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Expression of basic fibroblast growth factor (FGF-2)-associated with tumour proliferation in human pancreatic carcinoma

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Abstract Basic fibroblast growth factor (FGF-2) is one of the mitogens that facilitate epithelial proliferation and angiogenesis. We analysed the expression of FGF-2 and type I fibroblast growth factor receptor (FGFR1) in 20 selected cases of human pancreatic carcinoma (PC) in connection with proliferation of tumour cells and intratumour endothelial cells (ECs), using immunohistochemistry and in situ hybridization (ISH). By FGF-2 immunostaining, tumour cells were strongly positive in 10 cases (50%). By FGFR1 immunostaining, stromal fibroblasts and ECs occasionally showed positive staining. Tumour cells in 12 cases (60%) were strongly positive. Expression of FGF-2 mRNA, as examined by ISH, was detected in 12 cases (60%) of PC, and its distribution pattern was similar to that of FGF-2 immunostaining. We divided these cases into two groups according to the result of FGF-2 immunostaining, and examined the Ki67 labelling indices of tumour cells and ECs between these two groups. These two proliferative indices were significantly higher in FGF-2-positive than in FGF-2-negative cases ($P<0.05$, $P<0.05$, respectively). These findings suggest that the expression of FGF-2 in PC is strongly associated with the proliferation of tumour cells and ECs and its increased expression may give tumour a growth advantage.

Key words FGF-2 · Tumour proliferation · Angiogenesis · Hybridization · Human pancreatic carcinoma

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Introduction

Pancreatic carcinoma is one of the most devastating of neoplasms, because it is difficult to diagnose and can be resistant to therapy [11]. Although the background of its aggressive behaviour is still poorly understood, several circumferential risk factors [27] and various molecular alterations have been demonstrated, which may give carcinoma cells a growth advantage. For example, human pancreatic carcinoma exhibits a high mutation rate in the *Ki-ras* oncogene [3] and *p53* tumour suppressor gene [2]. In addition, frequent overexpression of some growth factors is also found [16, 17, 29, 30].

The fibroblast growth factor (FGF) family consists of a group of homologous growth-promoting polypeptides that have an affinity for heparin and glycosaminoglycans [4]. They have the potential to promote mitogenesis, angiogenesis and chemotaxis, thereby playing an important part in tissue development, differentiation and repair [7, 10, 20]. They are overexpressed in certain solid tumours and may be related to acceleration of neoplastic processes and poor prognosis of patients [30].

Basic FGF, known as FGF-2, is one member of this family. It is overexpressed in a significant proportion of human pancreatic carcinomas [14, 30], perhaps for modulating disease processes via autocrine and paracrine effects. It binds to high-affinity transmembrane receptors which contain intracellular tyrosine kinase domains [13], and the expression of these receptors has also been demonstrated in pancreatic carcinoma [16]. FGF-2 also promotes every phase of angiogenic processes, inducing synthesis of proteinases [21], stimulating endothelial cell migration and DNA synthesis [23], and promoting the formation of differentiated capillary tubes in vitro [22]. It thus appears probable that FGF-2 participates in tumour angiogenesis, an integral process in tumour cell growth.

We examined the expression of FGF-2 and high-affinity type I fibroblast growth factor receptor (FGFR1) immunohistochemically in paraffin-embedded sections of pancreatic carcinoma tissues. In addition, the presence of

FGF-2 mRNA was also investigated by in situ hybridization (ISH). To clarify the proliferation of tumour cells and vascular endothelial cells, we applied the mitotic index and expression of MIB-1 (Ki67 LI). We report here on the correlation between tumour cell proliferation and intratumour endothelial cell proliferation in pancreatic carcinoma; the possible role of FGF-2 will also be discussed.

Materials and methods

Twenty surgical cases of pancreatic carcinoma were selected from our lists (1992–1994). Their histopathological types were decided according to the classification of Kloeppel et al. [15]. Haematoxylin and eosin (H&E)-stained slides were reviewed, and paraformaldehyde fixed-paraffin-embedded tissues containing infiltrating tumour portions were selected. Non-tumoural pancreas tissues removed with carcinoma (10 cases) were used for control purposes.

