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Immunohistochemical Detection of Fibroblast Growth Factor Receptor 3 in Human Breast Cancer: Correlation with Clinicopathological/Molecular Parameters and Prognosis

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Key Words
Breast cancer · Prognosis · Fibroblast growth factor receptor 3 · Immunohistochemistry

Abstract
Objective: Reportedly, fibroblast growth factor receptor 3 (FGFR3) that regulates embryonic growth and development may function as an oncoprotein in certain malignancies. We aimed to investigate the biological significance of FGFR3 expression in invasive breast cancer. ***Methods:*** FGFR3 expression was investigated in 50 invasive breast cancer specimens by immunohistochemistry. The association between FGFR3 expression and clinicopathological/molecular parameters or prognosis was evaluated. ***Results:*** Weak FGFR3 expression was observed in myoepithelial cells, but not in duct epithelial cells, of the normal mammary ducts and lobules. FGFR3 expression in breast cancer cells was observed in 19 of 50 (38.0%) cases (9 weak positive and 10 strong positive). Besides the cytoplasm and cell membrane, nuclear staining was observed in 3 of 10 strong-positive cases. FGFR3 was further detected in non-neoplastic duct epithelial cells or duct papillomatosis in 5 strong-positive cases. No significant correlation was observed between FGFR3 expression and specific clinicopathological/molecular parameters. In contrast, FGFR3 expression was found to be significantly associated with overall survival in our cohort. ***Conclusions:*** FGFR3 expression in invasive breast cancer was not found to be significantly associated with specific clinicopathological/molecular parameters, but might be used as a candidate marker for a poor prognosis.

Introduction

Fibroblast growth factor receptor (FGFR) 3 is one of the four highly conserved and closely related cell membrane-associated tyrosine kinase receptors (FGFR1–4), which play important roles in the cell signaling pathways that regulate embryonic growth, development, cell proliferation, differentiation, and angiogenesis [1–4]. The FGFR proteins consist of an extracellular domain with three glycosylated immunoglobulin-like domains, a transmembrane domain, and a split intracellular tyrosine kinase domain [5]. The *FGFR3* gene, which localizes on chromosome 4p16.3, comprises 19 exons and 18 introns spanning 16.5 kb [6, 7]. It has been reported that a specific point mutation within the *FGFR3* gene leads to congenital anomalies such as achondroplasia and thanatophoric dysplasia [8, 9]. FGFR3 has further been suggested to act as an inhibitor of osteogenesis, and the mutations

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found in these human skeletal disorders result in constitutive activation of the tyrosine kinase domain in FGFR3 [10]. FGFR3 normally exists in two forms, FGFR3IIIc and FGFR3IIIb, which arise following an alternative splicing event in which either exon 8 or 9, respectively, is skipped [11, 12]. The FGFR3IIIb variant has been reported to be characteristic of the epithelial lineage. Another FGFR3IIIc variant, FGFR3IIIS, which is frequently expressed in tumorigenic but rarely in non-tumorigenic cells, has also been identified [13]. FGFR3IIIS is identical to FGFR3IIIc except for a 336-bp deletion resulting in loss of exons 9 and 10, and a 30-bp deletion in exon 7.

A possible oncogenic role of *FGFR3* has been uncovered by recent findings. Chromosomal translocations t(4;14)(p16.3;q32.3) resulting in a deregulated expression of FGFR3, as well as activating point mutations in the *FGFR3* gene, have been identified in a subset of multiple myeloma [14]. Activated FGFR3 has also been shown to function as an oncoprotein that acts through the MAP kinase pathway to transform NIH 3T3 cells [15]. Somatic activating mutations of the *FGFR3* gene have been additionally reported in a subset of epithelial malignancies, including cervical carcinoma, nasopharyngeal carcinoma, colorectal cancer and bladder cancer [16, 17]. In an initial study, *FGFR3* mutations were found in 3 of 12 (25%) cervical carcinomas and 9 of 26 (35%) bladder carcinomas, respectively [16]. However, an *FGFR3* mutation was subsequently found in only a single case (1.9%) from a larger study cohort of 51 carcinomas of the uterine cervix [18]. In contrast, several more recent studies involving larger series of bladder tumors have confirmed high frequencies of *FGFR3* mutations: 36, 47 and 31%, respectively, in three reports [19–21].

