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# Localization of Acidic and Basic Fibroblast Growth Factor mRNA in Human Brain Tumors

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**Acidic and basic fibroblast growth factors (aFGF and bFGF) are closely related peptide mitogens acting on both mesoderm- and neuroectoderm-derived cells, including fibroblasts, endothelial cells and glial cells. In order to identify the expression of mRNAs for these growth factors, *in situ* hybridization using human aFGF and bFGF RNA probes was performed in 24 human brain tumors. The mRNAs for aFGF and bFGF were expressed in the cells of various tumors (1/1 and 1/1 astrocytoma, 2/2 and 2/2 anaplastic astrocytomas, 6/6 and 6/6 glioblastomas, 4/4 and 4/4 meningiomas, 3/3 and 3/3 schwannomas, 1/2 and 1/2 pituitary adenomas, 4/4 and 4/4 metastatic carcinomas, 0/1 and 0/1 hemangioblastoma, 0/1 and 0/1 craniopharyngioma) and were also detected in endothelial cells and surrounding neuronal cells of brain tumors. These results suggest the possibilities that aFGF and bFGF contribute to the uncontrolled growth of tumor cells and the proliferation of endothelial cells in autocrine and paracrine manners, and that the expression of mRNAs for these growth factors in the surrounding neuronal cells results in enhancement of tumor growth.**

Key words: Fibroblast growth factor — Polymerase chain reaction — *In situ* hybridization — Human brain tumor

The growth of tumors depends not only on the biological characteristics of their component cells but also on various factors in the surrounding milieu. Growth factors and tumor vascularity are particularly important for tumor growth and regulation. Recently the role of acidic and basic fibroblast growth factors (aFGF and bFGF)[4] in these processes has also been highlighted.

aFGF and bFGF have been isolated from a wide variety of normal and malignant tissues and cells.[1] They are closely related peptide mitogens acting on both mesoderm- and neuroectoderm-derived cells, including fibroblasts, endothelial cells and glial cells.[2] FGFs exert their biological activities through high-affinity cell-surface receptors,[2,3] and it has been postulated that they may act as autocrine growth factors.[4–7] In gliomas, the genes encoding aFGF[4] and bFGF[8] are expressed, and human glioma cells have specific cell-surface receptors.[4,9]

On the other hand, since FGFs are the most potent angiogenic factors known, they also contribute to tumor angiogenesis.[10] In malignant gliomas, neovascularization is an especially common finding, and endothelial proliferation has been used as an indicator for pathological grading of the tumor. In order to clarify the correlation between tumor cell growth and angiogenesis in various types of brain tumors, the localization of aFGF and bFGF mRNA was examined by *in situ* hybridization, and an attempt was made to determine the cellular source of mRNA for these FGFs in human brain tumors.

## MATERIALS AND METHODS

**Tissue preparation** Human brain tumor specimens from 24 patients who had undergone surgery at The Nippon Medical School were used as materials (Table I). The diagnosis was based on morphological examination of formalin-fixed and deparaffinized sections stained with hematoxylin-eosin. The tissues were fixed in freshly prepared 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 16–18 h at 4°C. Then, the tissues were immersed in 30% (w/v) sucrose in PBS containing 0.02% (v/v) diethylpyrocarbonate (Sigma Chemical Co.) at 4°C for 16–18 h and frozen in OCT compound at −80°C. Frozen sections were cut with a cryostat at a thickness of 5–6 $\mu$m, and mounted on 3-aminopropylmethoxysilane-coated slides. The sections were rehydrated in PBS, and immersed in 0.2 *N* HCl for 20 min at room temperature (RT) followed by incubation in 1 $\mu$g/ml proteinase K (Sigma Chemical Co.) for 15 min at 37°C and immersion in freshly prepared 0.25% acetic anhydride in 0.1 *M* triethanolamine for 10 min at RT. Then, the sections were post-fixed in 4% PFA/PBS for 5 min at RT, and washed twice with 2 mg/ml glycine in PBS for 15 min at RT. The sections were kept in 50% (v/v) deionized formamide/2×SSC.

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[4] Abbreviations used: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; PFA, paraformaldehyde; PBS, phosphate-buffered saline; RT, room temperature; PCR, polymerase chain reaction.