Immunostaining was performed as previously described [16, 30]. Consecutive 4-μm-thick sections were dewaxed and incubated at 4°C for 15 h with primary antibodies. Monoclonal primary antibody against bovine FGF-2 (Upstate Biotechnology, Lake Placid, N.Y.) was used at a dilution of 1:50. This antibody is highly specific for FGF-2 from human, bovine and rodent sources. It reacts with the high-molecular-weight forms of human FGF-2 [16] and does not cross-react with other FGFs. Monoclonal antibody for bovine FGFR1 (Chemicon International, Temecula, Calif.) was also used at a dilution of 1:50. It can react with the human *flg* gene product and cross-react, to a lesser degree, with the *bek* gene product. Then the sections were incubated for 1 h at room temperature with a biotinylated anti-mouse immunoglobulin secondary antibody and incubated for 30 min with ABC according to the manufacturer·s instructions (Dako, Glostrup, Denmark). 3-Amino-9-ethylcarbazol (Dako) or New-Fuchsin (Dako) was used as a chromogenic substrate. Using these different chromogens, we presented FGF-2 as brown-staining and FGFR-1 as red-staining. Counterstaining was performed with Mayer's haematoxylin. One thousand tumour cells were counted, and when more than 10% of the cells clearly showed positive staining the case was recorded as positive [9]. The specification of the two antibodies was tested by inhibition of binding after preabsorption of antibodies with tenfold excess of recombinant human basic FGF (Oncogene Science, Uniondale, N.Y.) or bovine basic FGFR1 (Upstate Biotechnology), and the retention of staining after pre-absorption with bovine serum albumin.

In double staining we applied immunostaining for Ki67 subsequent to labelling for CD34, according to Vartanian and Weidner [26]. We applied primary monoclonal antibody for CD34 (NU4-A1, Nichirei, Tokyo, Japan) (dilution 1:50) and 3,3·-diaminobenzidine tetrahydrochloride as chromogenic substrate. The sections were immediately microwaved at 500 W for 15 min, and then Ki67 detection was performed by using MIB-1 antibody (Immunotech, Marseille, France) (dilution 1:100) as primary antibody and New-Fuchsin as chromogenic substrate. The remaining procedures were the same as described earlier. Using 3-amino-9-ethylcarbazol or New-Fuchsin, we presented Ki67 antigen as red-staining and CD34 antigen as brown-staining.

For ISH, we used the anti-sense oligonucleotide (5'-GCA GAA GAG AGA GGA GTT GTG TCT ATC AAA GGA GTG TGT GCT-3') complementary to the human FGF-2 mRNA coding for amino acids 66–79. This sequence was compared with all known primate nucleic acid sequences, including ribosomal RNA, entered in the translated GenBank database and was found to be unique. We also used sense oligo-DNA corresponding to these mRNA sequences. They were labelled with a complex of biotin-11-dUTP and dATP by means of a biotin-tailing kit (Sumitomo Metal, Tokyo, Japan). ISH was done according to the method reported by Furuta et al. [8] with some modifications: 4-μm-thick sections were placed on 3-aminopropyltriethoxyslane coated slides, digested with 0.05% pepsin (diluted with 0.2 N HCl) for 13 min, and prehybridized for 3 h at 37°C with a mixture consisting of 50% deionized formamide, 1x Denhardt's solution, 2x SSC, 100 μg/ml of denatured salmon sperm DNA, 100 mg/ml of transfer RNA, and 10% dextran sulphate. Hybridization was performed for 15 h at 37°C by adding 20 μl of hybridization mixtures containing 2 ng/μ l of biotinylated oligonucleotide probe. The slides were washed in 2x SSC and 50% formamide at 54°C three times for 1 h each, in 1x SSC twice for 15 min at 54°C, and then in Tris-buffer saline (pH 7.4) for 10 min at room temperature. The slides were then incubated for 45 min with ABC, and visualization was performed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indoxyl phosphate solution. The slides were counterstained with methyl green. For negative control, sections were treated with RNAase or hybridized with sense probe. Similar positive criteria to those used for the immunostaining for FGF-2 were applied.