FGFR3 mutations in bladder tumors seemed to correlate with a favorable outcome, and it has been reported that their frequency is higher in pTa tumors (74%) compared with those of CIS (0%), pT1 (21%), and pT2–4 (16%) [19]. In another study of 57 patients with superficial bladder cancer, a significant difference in recurrence was found between patients with a wild-type *FGFR3* genotype and those harboring mutations in this gene [20]. In this same study, 14 of 23 (61%) patients bearing the wild-type *FGFR3* gene developed recurrent bladder cancer, whereas only 7 of 34 (21%) patients bearing the mutant *FGFR3* had a recurrent cancer. In another study of 772 patients with bladder tumors, *FGFR3* mutations in exons 7 and 10 were found to be more common among low malignant potential neoplasms (77%) and TaG1/TaG2 tumors (61/58%) than among TaG3 (34%) or T1G3 (17%) tumors [21]. These findings suggested that *FGFR3* mutations characterize a subgroup of bladder cancers with a good prognosis. On the other hand, association of FGFR3 expression with the pathogenesis and/or prognosis of bladder cancer remains controversial [22–24].

The involvement of *FGFR3* mutations and/or expression in breast cancer has not been well investigated to date. Sibley et al. [25] have reported that no *FGFR3* mutation could be detected in 6 breast tumors. By examining 80 breast cancer specimens and 32 non-malignant tissue samples using immunohistochemistry (IHC), Zammit et al. [26] also reported no differences in the level of FGFR3 staining between malignant and non-malignant epithelial cells. However, they did describe an altered intracellular localization of FGFR3, namely predominant nuclear staining, in human breast cancer cells.

In our present study, we performed immunohistochemical analysis of FGFR3 protein expression in 50 patients with breast cancer, correlated its expression levels with other clinicopathological features of this disease, and then investigated its possible prognostic value.

Materials and Methods

Tumor Samples

Breast cancer patients examined in this study had undergone a surgical resection at Dokkyo Medical University Koshigaya Hospital between 1990 and 2002. Cases of invasive ductal carcinoma, for which surgical specimens had been properly processed and were suitable for IHC and fluorescence in situ hybridization (FISH) analysis, were selected. Cases without a sufficient amount of samples to permit a full investigation or that had incomplete medical records to enable survival analysis were excluded. A total of 50 cases were finally included in the study cohort. The study protocol was approved by the ethical review board of Dokkyo Medical University Koshigaya Hospital (No. 0616). Tumor specimens were fixed in 20% neutral-buffered formalin for 48 h, embedded in paraffin, and then cut into 4-μm-thick sections for IHC and 5-μm-thick sections for FISH. The sections were mounted on silane-coated glass slides. Hormone receptor analysis was outsourced to a commercial laboratory (SRL, Tokyo, Japan) to which snap-frozen samples had been sent for each patient at the time of surgery. The titers of the hormone receptors were analyzed using the routine enzyme immunoassay by SRL. The cutoff values for hormone receptor status positivity were determined to be 13 fmol/mg protein for estrogen receptor (ER) and 10 fmol/mg protein for progesterone receptor (PR), according to the manufacturer's recommendations. Clinicopathological classifications and stage groupings were performed based on the General Rules for Clinical and Pathological Recording of Breast Cancer (The 15th edition) by the Japanese Breast Cancer Society [27]. This study was performed following the recommendations for tumor marker prognostic studies (the REMARK criteria) [28].

All of the patients were female, ranging in age from 32 to 72 years (with a median of 51.5 years). Forty-one patients had a tu-

