Expression of basic fibroblast growth factor and its receptor in human pancreatic carcinomas

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Summary We examined the expression of basic fibroblast growth factor (FGF) and FGF receptor by immunohistochemistry in 32 human pancreatic ductal adenocarcinomas. Mild to marked basic FGF immunoreactivity was noted in 19 (59.4%) of the 32 tumors examined, and 30 (93.3%) of the tumors exhibited a cytoplasmic staining pattern against FGF receptor. The tumors were divided into two groups according to the proportion of positively stained tumour cells: a low expression group (positive cells <25%) and a high expression group (positive cells ≥25%). No statistically significant difference in tumour size, differentiation, metastases or stage was found between the low and high basic FGF expression groups. However, a significant correlation was found between FGF receptor expression level and the presence of retroperitoneal invasion, lymph node metastasis, and tumour stage. In addition, low FGF receptor expression was significantly associated with a longer post-operative survival as compared with high FGF receptor expression, whereas there was no significant difference in post-operative survival between the low and high basic FGF expression groups. Increased expression of FGF receptor is correlated with the extent of malignancy and post-operative survival in human pancreatic ductal adenocarcinomas. Thus, overexpression of FGF receptor may prove to be a more useful prognostic marker than basic FGF expression level in pancreatic cancer patients.

Keywords: basic fibroblast growth factor; fibroblast growth factor receptor; human pancreatic cancer

Members of the fibroblast growth factor (FGF, or heparin-binding growth factor) family are potent mitogens for a wide variety of mesodermal and neuroectodermal cells and have been isolated from a variety of tissue and cell sources including tumour cells (Thomas and Gimenez-Gallego, 1986; Gospodarowicz et al., 1987). To date, at least nine members have been identified from both normal and tumour tissues, including basic FGF, acidic FGF, the int-2 gene product (FGF-3), Kaposi FGF (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (FGF-7), androgen-induced growth factor, and FGF-9 (Klagsburn, 1989; Tanaka et al., 1992; Miyamoto et al., 1993).

Basic FGF is thought to induce fibrosis, angiogenesis, and tumour progression in human gastric carcinomas, renal cell carcinomas, brain tumours, and malignant melanoma through an autocrine mechanism (Becker et al., 1989; Takahashi et al., 1990; Zagzag et al., 1990; Tanimoto et al., 1991; Eguchi et al., 1992). Pancreatic carcinomas exhibit strong stromal reactions, or desmoplasia, and have an aggressive behaviour and poor prognosis (Ohta et al., 1993). Therefore, it is feasible that basic FGF is the factor responsible for desmoplasia and cancer cell proliferation in pancreatic carcinomas. This hypothesis is supported by a study in which basic FGF expression was detected in two human pancreatic carcinoma cell lines (Beauchamp et al., 1990). A recent study (Yamanaka et al., 1993; Leung et al., 1994) has also demonstrated the overexpression of basic FGF in human pancreatic carcinoma tissues. In addition, pancreatic carcinoma cells overexpress the FGF receptor which possesses intrinsic tyrosine kinase activity, raising the possibility that the abundance of basic FGF and its receptor may provide human pancreatic carcinoma cells with a considerable growth advantage (Kobrin et al., 1993; Leung et al., 1994). However, the tissue localisation of basic FGF and its receptor proteins have not been fully elucidated in human pancreatic carcinomas.

We examined the immunohistochemical localisation of basic FGF and its receptor in human pancreatic carcinomas and normal pancreatic tissues at the light and electron microscopically level, and determined the relevance of this growth factor system to malignant transformation and clinical parameters including prognosis.