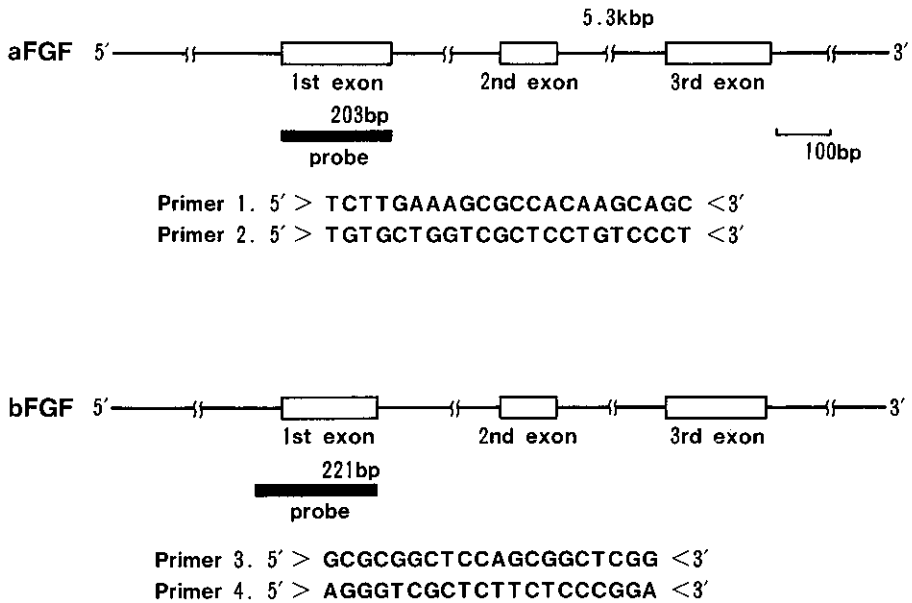


Fig. 1. Probes used in hybridization and the sequences of PCR primers for aFGF and bFGF. The lengths of probes for aFGF and bFGF were, respectively, 203 bp and 221 bp. The sequence of PCR primers conformed to that of the genomic DNA.[2,3]

**Preparation of digoxigenin-labeled RNA probes** (Fig. 1) DNA fragments of 203 bp and 221 bp, including the first exon of aFGF and bFGF based on previously reported sequence data for genomic DNA,[11,12] were synthesized using the polymerase chain reaction method (Takara Shuzo) followed by insertion in plasmid pGEM3z (Promega). The nucleotide sequence was then confirmed by the chain termination method of Sanger *et al.*[13] Oligonucleotides used as primers for the PCR method were synthesized by the phosphoramidite method on a synthesizer, and template DNA was derived from human liver genomic DNA. The RNA probes were labeled with digoxigenin-UTP by T7 or SP6 RNA polymerase using an RNA labeling kit (Boehringer Mannheim GmbH).[14]

**Hybridization** The hybridization buffer contained 0.3 *M* NaCl, 1 m*M* EDTA, 10 m*M* Tris-HCl (pH 7.6), 120 $\mu$g/ml herring sperm DNA (Boehringer Mannheim GmbH), 200 $\mu$g/ml yeast RNA (Boehringer Mannheim GmbH), 1×Denhart's solution, 10% (w/v) dextran sulfate (Sigma Chemical Co.), 50% (v/v) deionized formamide and 50–100 ng/ml anti-sense or sense labeled RNA probe.

Fifty microliters of the hybridization buffer was applied to each section, followed by incubation in a moist chamber for 16 h at 42°C for aFGF and at 50°C for bFGF. Then, the sections were washed three times in 50% formamide/2×SSC for 1 h at 42°C for aFGF and at 50°C for bFGF followed by once in 0.1×SSC for 1 h at 42°C for aFGF and at 50°C for bFGF, then once in 0.1×SSC for 30 min at RT and once in 2×SSC for 30 min at RT.

**Detection of digoxigenin-labeled probes** For immunological detection, a nucleic acid detection kit (Boehringer Mannheim GmbH) was employed. The sections were washed briefly with buffer 1 solution (100 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.6), and incubated with 0.5% (w/v) blocking reagent in buffer 1 solution for 30 min at RT. After washing again briefly with buffer 1 solution, the sections were incubated with a 1/500 dilution of polyclonal sheep anti-digoxigenin Fab fragment conjugated to alkaline phosphatase in buffer 1 solution for 30 min at RT. The sections were washed three times with buffer 1 solution for 10 min at RT, and equilibrated with buffer 3 solution containing 100 m*M* Tris-HCl, 100 m*M* NaCl, and 50 m*M* $MgCl_2$, pH 9.5, for 5 min. Then, the sections were incubated with color solution containing NBT and BCIP in a dark box for 40 min. The reaction was stopped with TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0), the sections were mounted in aqueous mounting medium (Daido Sangyo Co.) and observed by light microscopy.