We counted the number of lumina that were encircled by CD34-positive endothelial cells to determine the density of microvessels comprising capillaries and venules less than 100 μm in diameter. Intratumour microvessel density was measured in the densest areas, which were usually located at the infiltrating periphery of the carcinoma [26]. We assessed the density as the total number of microvessels in 1 mm^2.

The Ki67 labelling index (LI) was presented as the number of Ki67-positive nuclei per 1,000 tumour cell nuclei. In an adjacent

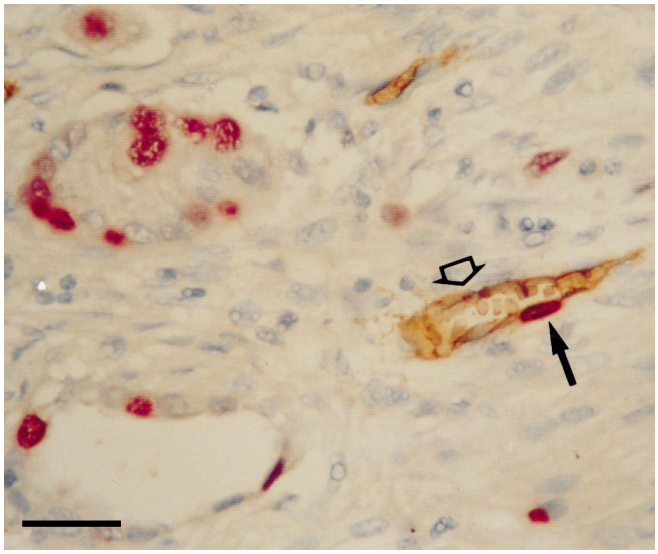
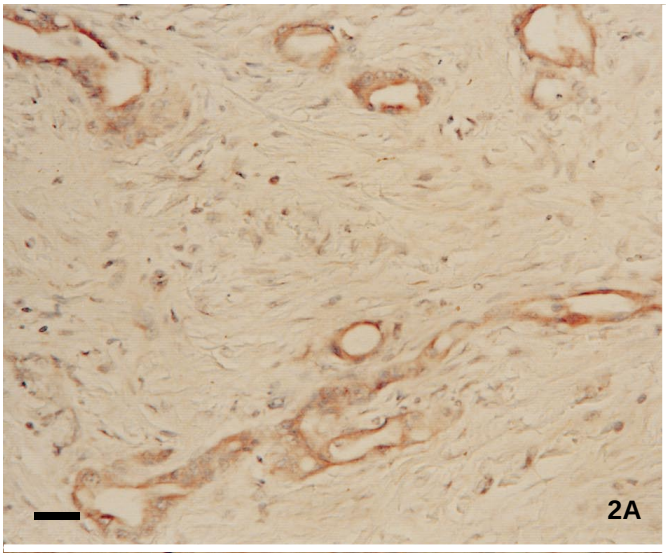


Fig. 1 Double immunostaining for Ki67 antigen and CD34 antigen. Many adenocarcinoma cells show Ki67-positive nuclear staining. CD34-positive endothelial cell (*open arrow*) also contains a Ki67-positive nucleus (*arrow*). *Bar* 100 μm

Fig. 2 **A** Positive immunostaining for FGF-2 in the cytoplasm of tumour cells. *Bar* 100 μm. **B** Atrophic acinar cells and ductal cells in pancreatitis show faint positive cytoplasmic staining for FGF-2. *Bar* 100 μm. **C, D** Codistribution of FGF-2 (**C**) and FGFR1 (**D**). Immunostaining of consecutive tissue sections of pancreatic carcinoma reveals presence of FGF-2 and FGFR1. Note similar distribution pattern of FGF-2 and FGFR1 in carcinoma cells. An endothelial cell shows faint immunoreactivity for FGFR1 (*arrow* in **D**). Stromal fibroblasts present no positive staining for either antibody. *Bar* 100 μm, ×400 ▶

