Expression of NGF family and their receptors in gastric carcinoma: A cDNA microarray study

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AIM: To investigate the expression of NGF family and their receptors in gastric carcinoma and normal gastric mucosa, and to elucidate their effects on gastric carcinoma.

METHODS: RNA of gastric cancer tissues and normal gastric tissues was respectively isolated and mRNA was purified. Probes of both mRNA reverse transcription product cDNAs labeled with α-33P dATP were respectively hybridized with Atlas Array membrane where NGF and their family genes were spotted on. Hybridized signal images on Atlas Array membranes of gastric cancer tissues and normal gastric mucosa, then their effects on gastric carcinoma were investigated.

RESULTS: Hybridization signal images on Atlas Array membrane appeared in a lower level of nonspecific hybridization. Both of NGF family and their receptors Trk family mRNA were expressed in gastric cancer and normal gastric mucosa. But adversely up-regulated expression in other tissues and organs. NGF, BDGF, NT-3, NT-4/5, NT-6 and TrkA, B and C were down-regulated simultaneously in gastric carcinoma in comparison with normal gastric mucosa. Degrees of down-regulation in NGF family were greater than those in their receptors Trk family. Down-regulation of NT-3 and BDGF was the most significant, and TrkC down-regulation level was the lowest in receptors Trk family.

CONCLUSION: Down-regulated expression of NGF family and their receptors Trk family mRNA in gastric cancer is confirmed. NGF family and their receptors Trk family probably play a unique role in gastric cancer cell apoptosis by a novel Ras or Raf signal transduction pathway. Their synchronous effects are closely associated with occurrence and development of gastric carcinoma induced by reduction of signal transduction of programmed cell death.

INTRODUCTION
Recently, NGF family and their receptors family have been found in other non-neural tissues of the body, and more and more attentions are being paid to their effects on these tissues, especially on tumor tissues. Gene expression of NGF and Trk families in gastric tissue and gastric carcinoma has been seldom documented. Simultaneous detection of NGF family and their receptors family expression has become possible since cDNA microarray was developed. As abnormal expression of many genes is involved in gastric carcinogenesis, it contributes to a better understanding of their roles in the occurrence and development of gastric cancer and helps reveal mRNA expression of the family genes in gastric tissue and gastric carcinoma.

MATERIALS AND METHODS
RNA extraction and mRNA purification
RNA of gastric cancer tissues and normal gastric mucosa was respectively isolated with Trizol (Gibco) in five cases of gastric cancer from Xijing Hospital, Fourth Military Medical University. To ensure good total RNA quality 28S/18S rRNA of gastric cancer tissues and normal gastric mucosa of each case was performed in liquid nitrogen after being removed intraoperatively, and trituration of the samples was performed in liquid nitrogen. Then, mRNA was purified in Oligotex mRNA Kit (Qiagen). An equal mRNA mixture of gastric cancer tissues and normal gastric mucosa from five patients respectively constituted gastric cancer group and normal gastric mucosa group. At last, reverse transcription product (the first stranded cDNAs) of mRNA mixture was electrophoresed to evaluate its size and quality.

Probe labeling
One µg of mRNA mixture of gastric cancer tissue and normal gastric mucosa from five patients was respectively transcribed into cDNAs as a probe labeled with 3.5 µL α-33P dATP (>2, 500 kCi·mol−1, 10 Ci·L−1, Dupont) and 1 µL CDS primer 1 (Clontech) in a mixture containing 1 µL MMLV, 1 µL 10x dNTP Mix (for dATP label), 0.5 µL DTT (100 mM), and 2 µL 5x reaction buffer in a final volume of 10 µL was incubated for 0.25 h at 50 °C using an unregulated heat block (Eppendorf). Labeled reaction was stopped by adding 1 µL 10x Termination Mix. To purify the labeled cDNA from unincorporated 33P-labeled nucleotides and small (<0.1 kb) cDNA fragments, the above probe synthetic reactions were diluted to 200 µL total volume with Buffer NT2 included in Atlas cDNA Expression Arrays (Clontech), then transferred to a NucleoSpin Extraction Spin Column. 400 µL Buffer NT3 was added into the column after centrifugation at 14 000 rpm for 1 min and the flowthrough
was discarded. The procedure was repeated twice. To elute the labeled probe, 100NE was added into the column, centrifuged at 14,000 rpm for 1 min. The flowthrough was obtained as the labeled probe.