Materials and methods

Patients and tissue specimens

The present study included 32 pancreatic ductal adenocarcinomas surgically resected between 1987 and 1993. All tumours were histologically proven to be pancreatic invasive tubular and/or papillary adenocarcinoma. There were no periampullary tumours or distal bile duct tumours not originating from the pancreatic duct. The patients were 22 men and ten women, ranging from 32 to 77 years of age, with a mean age of 63 years. Normal pancreatic tissues were obtained from two male and three female patients undergoing surgery for gastric cancer with combined resection of the distal pancreas and spleen. The resected specimens with attached peripancreatic lymph nodes and neural plexuses were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin, and cut into 5 mm stepwise tissue sections. Histological findings were evaluated according to the General Rules for Cancer of the Pancreas proposed by the Japanese Pancreatic Society (1986). All of the patients on the study were followed until December 1993. Four patients died within 60 days after surgery because of sepsis and hepato-renal failure, and 22 patients relapsed with carcinoma of the pancreas and died from progressive disease in the liver and/or peritoneum. Two patients died of other or unknown causes and four patients survived.

Three or more representative sections, including areas of associated chronic pancreatitis adjacent to the carcinoma, were used for immunohistochemical staining as described below. In addition, in two selected cases, parallel samples were fixed immediately with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 4 h. The tissue blocks were further rinsed overnight in a phosphate buffer containing 20% sucrose, then cut into 15–20 µm sections on a cryostat and mounted on poly-L-lysine-coated glass slides for immunoelectron microscopy of basic FGF.

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Antibodies

Monoclonal antibody against human basic FGF was obtained and purified as described previously (Matsuzaki et al., 1989; Yoshitake et al., 1991). This antibody is highly specific for basic FGF from human, bovine and rodent sources, and does not cross-react with acidic FGF. The anti-FGF receptor antibody was a polyclonal antibody raised in rabbits against purified human recombinant FGF receptor (Flg-5) extracellular domain (Austral Biologicals, CA, USA). This polyclonal antibody recognises recombinant human FGF receptor as evidenced by Western analysis (Figure 1).

Light microscopic immunohistochemistry

Immunohistochemistry was performed using a three-step indirect immunoperoxidase method (streptavidin–biotin–peroxidase complex) as previously reported (Hughes and Hall, 1993) with a slight modification. Briefly, 4 μm sections were mounted on poly-L-lysine-coated glass slides, air-dried, and deparaffinised with graded xylene and alcohol. For basic FGF staining, protease digestion was carried out using protease K (Boehringer Mannheim Biochemica, Germany) at a concentration of 40 μg ml⁻¹ for 5 min at 37°C to facilitate penetration of the primary antibody. Following a phosphate-buffered saline (PBS) rinse, the sections were immersed in absolute methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity, and incubated with normal goat serum at a 1:30 dilution for 30 min at room temperature to block non-specific binding. Each primary antibody was diluted in PBS/0.3% bovine serum albumin and used at the predetermined optimal dilution (10 μg ml⁻¹). After overnight incubation at 4°C, the sections were rinsed in PBS and incubated for 1 h at room temperature with a biotinylated goat anti-mouse or goat anti-rabbit IgG (Dakopatts, Copenhagen, Denmark). The peroxidase labelled streptavidin (Dakopatts, Copenhagen, Denmark) was then added for 30 min at room temperature. Reaction products were developed by immersing the sections in a 3.3'-diaminobenzidine tetrahydrochloride solution containing 0.1% hydrogen peroxide. Slides were counterstained lightly with methyl green. In each immunostaining run, the primary antibody was replaced by non-immune normal mouse serum (Dako, Santa Barbara, CA, USA) or PBS as negative controls, which resulted in no detectable staining. Sections from normal skin tissue specimens were used as positive controls which showed positive staining of sweat and sebaceous glands (Hughes and Hall, 1993).

Immunohistochemical quantification of staining with basic FGF or FGF receptor

The degree of primary antibody reactivity on individual tissue sections was scored semi-quantitatively (percentage of stained carcinoma cells in the section) by two authors (TO and YT) without knowledge of the patients’ outcome or clinicopathological features. Tumours with more than 5% stained cells were defined as positive and all others as negative. The proportion of positively stained tumour cells was subdivided as follows: minimal (+) denotes 5–25% of cells positive, moderate (+++) denotes 25–50% of cells positive, and marked (+++) denotes more than 50% of cells positive. In addition, staining intensity was evaluated visually and each specimen was assigned to one of the follow-

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Figure 1 Western blot analysis of the specificity of anti-FGF receptor polyclonal antibody. The recombinant human FGF receptor (Austral Biologicals, CA, USA) conjugated with BSA (0.1 μg per lane) was electrophoresed, blotted onto a nitrocellulose membrane, and immunoreacted with anti-FGF receptor antibody (a) and non-immune normal rabbit serum (b) at 1:200 dilution in PBS. As a result, the anti-FGF receptor antibody immunoreacted with recombinant human FGF receptor conjugated with BSA, forming a single major band of approximately 68 kDa. In contrast, non-immune normal rabbit serum showed no reaction with this antigen.