Fig. 3A, B In situ hybridization representing high levels of FGF-2 mRNA with a heterogeneous cytoplasmic pattern in adenocarcinoma cells. **A** Stromal fibroblasts and endothelial cells occasionally express faint FGF-2 mRNA. *Bar* 100 μm. **B** Acinar and ductal cells in pancreatitis weakly express FGF-2 mRNA, but their number is limited. *Bar* 100 μm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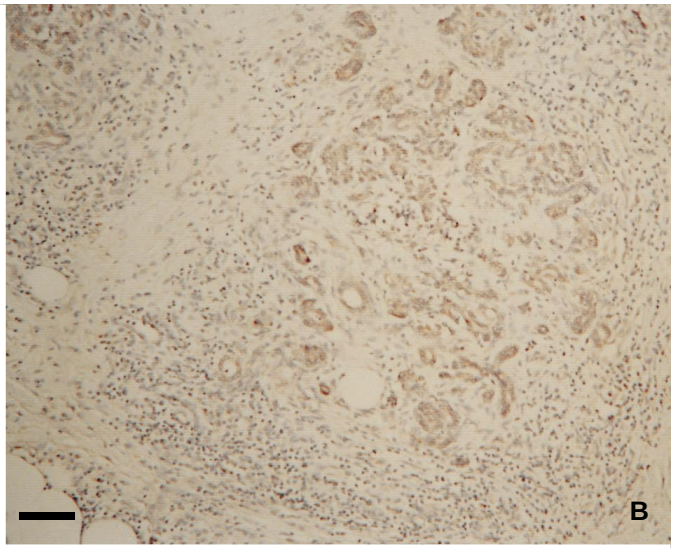