Prehybridization and hybridization
Prehybridization of Atlas Array membrane was carried out in 0.5 mg heat-denatured sheared salmon DNA, and 5 ml prewarmed ExpressHyb solution (Clontech) in a hybridization bottle was incubated for 0.5 h at 68 °C. Then, heat-denatured cDNA probes were added into the above prehybridization bottle together with 5 μl heat-denatured C(t)-1 DNA. The hybridization reactions were performed at 68 °C overnight. The next day, the Atlas Array membrane was washed three times in prewarmed wash solution 1 (2×SSC, 1 % SDS) with continuous agitation at 68 °C for 0.5 h, and in prewarmed wash solution 2 (0.1×SSC, 0.5 % SDS) at 68 °C for 0.5 h. The damp Atlas Array membrane was wrapped in a plastic wrap after the last washing in 2×SSC at room temperature for 5 min.

Exposure of hybridization signals to phosphorimager and result analyses
Atlas Array hybridization membrane was exposed to phosphor screen at room temperature overnight. The hybridization signals were analyzed with ArrayVersion 5.0 software (MD) after the phosphor screen was scanned with ImageQuant 5.1 software (MD). Quantitative data of each hybridization signals were obtained.

RESULTS
Identification of mRNA quality
Good total RNA quality was confirmed by 28S/18S >1.5. Size range of reverse transcription product cDNAs represented a smear from 0.2-4kb both in gastric cancer and normal gastric mucosa (Figure 1).

Image of scan on hybridization signals of phosphor screen
Hybridization signal images on Atlas Array membrane appeared in lower levels of nonspecific hybridization (Figure 2).

Expression of NGF family and their receptors Trk family mRNA
To quantify hybridization signals, signal intensity was detected after hybridized signal normalization of two hybridization Atlas Array membranes between gastric cancer and the normal mucosa. Signal intensity of NGF family and their receptors Trk family on Atlas Array membranes represented their mRNA expression level. NGF, BDGF, NT-3, NT-4/5, NT-6 and TrkA, B and C were down-regulated in gastric carcinoma in comparison with normal gastric mucosa. Degrees of down-regulation in NGF family were greater than those in their receptors Trk family. Down-regulation of NT-3 and BDGF was the most significant, and TrkC down-regulation level was the lowest in receptors Trk family (Table 1).

Table 1 Signal intensity of NGF family and their receptors Trk family

| Dot | Gene         | CA VOL | NOrn VOL | NOrn VOL / CA n VOL |
|-----|--------------|--------|----------|---------------------|
| D07n| NT-3,BDGF    | 0.031  | 0.311    | 10.128              |
| D11n| NGF          | 0.045  | 0.272    | 6.036               |
| D08n| NT-4/5, NT-6 | 0.06   | 0.352    | 5.822               |
| D02i| TrkC         | 0.029  | 0.119    | 4.172               |
| D03k| Trk          | 0.038  | 0.157    | 4.093               |
| D14h| TrkA         | 0.043  | 0.141    | 3.252               |
| D01i| TrkB         | 0.026  | 0.051    | 1.926               |

DISCUSSION
Gene microarray has been rapidly and extensively used in detecting expression of genes, DNA sequence, novel genes and gene mutants, DNA polymorphism, and in screening drugs, diagnosing diseases and mapping gene library since Schena reported it in 1995[12-28]. Profiling of differentially expressed genes in human gastric carcinoma by cDNA expression array was also reported[29]. The study detected expression of NGF family and their receptors Trk family mRNA by using cDNA microarray. The Atlas Array membranes were provided by Clontech. A set of housekeeping genes was included on the Atlas Array membranes to normalize mRNA expression levels. Our good total RNA and mRNA quality, as well as successful synthesis and labeling of a cDNA probe with highly specific activity ensured the best possible results. ExpressHybTM hybridization solution was used in our hybridization experiments, a low-viscosity hybridization solution that significantly enhances the sensitivity of detection and reduces background.