Figure 2 Light microscopic immunostaining for basic FGF and FGF receptor in normal human pancreas. (a) Basic FGF immunoreactivity is present in a heterogenous pattern in acinar cells, and is rarely present in ductal cells (×140); (b) FGF receptor is present in ductal cells and centroacinar cells, however, there is no staining in the acinar cells. Endothelial cells in the stroma (arrows) occasionally show FGF receptor immunoreactivity (×140).
ing categories: no staining (−), weak staining (W), and strong staining (S).

To determine the relationship between the overexpression of basic FGF or the FGF receptor and the biological behaviour of invasive ductal adenocarcinoma of the pancreas, the 32 patients were classified into two groups according to the proportion of positively stained tumour cells: group 1, patients with no staining or with less than 25% positive tumour cells (low-expression group); group 2, patients with more than 25% positive tumour cells (high-expression group).

Electron microscopic immunocytochemistry
Sections immunostained using the three-step indirect immunoperoxidase method described above were post-fixed with 0.5% osmium tetroxide for 20 min at room temperature. After block-staining with uranyl acetate, the sections were dehydrated in graded ethanol, embedded in Epon 812, and cut into ultrathin sections.

Statistical analysis
Statistical comparisons on baseline data between the two groups were performed by the chi-square test. The cumulative survival rate was calculated by the Kaplan–Meier method. This was done under the consideration that the number of cases in each group was not large. Statistical analysis of differences between the two groups was made by the log-rank test. The difference was considered to be significant when \( P < 0.05 \).

Results
Light microscopic immunohistochemistry for basic FGF
In most sections of normal pancreas, moderate basic FGF immunoreactivity was present in a heterogeneous pattern in acinar cells. It was most important at the basal aspect of the acinar cells (Figure 2a). Relatively weak cytoplasmic staining of some intralobular and interlobular duct cells was also seen. However, immunostaining was rarely present in islet cells or stromal cells.

Nineteen of the 32 pancreatic ductal adenocarcinomas (59.4%) showed minimal to marked immunoreactivity for basic FGF (Table I). Eleven of the 19 positively stained tumours exhibited cytoplasmic immunoreactivity (Figure 3a,b), while the other eight showed predominantly nuclear immunoreactivity, a phenomenon which was not observed in the normal pancreas (Figure 3c). Twelve of the adenocarcinomas (40.6%) showed little or no immunostaining in the carcinoma cells. However, intense basic FGF immunoreactivity was seen in many surrounding stromal cells including fibroblasts and macrophages (Figure 3d). In areas of associated chronic pancreatitis, there was a considerable increase in basic FGF immunoreactivity in the atrophied acinar and ductal cells in comparison with normal pancreas.

Light microscopic immunohistochemistry for FGF receptor
Most sections of normal pancreas showed intense cytoplasmic staining for FGF receptor in intralobular, interlobular

