Expression of Tyrosine-kinase Receptors and Neurotrophins in Human Neuroblastomas

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This work was supported by the National Natural Science Foundation of China (No.39470739).

Received June 21, 2005; accepted November 20, 2005.

OBJECTIVE The aim of this study was to investigate mRNA expression of tyrosine-kinase receptors (TRKs) and neurotrophins (NTs) in human neuroblastomas.

METHODS Expression of TrkA, TrkB, TrkC and BDNF was quantitatively examined by reverse transcription-polymerase chain reaction (RT-PCR) in 27 cases of neuroblastomas.

RESULTS The high and total rates of TrkA were expressed in significantly more tumors in a lower-stage group compared to a higher-stage group ($P<0.05$) and the high level of TrkA expression was correlated positively with the 2-year cumulative-survival rate of the patients ($P<0.01$). The high and total rates of TrkB were expressed in significantly more tumors in a higher-stage group compared to a lower-stage group ($P<0.05$). All 3 rates of BDNF expression between the 2 groups showed no statistical difference ($P>0.05$), but the co-expression ratio of TrkB and BDNF showed a remarkable significance in the higher-stage group more than in the lower-stage group ($P<0.05$). TrkC expression was usually accompanied by TrkA expression, but there was only a non-significant trend between TrkC expression and TrkA expression.

CONCLUSION RT-PCR for mRNA expression of TRKs and NTs has important clinical significance relating to the tumor stage and outcome for patients with neuroblastomas.

KEYWORDS: neuroblastoma, tyrosine-kinase receptor, neurotrophin, RT-PCR.

NGF is a member of a family homologous neurotrophins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5(NT-4).[1-3] Recently, 3 tyrosine kinase receptor genes (TRKs) for the neurotrophic factors of the NGF family have been cloned. The genes TrkA, TrkB, and TrkC encode the primary receptors for NGF, BDNF and NT-3, respectively.[4-6] There is increasing evidence that TRK genes play an important role in the biology and clinical behavior of neuroblastomas, tumors of the peripheral nervous system.

Neuroblastomas are one of the most common pediatric neoplasmas which are derived from the sympathoadrenal lineage of the neural crest. Neurotrophic factors and their receptors have been implicated in the pathogenesis of neuroblastoma, but their role has been unclear. To evaluate the clinical significance of expression of these genes in neuroblastomas, we studied the expression level of these genes detected by the reverse transcription-polymerase chain reaction (RT-PCR) in 27 cases of neuroblastomas.

MATERIALS AND METHODS

Patient materials

Pertinent clinical features and materials were obtained from 27 patients registered and treated for 27 cases of neuroblastomas in our own hospital from 1998 to 2003. All diagnoses of neuroblastoma were confirmed by histologic assessment of a tumor specimen obtained at surgery. The tumor tissues were immediately frozen and stored at -70℃ until used. The tumor stage was determined clinically or at the time of surgical biopsy or resection according to the criteria of Evans. We classified these patients into the 2 groups: a lower-stage group (stage I, II and IVS) and a higher-stage group (stage III and IV). At a median follow-up of 26 months, all 12 patients diagnosed with stage I, II and IVS disease were alive. Of the 15 patients with stage III and IV disease, 10 cases have died (66.7%), 3 cases are alive with active disease, and 2 cases remained free of disease at a median follow-up of 23 months.

RNA extraction and cDNA synthesis

Cryopreserved tumor samples were used. Total RNAs were extracted and reverse transcription (RT) was performed with a first-strand cDNA synthesis using random hexanucleotide primers. A Trizol Kit for extracting total RNAs and a RT-PCR Kit from Takara company in Japan were used for the procedures.