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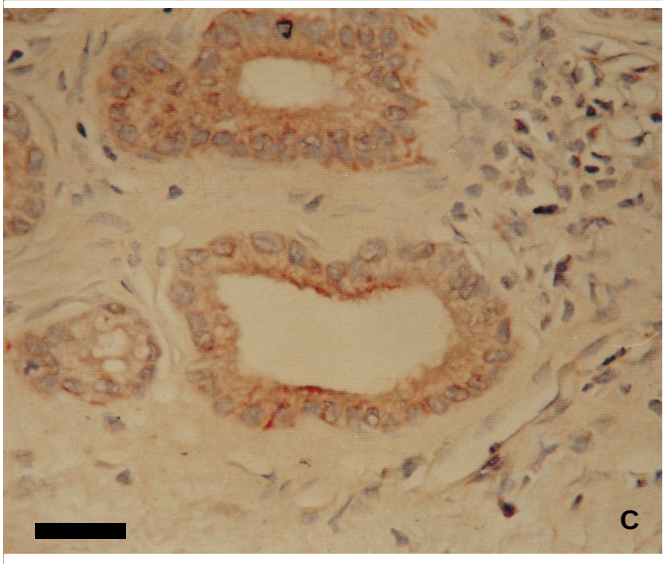
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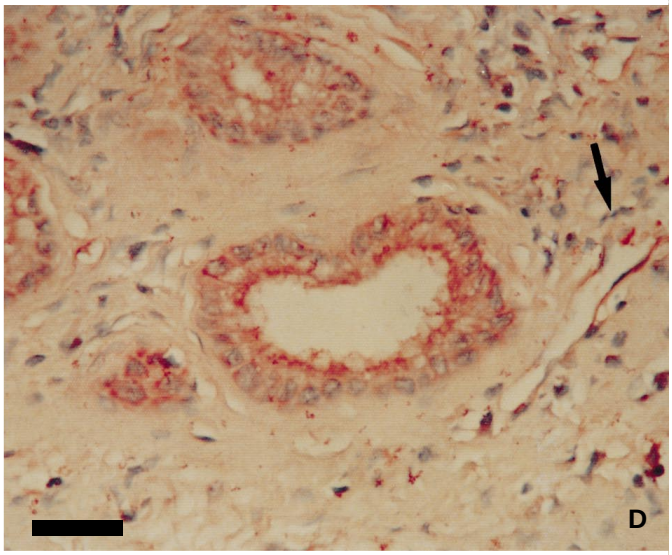
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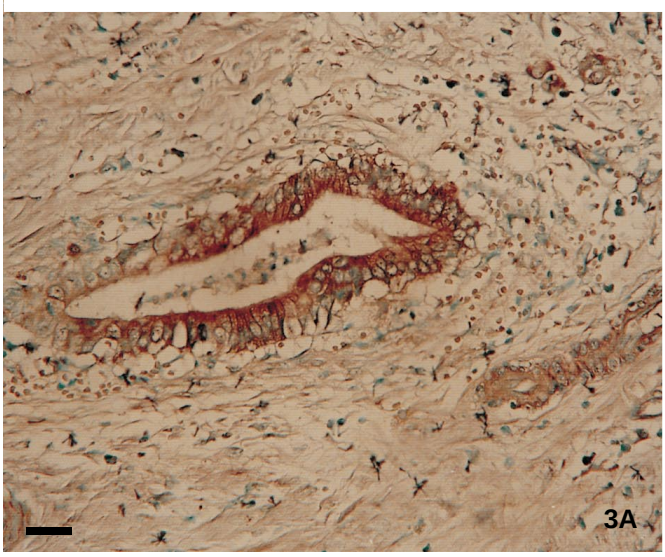
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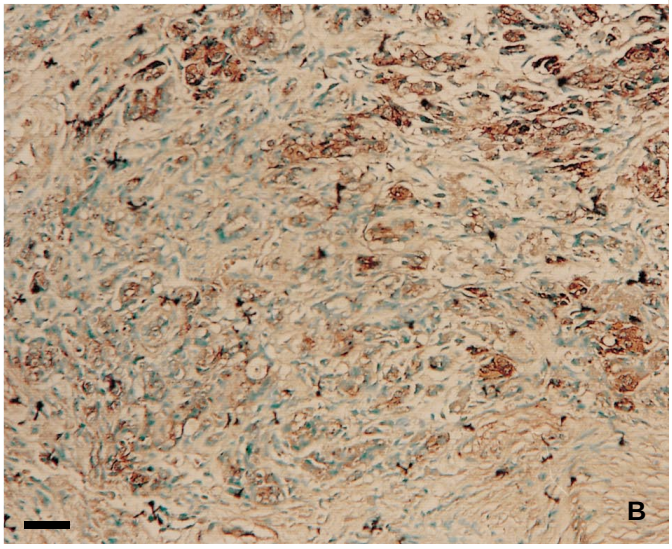
B