![Figure 3](image-url)
Table I  Immunostaining of human pancreatic cancer specimens with anti-basic FGF and anti-FGF receptor antibodies

| Case Number | Stained proportion | Basic FGF Staining intensity | Staining pattern | FGF receptor | Stained proportion | Stained intensity |
|-------------|--------------------|-----------------------------|------------------|--------------|--------------------|------------------|
| 1           | +++                | ++                          | W                | N            | +++                | S                |
| 2           | ++                 | +                            | W                | C            | ++                 | S                |
| 3           | + +                | +                            | S                | C            | + +                | S                |
| 4           | + + +              | + +                          | S                | N            | + + +              | S                |
| 5           | ++                 | +                            | W                | C            | ++                 | S                |
| 6           | + + +              | + +                          | S                | N            | + + +              | S                |
| 7           | + + +              | + +                          | S                | N            | + + +              | S                |
| 8           | + + +              | + +                          | S                | N            | + + +              | S                |
| 9           | + + +              | + +                          | S                | C            | + + +              | S                |
| 10          | + +                | +                            | S                | C            | + +                | S                |
| 11          | + +                | +                            | W                | C            | + +                | S                |
| 12          | + + +              | + +                          | W                | C            | + + +              | S                |
| 13          | + +                | +                            | W                | C            | ++                 | S                |
| 14          | + +                | +                            | S                | N            | + +                | S                |
| 15          | + +                | +                            | W                | C            | + +                | S                |
| 16          | + +                | +                            | S                | N            | + +                | S                |
| 17          | + +                | +                            | S                | N            | + +                | S                |
| 18          | + +                | +                            | W                | C            | + +                | S                |
| 19          | + +                | +                            | W                | C            | + +                | S                |
| 20          | + +                | +                            | W                | C            | + +                | S                |
| 21          | + +                | +                            | W                | C            | + +                | S                |
| 22          | + +                | +                            | W                | C            | + +                | S                |
| 23          | + +                | +                            | W                | C            | + +                | S                |
| 24          | + +                | +                            | W                | C            | + +                | S                |
| 25          | + +                | +                            | W                | C            | + +                | S                |
| 26          | + +                | +                            | W                | C            | + +                | S                |
| 27          | + +                | +                            | W                | C            | + +                | S                |
| 28          | + +                | +                            | W                | C            | + +                | S                |
| 29          | + +                | +                            | W                | C            | + +                | S                |
| 30          | + +                | +                            | W                | C            | + +                | S                |
| 31          | + +                | +                            | W                | C            | + +                | S                |
| 32          | + +                | +                            | W                | C            | + +                | S                |

Stained proportion: --, all cells negative or <5% of cells positive; +, 5–20% of cells positive; ++, 25–50% of cells positive; ++++, 50–100% of cells positive. Staining intensity: --, no staining; W, weak intensity; S, strong intensity. Staining pattern: N, nuclear staining type; C, cytoplasmic staining type.

Figure 4 Light microscopic immunostaining for FGF receptor in human pancreatic ductal adenocarcinomas. FGF receptor is found on both the cell surface and in the cytoplasm, and is especially prominent at the apical surfaces of carcinoma cells (a, ×70; b, ×182).

Relationship between basic FGF or FGF receptor expression levels and clinicopathological features in pancreatic cancers

No statistically significant difference in tumour size, tumour location, anterior capsular invasion, retroperitoneal invasion, histological differentiation, presence of lymph node metastases, presence of liver metastases, or tumour stage were found between the low and high basic FGF expression groups (Table II). In contrast, significant difference in retroperitoneal invasion (P<0.05), lymph node metastasis (P<0.05), and tumour stage (P<0.01) was found between the low and high FGF receptor groups (Table II).

Survival analysis

Survival data were available for 28 of the 32 patients. There was no significant difference in post-operative survival between the low and high basic FGF expression groups (Figure 6). In contrast, low FGF receptor expression was associated with longer post-operative survival as compared with high FGF receptor expression and this difference was statistically significant (P<0.01), although the low FGF receptor expression group represented only a small subgroup of the total population (Figure 7).

Discussion

The detection of small pancreatic cancers in Japan has been increasing with improvements in diagnostic methods and the
discovery of tumour markers for pancreatic cancer (Ariyama et al., 1990; Satake et al., 1991). However, even if pancreatic ductal adenocarcinomas, excluding an intraductal variant of mucin-producing pancreatic tumour (Morohoshi et al., 1989), are detected early and completely resected, the incidence of recurrence after pancreatectomy is high and the prognosis is poor (Kayahara et al., 1993; Ohta et al., 1993). This may be due to the aggressive biological behaviour of this cancer.