## RESULTS

*In situ* hybridization studies for aFGF and bFGF were performed under high-stringency conditions for all 24 cases of human brain tumors (Table I). *In situ* hybridiza-

Table I. Expression of mRNAs for aFGF and bFGF in Human Brain Tumors

| Case No. | Sex | Age | Diagnosis | P/R[a] | mRNA expression | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Tumor cells | | Vascular cells | |
| | | | | | aFGF | bFGF | aFGF | bFGF |
| | | | Gliomas | | | | | |
| 1 | M | 41 | astrocytoma | P | +[b] | + | − | − |
| 2 | M | 60 | anaplastic | R | ++ | ++ | ++ | ++ |
| 3 | M | 33 | anaplastic | R | +++ | +++ | ++ | ++ |
| 4 | M | 47 | glioblastoma | P | + | ++ | + | + |
| 5 | M | 68 | glioblastoma | P | +++ | +++ | +++ | +++ |
| 6 | M | 52 | glioblastoma | P | + | ++ | + | ++ |
| 7 | F | 61 | glioblastoma | P&R | +++ | +++ | ++ | ++ |
| 8 | M | 75 | glioblastoma | P | ++ | ++ | + | ++ |
| 9 | M | 17 | glioblastoma[c] | R | +++ | +++ | +++ | +++ |
| | | | Meningiomas | | | | | |
| 10 | M | 48 | meningotheliomatous | P | ++ | +++ | ++ | +++ |
| 11 | F | 61 | meningotheliomatous | P | + | ++ | + | + |
| 12 | M | 58 | angiomatous | P | ++ | ++ | ++ | ++ |
| 13 | F | 63 | meningotheliomatous | P | + | ++ | + | ++ |
| | | | Schwannomas | | | | | |
| 14 | F | 39 | acoustic neurinoma | P | + | + | + | ++ |
| 15 | F | 49 | acoustic neurinoma | P | + | + | + | + |
| 16 | F | 35 | acoustic neurinoma | P | + | ++ | + | ++ |
| | | | Pituitary adenomas | | | | | |
| 17 | M | 53 | non-producing | P | ++ | ++ | + | + |
| 18 | F | 63 | non-producing | P | − | − | − | − |
| | | | Metastasis | | | | | |
| 19 | M | 41 | adenocarcinoma[d] | P | + | + | + | + |
| 20 | F | 52 | adenocarcinoma[e] | P | ++ | ++ | ++ | ++ |
| 21 | M | 45 | squamous cell carcinoma[d] | P | ++ | ++ | ++ | ++ |
| 22 | M | 71 | adenocarcinoma[d] | P | + | + | + | + |
| | | | Others | | | | | |
| 23 | F | 30 | hemangioblastoma | P | − | − | − | − |
| 24 | M | 47 | craniopharyngioma | P | − | − | − | − |

*a*) P/R: Primary or recurrence.
*b*) Percentage of positive cells in a hundred tumor cells in the positive area was expressed as: −, negative; +, < 30%; ++, 30%–60%; +++, > 60%.
*c*) Giant-cell glioblastoma. *d*) Metastasis from lung. *e*) Metastasis from uterus.

tion with the sense RNA probe for both FGFs always showed negative staining (Fig. 2A). However, *in situ* hybridization with the anti-sense RNA probes for aFGF and bFGF usually showed dark violet homogeneous or granular staining in the cytoplasm, especially in the perinuclear region of tumor cells, and staining intensity was stronger for bFGF than for aFGF in most of the cases (Fig. 2B and 2C). Both FGF mRNAs were homogeneously distributed throughout the cytoplasm of endothelial cells (Fig. 2D).

The staining intensity for both types of FGF mRNA was strongest in glioblastomas and anaplastic astrocytomas and the percentage of positive tumor cells was also highest in these tumors as shown in Table I. These positive tumor cells were usually distributed diffusely, but were localized much more in perivascular regions than in other areas. In the boundary region, positive tumor cells were scattered among normal glial and neuronal cells, and some neuronal cells were positive. In a giant cell glioblastoma, many giant cells and small round cells showed positive staining (Fig. 3A and 3B). However, in a benign astrocytoma, only a few positive cells were interspersed and some neuronal cells were positive. A large number of endothelial cells as well as tumor cells