H&E-stained section, the mitotic figures of tumour cells were counted in a similar manner.

The endothelial cell proliferation index (ECPI) was obtained by counting Ki67-positive (red) nuclei of CD34-positive endothelial cells of microvessels (Fig. 1). For this, 1,000 endothelial cell nuclei were counted. A similar assessment was also carried out in the non-tumour pancreas in 10 cases.

Correlation analysis was done according to Pearson's correlation coefficients. We used a linear regression model to examine statistical correlations among intratumour microvessel density, mitotic index, Ki67 LI and ECPI. The Mann-Whitney U-test was performed to examine the relationship between the expression of FGF-2 and the other parameters. Significance was defined as $P<0.05$.

Results

The cases used in this study were divided into 1 (5%) adenosquamous carcinoma and 19 ductal adenocarcinomas, divided in turn into 13 (65.0%) well-differentiated types and 6 (30.0%) moderately differentiated types.

In carcinoma, positive immunostaining for FGF-2 was usually present in the cytoplasm of tumour cells (Fig. 2A, C). In non-tumour tissues, only atrophic acinar cells and ductal cells in pancreatitis showed focal and faint positive cytoplasmic staining for FGF-2 (Fig. 2B). Both fibroblasts and endothelial cells, in tumour and non-tumour tissues, were rarely positive for FGF-2.

Positive immunostaining for FGFR1 was also present in the cytoplasm of tumour cells (Fig. 2D). Furthermore, FGF-2 and FGFR1 often coexisted and took on a similar distribution pattern, as confirmed by immunostaining of serial sections (Fig. 2C, D). In addition, intratumour microvessels occasionally showed positive staining for FGFR1 (Fig. 2D), and stromal fibroblasts in some tumour tissues occasionally showed focal, faintly positive staining in their cytoplasm. In addition, pre-absorption of the antibodies with bovine serum did not eliminate the staining, and pre-absorption with antibodies resulted in inhibition of positive staining.

In situ hybridization showed FGF-2 mRNA expressed at a high level in carcinoma cells (Fig. 3A). Its distribution pattern was similar to that of FGF-2 immunostaining. In contrast, few ISH signals were present in non-tumour tissues. Acinar and ductal cells in pancreatitis weakly expressed FGF-2 mRNA, but their number was limited (Fig. 3B). Stromal fibroblasts and endothelial cells occasionally expressed ISH signals, but only in the tumour. Sections for negative control contained no specific signal.

As shown in Table 1, 10 (50.0%) and 12 (60.0%) of 20 tumour samples were positive for FGF-2 and FGFR1, respectively. All the positive cases revealed more than 30% (max. 100%) of the tumour cells with clear positive staining, and negative cases revealed very rare staining, or none at all, for each of the antibodies. Ten of the 20 cases were positive for both antibodies, and 10 were negative for both. According to ISH, 12 samples (60.0%) were positive for FGF-2 mRNA. Of these, all the tumours that expressed FGF-2 also expressed FGF-2 mRNA. In carcinoma, the expression of FGF-2 did not statistically correlate with the histological grade or the microvessel density.

We used two indices for examining the proliferative activities of tumour cells, the mitotic index and Ki67 LI by MIB-1 immunostaining. As shown in Table 1, the mitotic index, expressing the number of tumour cells undergoing mitosis, ranged from 3 to 27, with a mean value of 11.26 (median 9.75, SD ± 6.05). MIB-1-positive tumour cells were scattered throughout carcinoma tissues. The mean Ki67 LI was 117.3 (median 116.0, SD ± 58.4) and ranged from 12 to 220. These two indices of tumour cells were significantly correlated ($P<0.001$), but they did not statistically correlate with histological grade or the microvessel density.

Table 1 FGF-2 and FGFR-1 expression, cell proliferation indices, and microvessel density in pancreatic carcinoma (*FGF-2* basic fibroblast, *g* growth factor, *FGFR-1* type 1 FGF receptor, *IS* immunostaining, *ISH* in situ hybridization, *Ki67 LI* Ki67 labeling index, *ECPI* endothelial cell proliferation index)

Case	IS of FGF-2	ISH of FGF-2	IS of FGFR-1	Mitotic Index	Ki67 LI	ECPI	Microvessel density
1	–	–	–	13	91	4	134
2	+	+	+	27	178	12	50
3	–	+	+	8	129	4	133
4	+	+	+	13	149	6	68
5	+	+	+	17	158	3	118
6	+	+	+	11	136	0	216
7	+	+	+	16	160	8	157
8	–	–	–	5	76	4	90
9	+	+	+	4	91	5	61
10	+	+	+	17	70	7	36
11	+	+	+	7	162	15	50
12	–	–	–	6	83	0	134
13	–	–	–	5	73	2	16
14	–	+	+	7	72	8	22
15	–	–	–	8	78	0	26
16	+	+	+	13	200	7	69
17	+	+	+	18	220	13	59
18	–	–	–	8	142	4	85
19	–	–	–	23	198	6	127
20	–	–	–	3	12	0	52