Recently, various prognostic factors for pancreatic cancers, including DNA nuclear content analysis, argyrophilic nucleolar organiser region (Ag-NOR) counts, and the presence or absence of overexpression of various proto-oncogenes, growth factors, and their receptors have been investigated. However, there have been only a few reports of reliable prognostic factors for pancreatic cancers (Alanen et al., 1990; Motojima et al., 1991; Tian et al., 1992; Nakamori et al., 1993). Therefore, it is essential to examine resected specimens for features that might correlate with survival. These features, if identified, would be a guide to prognosis after operation.

Basic FGF has been implicated in tumour angiogenesis through its ability to stimulate the growth of endothelial cells (Folkman and Klagesburn, 1987). Additionally, this growth factor stimulates fibroblast and epithelial cell growth (Rizzino et al., 1986; Ristow and Messmer, 1988). Basic FGF mediates its biological effects by binding to a high-affinity cell surface receptor (FGF receptor) containing an intracellular tyrosine kinase domain (Fresel et al., 1986; Olwin and Hauschka, 1989; Klagesburn and Baird, 1991). Schweigerer et al. (1987b) reported that basic FGF is an autocrine growth factor for human embryonal rhabdomyosarcoma cells. In addition, human gastric cancers, gliomas, meningiomas and renal cell carcinomas have been reported to express basic FGF mRNA (Takahashi et al., 1990; Zaggag et al., 1990; Tanimoto et al., 1991; Eguchi et al., 1992), and Kaposi's sarcoma cells have been reported to release basic FGF into their medium (Ensoli et al., 1989). However, basic FGF lacks a typical signal peptide region which facilitates secretion (Gospodarowicz et al., 1987) and its release mechanism remains unknown. Cell lysis or leakage may be involved in the release of basic FGF as the existence of similar mechanisms has been proposed for interleukin-1, another growth factor that lacks a signal peptide (Auron et al., 1984; March et al., 1985; Schweigerer et al., 1987a; Lemoine et al., 1993).

Previous studies have demonstrated that human pancreatic carcinoma cell lines overexpress basic FGF and the FGF receptor (Beauchamp et al., 1990; Lemoine et al., 1993). In addition, a recent study has indicated that there are increased levels of basic FGF and FGF receptor in human pancreatic cancers as compared with normal human pancreatic tissues, using immunohistochemical staining, northern blotting, and in situ hybridisation (Kobrin et al., 1993; Yamanaka et al., 1993; Leung et al., 1994). In the present study, we demonstrated the presence of basic FGF and FGF receptor expression in human pancreatic cancers and normal pancreatic tissues by immunocyto- and immunohistochemistry. In the normal pancreas, moderate to marked basic FGF immunoreactivity was present in a heterogeneous pattern at the basal aspect of acinar cells, and intense cytoplasmic FGF receptor immunoreactivity was seen in intralobular, interlobular and main pancreatic duct cells. Additionally, in the human pancreatic cancers minimal to marked basic FGF immunoreactivity was noted in 19 (59.4%) of the 32 tumours and 30 (93.8%) tumours showed minimal to marked cytoplasmic staining for FGF receptor. This suggests that there is concomitant expression of basic FGF and FGF receptor in pancreatic ductal adenocarcinomas, which may allow for excessive autocrine growth stimulation. Furthermore, eight (25%) tumours had nuclear staining for basic FGF, supporting the concept of an intracellular stimulating effect like that of the protein (Yamamoto et al., 1991; Nakazaki et al., 1992), i.e. the presence of basic FGF protein in the nucleus has raised the possibility of specific nuclear functions for this molecule in addition to signalling at the cell surface (Mason, 1994). Thus, tumour-derived basic FGF may play a role as a

