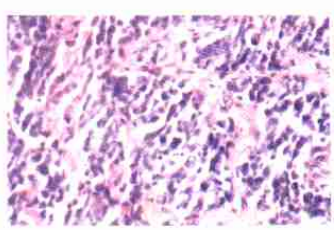
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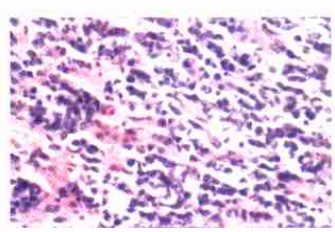
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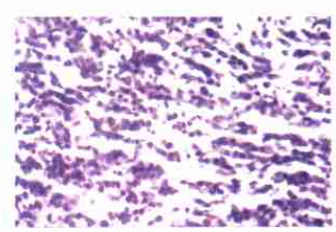
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Figure 1 Expression of TrkAmRNA
T: SY5Y-TrkA cell line; E: SY5Y-Vec cell line; C: SY5Y cell line; M: Marker

Figure 2 Expression of VEGFmRNA
M: Marker; C: Control group; E: Empty-Vec group; T: Experiment group

Figure 3 Experssion of VEGF protein in the tumor tissue of the Control group (DAB 40 ×)

Figure 4 Expression of VEGF protein in the tumor tissue of the Empty-Vec group (DAB 40 ×)

Figure 5 Expression of VEGF protein in the tumor tissue of the Experiment group (DAB 40 ×)

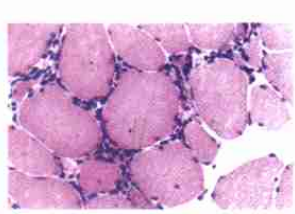
Figure 6 MVD of the Control group (HE)

Figure 7 MVD of the Empty-Vec group (HE)

Figure 8 MVD of the Experiment group (HE)

儿童型肌炎 / 皮肌炎临床和病理研究

（正文见第 117 页）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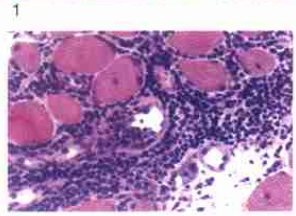
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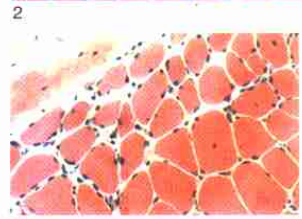
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图 1　JPM 肌纤维大小不等、肌内膜炎（HE 染色，× 400）

Figure 1 Inequality of muscle fiber in size and endomysial inflammation infiltration in JPM (HE × 400)

图 2　JPM 肌束内血管炎（HE 染色，× 400）

Figure 2 Fascicular vasculitis in JPM (HE × 400)

图 3　JDM 肌束周、肌内膜及血管周广泛炎症，肌纤维被吞噬，部分血管狭窄闭塞（HE 染色，× 400）

Figure 3 Extensive perifascicular, endomysial and vascular inflammation infiltration, phagocytized muscle fiber, and constriction or emphraxis in part of vessels in JDM (HE × 400)

图 4　JPM 肌束周萎缩（HE 染色，× 400）

Figure 4 Perifascicular atrophy in JPM (HE × 400)

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