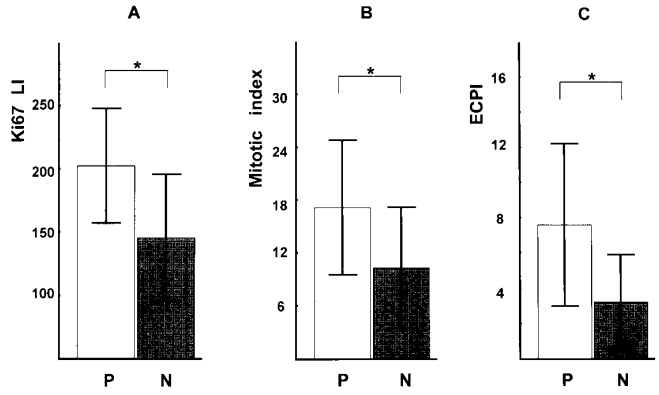

Fig. 4A–C Proliferative indices in FGF-2-positive and -negative cases. **A** Ki67 labelling index of tumour cells; **B** mitotic index of tumour cells; (C) ECPI in tumour tissue. *Column* represents average of each proliferative index. *Bars* SD. *ECPI* endothelial cell proliferation index, *P* positive cases for FGF-2 immunostaining; *N* negative cases for FGF-2 immunostaining. *Differences observed between the positive and negative cases are statistically significant ($P<0.05$)

Ki67 LI was also used for evaluating ECPI. As shown in Table 1, the mean value of ECPI in tumour tissues was 5.5 (median 5.5, SD ± 4.0), whereas it was 0.1 (median 0.0, SD ± 0.1) in non-tumour pancreatic tissue (data not shown). Intratumour microvessel density was 122.2 (median 68.5, SD ± 52.5).

ECPI in tumour tissue was significantly greater than that in the non-tumour pancreas ($P<0.001$). There were statistical correlations between ECPI and both Ki67 LI and the mitotic index of tumour cells ($P<0.01$, $P<0.05$). However, the intratumour microvessel density did not correlate with the proliferative activity indices of tumour cells and endothelial cells.

Statistical analysis was also performed to compare Ki67 LI, the mitotic index and ECPI between positive and negative carcinoma cases for FGF-2 immunostaining. We found that the Ki67 LI of tumour cells was significantly greater in FGF-2-positive cases ($P<0.05$) (Fig. 4A), as was the mitotic count ($P<0.05$) (Fig. 4B). ECPI was also significantly greater ($P<0.05$) in FGF-2-positive cases (Fig. 4C). The microvessel density did not correlate with ECPI.

Discussion

It has become apparent that FGF-2 is an important mediator of tumour angiogenesis through its stimulatory effect on endothelial cell proliferation [7]. Several biochemical analyses have also revealed its similar effect on epithelial cell growth [5, 25]. In human pancreatic carcinoma, expression of FGF-2 and its receptor has been confirmed in resected human specimens [19, 24, 30]. It remains unclear, however, whether production of FGF-2 by carcinoma cells is crucial for their unrestrained proliferation or for stimulation of other cell functions related to tumour progression, such as tumour invasion, metastatic spread and angiogenesis. This uncertainty comes principally from a lack of substantial information concerning the in vivo expression of FGF-2 and its receptors in connection with cellular activities.