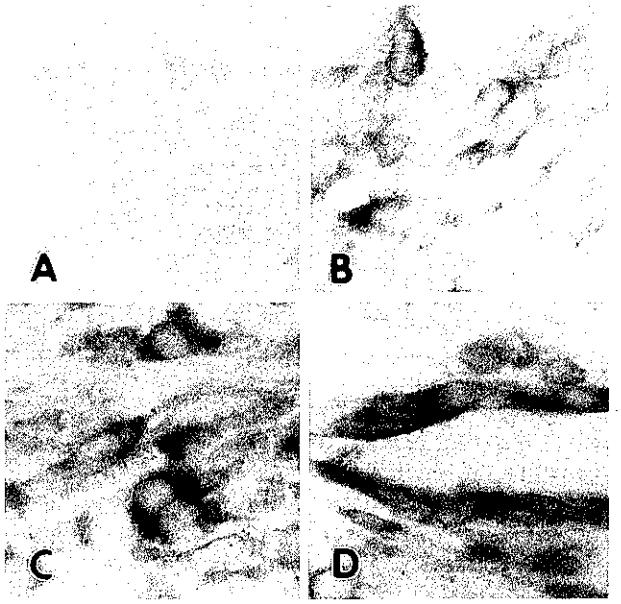


Fig. 2. *In situ* hybridization with the sense RNA probe for aFGF in case 3 shows no positive staining (A). *In situ* hybridization with the anti-sense RNA probes for aFGF (B) and bFGF (C) in case 3 show positive staining in the cytoplasm of tumor cells. *In situ* hybridization with the anti-sense RNA probe for bFGF in case 3 shows homogenous positive staining throughout the cytoplasm of endothelial cells (D). (A, B, C, D: ×200)

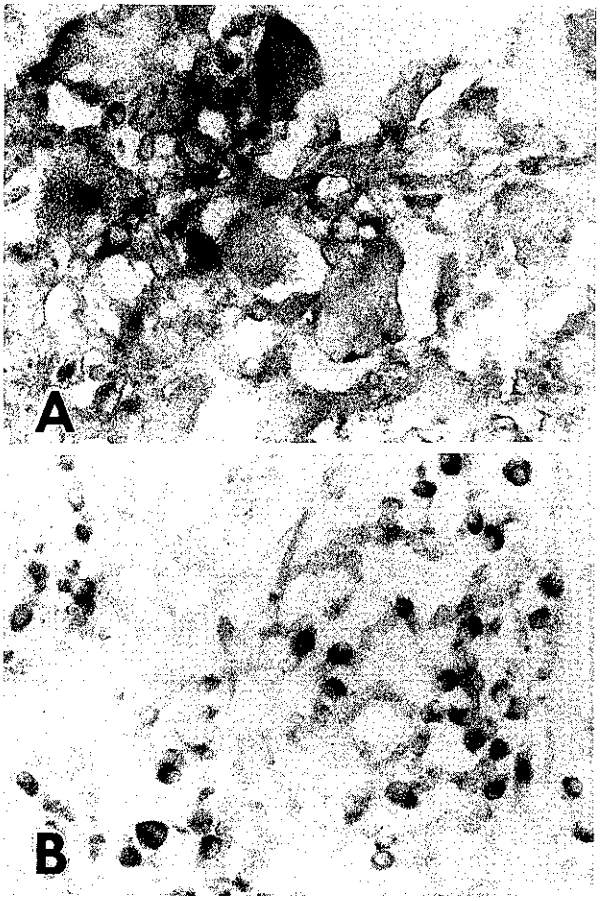


Fig. 3. *In situ* hybridization with the anti-sense RNA probe for bFGF in case 9 shows granular or homogenous positive staining in the cytoplasm of giant cells (A) and small round cells (B). (A, B: ×100)

expressed aFGF and bFGF mRNA in glioblastomas and anaplastic astrocytomas, but no positive endothelial cells were seen in an astrocytoma.

Meningioma cells showed diffuse weak positivity for both types of FGF mRNA, but the staining intensity was higher for bFGF than for aFGF. Schwannomas demonstrated scattered positive cells for bFGF and aFGF mRNA, not only in Antoni A areas but also in some Antoni B areas (Fig. 4A and 4B); staining intensity and the percentage of positive tumor cells were higher for bFGF mRNA than for aFGF mRNA. Only one pituitary adenoma showed a small area with weakly positive tumor cells. All metastatic carcinomas demonstrated positive staining in carcinoma cells, and weak staining in endothelial cells; positive tumor cells were especially abundant around the vessels. Hemangioblastoma and craniopharyngioma showed no positive staining in tumor or vascular cells.