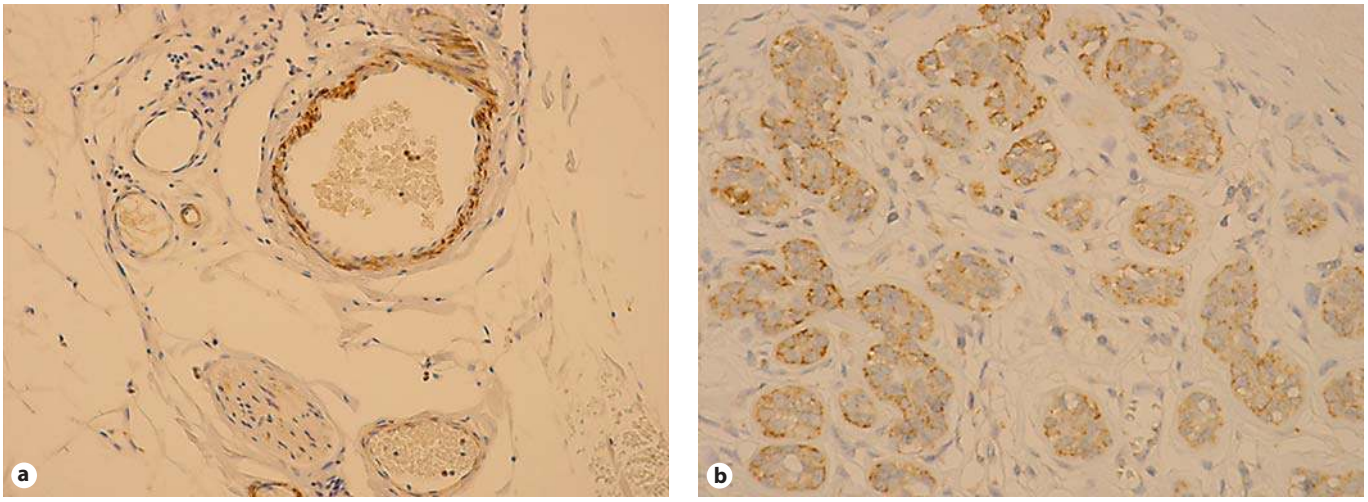

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Fig. 1. FGFR3 protein expression in normal breast tissue. Formalin-fixed, paraffin-embedded tissue samples were analyzed by IHC. Positive FGFR3 staining was observed in cytoplasm and/or cell membrane. **a** FGFR3 expression in the smooth muscle cells of the media of vessel walls (original magnification, ×4). **b** FGFR3 expression in myoepithelial cells of the lobules of the mammary gland (original magnification, ×4).

mor stage of pT1/pT2 and 9 patients were pT3/pT4. Lymph node involvement, all of which was in grade n1, was found in 28 patients. In addition to surgery, additional chemotherapies were performed in 40 patients, 2 of whom also received preoperative chemotherapy. All of the patients with lymph node metastasis or with a pT3/pT4 tumor stage received chemotherapy. Hormonal therapy was performed in 43 patients, 2 of whom also received preoperative hormonal therapy. Irradiation was performed in 39 patients, 2 of whom also received preoperative radiotherapy. Twenty-four of 28 patients with lymph node metastasis and all patients with pT3/pT4 received irradiation. Microscopically, 14 cases of papillotubular carcinoma, 12 cases of solid-tubular carcinoma, and 24 cases of scirrhous carcinoma were observed. Clinical follow-ups were undertaken for all patients for an additional 10 years after surgery.

Immunohistochemistry

FGFR3 protein expression was immunohistochemically detected using a rabbit polyclonal antibody raised against human FGFR3 (ready-to-use, LabVision, Fremont, Calif., USA). Expression of the *p53* tumor suppressor gene product (the p53 protein) was investigated using a specific monoclonal antibody (clone DO-7, 5 μg/ml, Nichirei, Tokyo, Japan). FGFR3 staining was observed predominantly in the cytoplasm and/or cell membrane, but also very occasionally in the nucleus of cells. The staining was scored as negative or as positive. Positive staining consisted of weak staining (similar or somewhat higher levels compared with smooth muscle cells or myoepithelial cells) and intense staining. For the scoring of p53 immunoreactivity, more than 500 tumor cells were counted. The percentage of cells exhibiting p53 immunoreactivity was then determined, and p53 staining was scored as either negative (<10%) or positive (>10%). The specificity of the antibody was confirmed by negative control staining using non-immune serum.

Fluorescence in situ Hybridization

FISH was performed according to the PathVysion (Vysis, Inc., Downers Grove, Ill., USA) protocol. Briefly, paraffin-embedded, 5-μm-thick tumor tissue sections were dewaxed, rehydrated, and air-dried. The sections were then boiled in 10 mM citrate buffer (pH 6.0) for 15 min in a microwave, digested with 0.2% pepsin (37°C, 10 min), and hybridized with fluorescence-labeled probes for *HER-2/neu* gene and alpha-satellite DNA for chromosome 17. The locus-specific identifier *HER-2/neu* probe is a 190-kb SpectrumOrange (Vysis) directly labeled fluorescent DNA fragment that is specific for the *HER-2/neu* gene locus (17q11.2–q12). The chromosome enumeration probe (CEP) 17 is a 5.4-kb SpectrumGreen (Vysis) directly labeled fluorescent DNA probe specific for the alpha satellite sequence at the centromeric region of chromosome 17 (17p11.1–q11.1). The probes were premixed and predenatured in hybridization buffer for ease of use. The nuclei were counterstained with 4′-6′-diamidino-2′-phenylindole. Positive controls were included in each experiment and consisted of freshly cut paraffin sections of cases known by FISH to be amplified for the *HER-2/neu* gene. The total signal numbers for HER-2 and CEP17 in 60 cancer cell nuclei were counted, and the value obtained by dividing the total number of HER-2 signals by the total number of CEP17 signals was defined as the HER-2/CEP17 ratio. A HER-2/CEP17 ratio of 2.0 or higher was judged to indicate HER-2 amplification (FISH positive), whereas samples with a ratio below 2.0 were evaluated as FISH negative [29].