Quantitative PCR

Expression of TrkA, TrkB, TrkC and BDNF was measured by the reverse transcription-polymerase chain reaction (RT-PCR) and β-actin was used as an internal control gene to analyze gene-expression ratios (TRKs/β-actin). The information on the these gene sequences was obtained from a GenBank database search. These primers were synthesized by the Gibco Co. in the USA. The sequences of the PCR primers were as follows: *TrkA: forward primer 5'-CTG GGC GGA GTG CCT GAA-3'*, reverse primer 5'-GGC TGC GGC TCC AGG AA-3'; TrkB: forward primer 5'-ATA TGC AGC ATC TGC GAC TG-3', reverse primer 5'-AGC ATG AGC ACA TCG TCA AG-3'; TrkC: forward primer 5'-GTG CTG AAG CGA GAA CTG-3'; reverse primer 5'-TCT TTA ACC CTG CTG GTG-3'; BDNF: forward primer 5'-AGA AGA GGA GGC TCC AAA GG-3'; reverse primer 5'-GGC TGC GGC TCC AGG AA-3'; β-actin: *forward primer 5'-TCG TCA CCA ACT GGG ACG ACA-3'*; reverse primer 5'-GAT CTT GAT CTT CAT TGT GCT-3'. Quantitative PCR was performed in a final volume of 25 μl, and each sample was analyzed in duplicate. After 35 cycles of PCR, the amplified products were gel- electrophoresed and transferred to nylon membranes for chemiluminescent detection. The background-subtracted and band intensities were a quantified by densitometric analysis program. After normalisation to the β-actin gene, mRNA expression was expressed as arbitrary density units (d.u.) and converted to the following scale: negative (<0.5 d.u.), low expression (0.5~1.0 d.u.) and high expression (>1.0 d.u.).

Statistical analysis

The expression levels of these genes in the subgroups were represented by percentiles. A comparison of the gene dosage and expression in relation to clinical and genetic parameters was made using the Chi-Square test.

RESULTS

Expression of TrkA in neuroblastomas

The low, high and total ratios of TrkA expression in the lower-stage group (12 cases) were 25.0%, 66.7% and 91.7% respectively, and those ratios in the higher-stage group (15 cases) were 26.7%, 20.0% and 46.7% respectively. The high and total rates of TrkA were expressed in significantly more tumors in the lower-stage group compared to the higher-stage group ($P<0.05$). TrkA expression correlated strongly with survival: the 2-year cumulative-survival rates of the group with the high, low and negative level of TrkA expression were 90.9%, 71.4% and 22.2%, whereas the high level of TrkA expression was correlated positively with the 2-year cumulative-survival rate of the patients ($P<0.01$). The NGF mRNA was not detectable by RT-PCR in any of the primary tumors.

Expression of TrkB and BDNF in neuroblastoms

The low, high and total ratios of TrkB expression in the lower-stage group (12 cases) were 25.0%, 8.3% and 33.3% respectively, and those ratios in the higher-stage group (15 cases) were 13.3%, 66.7% and 80.0% respectively. The high and total rates of TrkB were expressed in significantly more tumors in the lower-stage group than in the higher-stage group ($P<0.05$). The low, high and total rates of BDNF expression in the lower-stage group (12 cases) were 25.0%, 33.3% and 58.3% respectively, and those ratios in the higher-stage group (15 cases) were 33.3%, 46.7% and 80.0% respectively, but all 3 rates of BDNF expression between the 2 groups showed no statistical difference ($P>0.05$). Overall, 51.9% of the neuroblastomas had

concordant expression of both TrkB and BDNF, but the co-expression ratio of TrkB and BDNF showed a remarkable significance in the higher-stage group compared to the lower-stage group ($P<0.05$).

Expression of TrkC in neuroblastomas

The low, high and total rates of TrkC expression in the lower-stage group (12 cases) were 33.3%, 16.7% and 50.0% respectively, and the ratios in the higher-stage group (15 cases) were 13.3%, 13.3% and 26.6% respectively, but all 3 rates of TrkC expression between the 2 groups weren not statistically different ($P>0.05$). TrkC expression in 6/10 (60%) patients was accompanied by TrkA expression, but there was only a non-significant trend between TrkC expression and TrkA expression. Furthermore, none of the primary tumors expressed NT-3 as determined by RT-PCR (Table 1).