Immunohistochemically, we confirmed that there was concomitant expression of FGF-2 and its high-affinity receptor (FGFR1) in a certain proportion of human pancreatic carcinoma cells. Moreover, proliferative indices of tumour cells in the FGF-2-positive cases were significantly higher than those in the FGF-2-negative cases. By means of ISH, we also clarified that FGF-2 mRNA was expressed at a high level in carcinoma cells, with a distribution pattern similar to that of its corresponding protein. Taking these immunohistological data together, it is suggested that the tumour cells expressed excessive amounts of FGF-2 because of their accelerated synthesis, and that FGF-2 was involved in tumour cell growth in an autocrine or paracrine manner. The concomitant existance of FGF-2 and FGFR-1 suggests that either a paracrine or an autocrine pathway may exist. Besides the fact that FGF-2 lacks a sequence for nuclear translocation, pancreatic carcinoma cells expressing FGFRs may respond to FGFs produced by other cell types or released from the extracellular matrix in paracrine fashion [14, 18]. Although the mechanism of FGF-2 externalization is still poorly understood, it has been suggested that it is released from the cell by exocytosis independent of the ER-Golgi pathway [21] or by cell lysis through apoptosis or cell injury. Recently, Friess et al. have reported that FGF-1 and FGF-2 are overexpressed in both acinar cell and ductal cells in pancreatitis [6], and in our study, those cells that appeared to be undergoing regressive changes also showed faint expression of FGF-2 and its mRNA. It has been proposed that cell injury results in the excessive production and release of FGF-2, which may exert paracrine effects on cell growth and differentiation.

When exogenous FGF-2 is applied to cells with FGFR, they respond well [13]. It is suggested that addition of FGFR tyrosine kinase was required in addition to the nuclear binding of FGF-2 to accomplish mitogenesis [12]. These findings suggest that FGF-2 overexpression may give tumour cells a distinct growth advantage through either autocrine or paracrine actions in pancreatic carcinoma.

Angiogenesis, the sprouting growth of new vasculature from pre-existing blood vessels, is a complex process comprising migration, proliferation and reorganization of endothelial cells [1]. Although the exact mechanism is poorly understood, it has been indicated that neovascularization can be induced by certain growth factors. Notably, FGF-2 is a potent mitogen and chemotactic factor for capillary endothelial cells [31]. In the presence of human pancreatic carcinoma, a relatively hypovascular tumour, we demonstrated that endothelial cell proliferation was significantly activated in the tumour compared with the non-tumour pancreas. Moreover, the ECPI of pancreatic carcinoma was significantly correlated with tumour cell proliferative indices, and ECPI of FGF-2-

positive cases was significantly greater than that of negative cases. For the reasons mentioned above, we speculate that FGF-2 secreted from tumour cells somehow enhanced endothelial cell proliferation.

The overexpression of FGFR1 in the tumour cells of human pancreatic carcinoma has recently been reported [16, 18]. In the present study, intratumour microvessels sometimes showed positive staining for FGFR1, although its expression was not so frequent and intense as that of carcinoma cells. We also showed that the microvessel density did not correlate with either the expression of FGF-2 or the proliferative indices of the carcinoma cells or endothelial cells. If FGF-2 produced by carcinoma cells plays a major part in direct stimulation of intratumour endothelial cell proliferation, it may be expected that the expression of FGFR1 protein will be more clearly demonstrated in endothelial cells. This difference requires further study, particularly from a technical point of view, with consideration of the type of fixative, the specificity of antibodies, the level of background, and the properties of the materials used.

In the present study, FGF-2-positive cases presented the highest proliferative indices for tumour and endothelial cells. Significant inverse correlations between high Ki67 LI and survival rate have been shown in many tumours [28, 30]. From previously reported data and our own, MIB-1 immunostaining seems to assess the malignant potential of carcinomas sastisfactorily. These findings suggest that FGF-2 overexpression may not only give tumour cells a distinct growth advantage through autocrine or paracrine mechanisms, but also stimulate angiogenesis directly or indirectly in pancreatic carcinoma. In a recent study, Yamanaka et al. [30] have reported that overexpression of FGF-2 in pancreatic carcinoma is associated with a poor prognosis. Their results may be explained by the existence of the pathway mentioned above. If this is so, the growth of pancreatic carcinoma cells and tumour angiogenesis could be inhibited by suppressing endogenous FGF-2 synthesis. Further studies on in vivo expression of FGF-2 and other growth factors and their receptors will clarify the mechanisms of tumour growth in human pancreatic carcinoma.

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