## DISCUSSION

We have demonstrated in this study that tumor cells and endothelial cells in more than 80% of human brain tumors and some neurons in the adjacent brain tissue express aFGF and bFGF mRNA *in vivo*. These results indicate that aFGF and bFGF may contribute to the uncontrolled growth of tumor cells and the proliferation of endothelial cells in autocrine and paracrine manners. Previous reports have demonstrated that some human brain tumors express the genes encoding aFGF[4, 8, 11] and bFGF,[8, 15] and possess specific cell-surface receptors for FGFs.[4, 9] Schweigerer *et al.*[5] reported that bFGF derived from capillary endothelial cells was able to stimulate proliferation of the cells in an autocrine manner via specific cell-surface receptors.[16] Our results suggest that aFGF derived from endothelial cells in human brain tumors can also stimulate cell proliferation in a similar way. The synthesis of FGF in the endothelium and glioma cell in malignant glioma may be a pathological manifestation of angiogenesis, and cell proliferation domi-

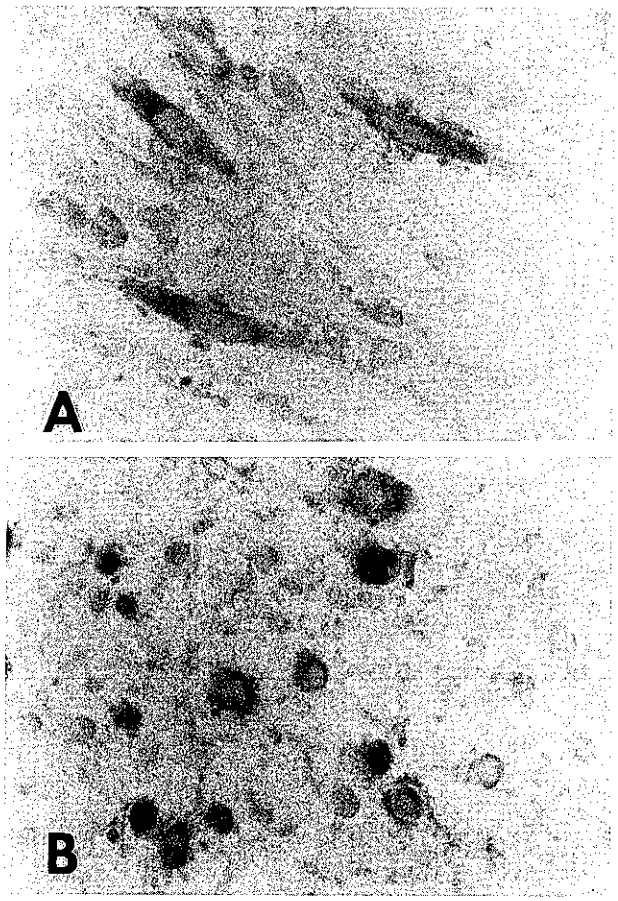


Fig. 4. *In situ* hybridization with the anti-sense RNA probe for bFGF in case 16 shows positive staining in schwannoma cells of both Antoni A area (A) and Antoni B area (B). (A, B: ×200)

nates at the expense of cell migration and differentiation, required for the formation of functioning vessels.[17]

Paulus *et al.*[18] observed positive immunoreactivity for bFGF in neurons, reactive astrocytes and endothelial cells in peritumoral tissues of human brain tumors. On the other hand, aFGF and bFGF may play an important role in wound healing in the central nervous system, and an increase of bFGF following brain injury in rats was found at the lesion boundary and localized in reactive astroglia.[19, 20] We demonstrated in the present study that some neurons and reactive astrocytes in the brain tissues adjacent to several brain tumors expressed aFGF and bFGF mRNA. However, it is very difficult to distinguish tumor cells from reactive astrocytes.

Previous reports have revealed that aFGF and bFGF mRNAs were barely detectable or absent in metastatic brain tumors.[8, 21] However, we showed that tumor cells in several metastatic brain tumors expressed the genes encoding aFGF and bFGF. Tumor-derived bFGF may have a dual influence by the autocrine growth stimulation of tumor cells, associated with endothelial cell proliferation by paracrine and autocrine stimulation.[4]

Our study revealed that the vascularity and pathological findings of endothelial cells in human brain tumors were not necessarily in accord with the staining intensity and percentage of positive tumor cells for FGF mRNA. Two possible reasons for this can be considered. First, platelet-derived growth factor[17] or another angiogenic factor such as angiogenin, or transforming growth factor $\alpha$ and $\beta$, may induce tumor angiogenesis. Second, tumor angiogenesis induced by FGF may be regulated by means of protein release or the number of receptors expressed.

This study suggests that FGFs in tumor cells, endothelium and surrounding neuronal cells may promote brain tumor growth in autocrine and paracrine manners.

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