DISCUSSION

Several studies have demonstrated that a high expression of TrkA in NB tumors is correlated with good prognosis, whereas negative and low expression of TrkA in NB tumors have been correlated with poor prognosis. Furthermore, TrkA expression in NB is a powerful predictor of the tumor stage and outcome.[7-9] The present studies indicated that the high and total rates of TrkA were expressed in significantly more of the tumors in the lower-stage group compared to the higher-stage group ($P<0.05$) and the high level of TrkA expression was correlated positively with the 2-year cumulative-survival rate of the patients ($P<0.01$). Our results showed that TrkA expression in NB is highly correlated with the tumor stage and prognosis. Recent studies have stated that signaling endosomes containing activated Trk-ligand complexes transmit a neurotrophic signal from the nerve terminals to remote cell bodies, where they selectively activate a novel MAP kinase, Erk5, as well as PI3 kinase, and thereby stimulate neuronal survival and differentiation.[10] The NGF/TrkA signal transduction pathway has played a important role in regulating the growth and differentiation of neuroblastoma.[7-11]

Expression of TrkB or BDNF genes in NB tumors has been reported to be associated with a poor prognosis.[12-14] The present studies demonstrate that the high and total rates of TrkB or BDNF were expressed in the higher-stage group more than in the lower-stage group, but both rates of TrkB expression in the 2 groups showed a statistical difference ($P<0.05$). The low, high and total rates of BDNF expression in the 2 groups showed no statistical difference ($P>0.05$), but the co-expression ratio of TrkB and BDNF showed a remarkable significance in the higher-stage group more than in the lower-stage group ($P<0.05$). These results demonstrate that expression of TrkB or BDNF is correlated with the tumor stage and prognosis and co-expression of both genes can suggest a unfavorable outcome for the patient. The association of aggressive tumor phenotype, high metastatic ability and chemoresistant phenotypes with the BDNF/TrkB signal transduction pathway in NB cells has been reported.[13-16] It is not clear whether TrkB expression in differentiated tumors is derived from the differentiated neuroblastic tumor cells or from Schwannian elements, because Schwann cells can express TrkB and BDNF.[17] In addition, Schwann cells were reported to promote neuroblastoma differentiation.[18]

Some studies have reported that TrkC expression showed a significant correlation between favourable stage and better prognosis. Localised neuroblastomas seemed to co-express TrkA and full-length TrkC recepors.[19-21] The present study suggests that the low, high and total rates of TrkC were expressed in slightly more of the lower-stage group compared to the higher-stage group ($P>0.05$). TrkC expression was usually accompanied by TrkA expression , but there was only a

Table 1. TrkA, TrkB, TrkC and BDNF mRNA expression ratios in various groups.

Groups	Case (n)	TrkA expression (%)			TrkB expression (%)			TrkC expression (%)			BDNF expression (%)		
		Low	High	All	Low	High	All	Low	High	All	Low	High	All
Lower-stage	12	25.0	66.7*	91.7*	25.0	8.3#	33.3*	33.3	16.7	50.0	25.0	33.3	58.3
Higher-stage	15	26.7	20.0	46.7	13.3	66.7	80.0	13.3	13.3	26.6	33.3	46.7	80.0
Total ratio	27	25.9	40.7	66.7	18.5	40.7	59.3	22.2	14.8	37.0	29.6	40.7	70.4

*Comparison between two groups, $P<0.05$. #Comparison between two groups, $P<0.01$.

non-significant trend between TrkC expression and TrkA expression. These results demonstrate that TrkC expression dose not significantly correlate with the tumor stage and outcome. Co-expression of TrkC and TrkA suggests a favourable stage and good prognosis for the patient, but TrkC expression is not significantly correlated with TrkA expression. Our findings which are different from the previous reports may be related with our limitted patients or not having truncated a TrkC probe. Recent studies have indicated that the NT-3/ TrkC signal transduction pathway is involved with neuroblastomas having favourable growth patterns, but further work will be needed to confirm concrete functions.[20,21]

In conclusion, we have learned a great deal concerning the possible role of the TrkA, TrkB and TrkC pathways in regulating survival, growth and differentiation of neuroblastomas. In addition, expression of these genes may provide important prognostic information, which in turn may permit the choice and intensity of therapy to be tailored to the biology of the tumor for each individual patient. That is why RT-PCR for mRNA expression of TRKs and NTs has important clinical significance relating to the tumor stage and outcome for patients with neuroblastomas. However, our current knowledge about the specific role of these receptors in mediating the clinical behavior of the tumors at the present time is speculative. We must study more patients with neuroblastomas to confirm the validity of these conclusions.

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