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Figure 5 Immuno electron micrograph for basic FGF in human pancreatic ductal adenocarcinoma. (a) Immunoreactivity in a fibroblast adjacent to carcinoma cells is mainly located in the cytosol adjacent to the rough endoplasmic reticulum (× 8800); (b) Carcinoma cell with intense immunoreactivity in the cytosol, and rarely in the rough endoplasmic reticulum and Golgi apparatus (× 16 000).
Table II Relationship between basic FGF or FGF receptor expression level and clinicopathological features in human pancreatic cancers

| Variablesa | Basic FGF       | FGF receptor  |
|------------|-----------------|---------------|
|            | Low expression group (%) | High expression group (%) | Low expression group (%) | High expression group (%) |
| No. of patients | 15               | 17            | 7              | 25             |
| Tumour size  | <=3.0 cm        | >3.0 cm       |
|             | 3 (20)          | 4 (24)        | 3 (43)         | 4 (16)         |
| Tumour location | Head        | Body and tail |
|             | 13 (87)         | 11 (65)       | 4 (57)         | 19 (76)        |
| Anterior capsular invasion | Negative | Positive |
|             | 8 (53)          | 7 (41)        | 4 (57)         | 11 (44)        |
| Retropertioneal invasion | Negative | Positive |
|             | 3 (20)          | 3 (18)        | 4 (57)         | 2 (8)          |
| Histological differentiation | Well/moderately | Poorly |
|             | 14 (93)         | 15 (88)       | 7 (100)        | 22 (88)        |
| Lymph node metastasis | Negative | Positive |
|             | 3 (20)          | 1 (6)         | 3 (43)         | 1 (4)          |
| Liver metastasis | Negative | Positive |
|             | 12 (80)         | 16 (94)       | 4 (57)b        | 24 (96)b       |
| Tumour stage | I/II | III/IV |
|             | 3 (20)          | 4 (24)        | 1 (14)         | 6 (24)         |

*Histological findings are evaluated according to the General Rules for Cancer of the Pancreas proposed by the Japanese Pancreatic Society (1986). Analysed by chi-square test. P<0.05.

Figure 6 Cumulative survival curves of patients with resected pancreatic ductal adenocarcinomas, subdivided according to the basic FGF expression level. O—O, High-expression group (positive cells >25%); ●—●, low-expression group (positive cells <25%). There is no significant difference in post-operative survival between the low and high basic FGF expression groups.

Figure 7 Cumulative survival curves of patients with resected pancreatic ductal adenocarcinomas, subdivided according to the FGF receptor expression level. O—O, High-expression group (positive cells >25%); ●—●, low-expression group (positive cells <25%). Low FGF receptor expression is significantly associated with longer post-operative survival (P<0.01).

potent mitogen in tumour growth and desmoplastic response; however, the main function of this protein in human pancreatic ductal cancers may not be to promote angiogenesis because pancreatic ductal cancers are almost invariably hypovascular. In contrast, brain tumours are known to have more intense neovascularisation than other tumours and produce basic FGF as a potent angiogenic mediator (Li et al., 1994). Additionally, although 13 tumours (40.6%) showed no basic FGF immunoreactivity, intense basic FGF immunoreactivity was seen in the adjacent fibroblasts in all basic FGF negative tumours, and 11 of 13 basic FGF negative tumours (84.6%) displayed mild to marked immunoreactivity to the FGF receptor. These findings suggest that basic FGF-negative carcinoma cells could be targets for paracrine growth control by basic FGF produced by stromal components. This hypothesis is supported by several experimental studies suggesting the importance of contacts between tumour cells and fibroblasts (Tanaka et al., 1988; Coucke et al., 1992; Gartner et al., 1992).

In the present study, high levels of FGF receptor expression was associated with the presence of retropertitoneal invasion and lymph node metastasis, and with advancing tumour stage, although no statistically significant difference in variable clinicopathological factors was found between the low and high basic FGF expression groups. In addition, low FGF receptor expression was significantly associated with longer post-operative survival, whereas there was no significant difference in post-operative survival between the low and high basic FGF expression groups. Thus, overexpression of FGF receptor may prove to be a more useful prognostic marker than basic FGF expression in pancreatic cancer patients. However, a recent study (Yamanaka et al.,
including a multivariate analysis, are needed to determine whether expression of basic FGF or of the FGF receptor is a better prognostic marker for patients with completely resected adenocarcinoma of the pancreas.

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