NGF is composed of three subunit proteins (α, β and γ) among which β subunit represents an active form. NGF produced by targets of sympathetic neuron, sensory central neuron exerts an important effect on growth, development, differentiation of these neurons. Brain-derived nerve growth factor (BDNF), NT-3, NT-4/5 and NT-6 are members of the
NGF family, and NGF has 50 % of homology with BDNF. Difference between members of the family comes from the distribution of tissues, the early and/or later expression, and different receptors. NGF family receptors are subdivided into three types: Trk A, TrkB and TrkC. The structure of the three receptors consists of cellular external region, transcellular membrane region and cellular internal region. The receptors all are tyrosine kinase, and there is 66-68 % of homology between them. NGF binds TrkA, and BDNF, NT-3, NT-4/5 and NT-6 bind TrkB, in which binding of NT-3 is weaker and mainly with TrkC. As the functions of NGF family, TrkA, TrkB and TrkC can regulate growth, development and differentiation of corresponding neurons while receiving signals of NGF, BDNF, NT-3, NT-4/5 and NT-6. NT-3 and its receptor TrkC play a role in early growth, development and differentiation of neural systems.

Recently NGF family and their receptors family were found in other non-nerve tissues of the body, and more and more attentions are being paid to their effects. It was reported that dermal pigment cells expressed NGF and Trk[32], hepatic cells expressed BDNF, NT-3, NT-4/5 as well as TrkA, which were related to liver pathophysiology. Hepatic stellate cells expressed BDNF, NT-3, NT-4/5, TrkB and TrkC that were involved in liver remodeling[33]. NT-3, NT-4/5, TrkB and TrkC expressed by microphages played a role in tissue inflammatory reaction and repair[34]. Cardiac myocytes expressed TrkC and NT-3, and early growth and development of the heart were retarded when blockage of TrkC was used[35]. Schneider et al [36] demonstrated TrkA and TrkC expression in pancreatic ducts and pancreatic islets. TrkB in apha-cells of islets, NGF in pancreatic ducts and pancreatic acinar cells, NT-3 and NT-4 respectively in capillary endothelia and ductule cells. Additionally, TrkB and TrkC were found in endocrine cells of gut epithelium and neural tissues in fish[35-36], and TrkA, TrkB and TrkC were all expressed in testis of rats. A current study also indicated NGF family and their receptors played an essential role in the development of tubulogenesis in embryonic kidney, spermatogenesis, hair follicle, heart and vascular differentiation and maintenance of blood and immune cells[37].

Similarly, more attentions are being paid to their effects on tumors. Antagonists of NGF family and their receptors are being applied to kill tumor cells experimentally. It was noted that NGF family and their receptors were not expressed in some normal tissues, but expressed in their corresponding tumors. For example, up-regulated expression of Trk was present in tumors originating from thyroid and ovary while absent in the normal tissues. When chromosomal translocation occurred, a fusion protein of Trk-T1 composed of carboxyl terminal tyrosine kinase domain of NTRK1 and amino terminal portion of TRP (translocated promoter region) was formed. Trk-T1 was oncogenic in vivo and contributed to the papillary neoplastic transformation of the thyroid[38]. These results suggest that NGF family and their receptors are involved in tumorigenicity. A recent study showed that pancreatic carcinoma cells could express NGF, TrkA and TrkC[39]. It is interesting that Trk was highly expressed in esophageal carcinoma, thyroid carcinoma and prostate carcinoma, and adversely Trk expression revealed significantly lower in gastric carcinoma and colon carcinoma[40,41]. Difference between NGF and Trk expression in various tumors suggests that NGF family and their receptors may play a different role or have directly reverse effects on various carcinoma originating from different tissues. Some studies verified the assumption that occurrence of apoptosis could be inhibited and/or enhanced while cascade effect was brought out by intracellular signal transduction pathway blocking or inducing programmed cell death[42].

Adversely, other novel Ras and/or Raf pathways participate in signal transduction paths mediating programmed cell death. For example, prostate growth depends on autocrine NGF interaction with Trk expressed by itself, otherwise, apoptosis occurred in medulloblastoma when NGF was bound to Trk[43].

The study showed that expression of NGF family and their receptors were simultaneously down-regulated in gastric cancer tissues. NT-3 and BDGF down-regulation was the lowest in NGF family, Trk C down-regulation was the lowest in Trk family. The evidences support that NGF family and their receptors Trk family may play a unique apoptotic role in gastric cancer by a new Ras or Raf signal transduction pathway. These further substantiate that NGF family and Trk family have synchronous effects on the occurrence and development of gastric carcinoma induced by reduction in signal transduction of programmed cell death brought out by simultaneously down-regulated expression of NGF family and Trk family. It remains unclear that which cells in gastric mucosa secret NGF family, and whether NGF plays a role in the occurrence and development of gastric carcinoma in autocrine or paracrine way.

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