Statistics

Correlations between FGFR3 expression levels and clinicopathological features or molecular markers were determined using the χ^2 test with or without a Yates' correction and/or a two-sided Fisher's exact probability test when needed. An association between candidate prognostic factors and prognosis was first analyzed using the Kaplan-Meier method linked with the log-rank

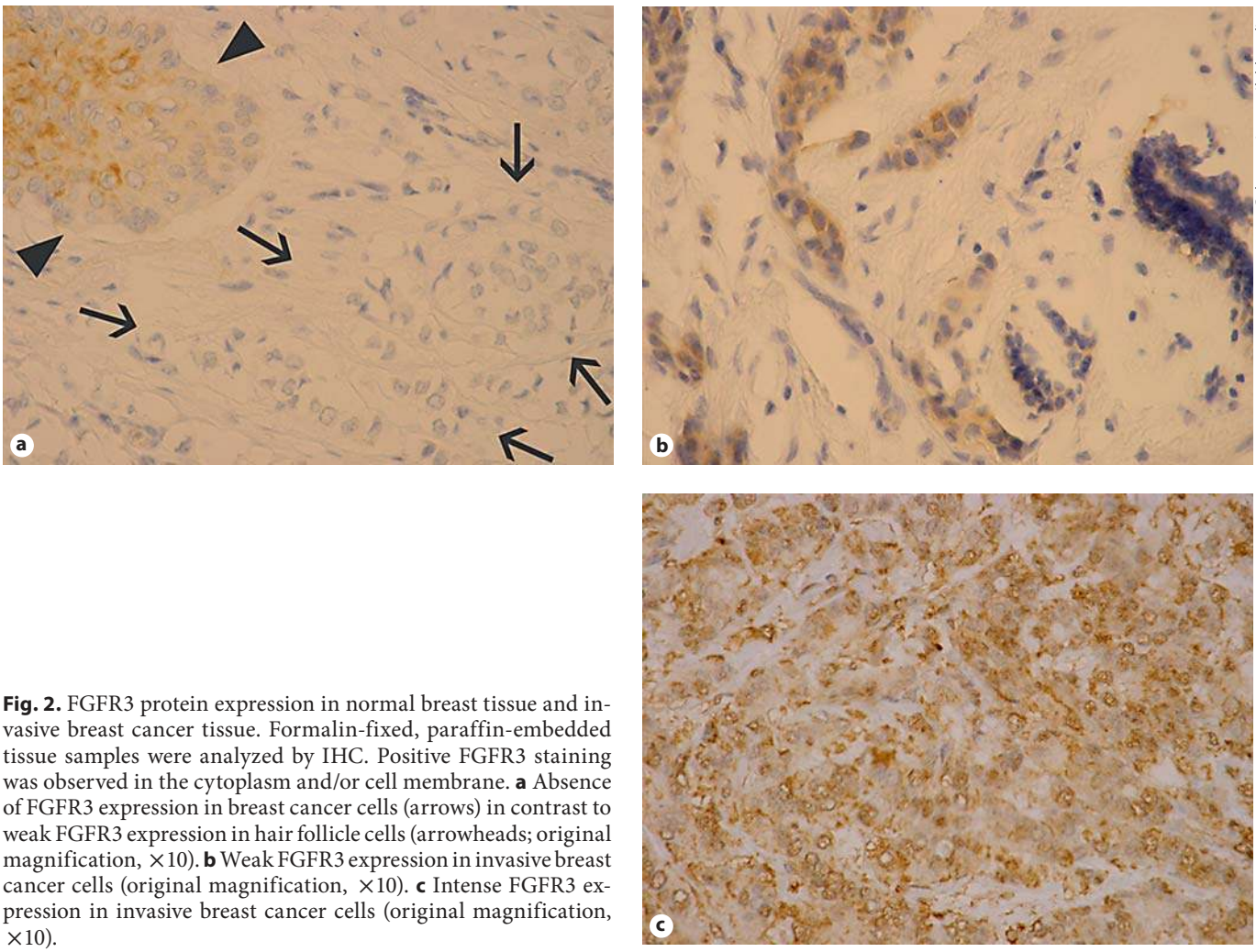

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Fig. 2. FGFR3 protein expression in normal breast tissue and invasive breast cancer tissue. Formalin-fixed, paraffin-embedded tissue samples were analyzed by IHC. Positive FGFR3 staining was observed in the cytoplasm and/or cell membrane. **a** Absence of FGFR3 expression in breast cancer cells (arrows) in contrast to weak FGFR3 expression in hair follicle cells (arrowheads; original magnification, ×10). **b** Weak FGFR3 expression in invasive breast cancer cells (original magnification, ×10). **c** Intense FGFR3 expression in invasive breast cancer cells (original magnification, ×10).

test. Parameters for which p values were found to be less than 0.20 were further tested using the multivariate Cox regression analysis (forced entry method). However, hormone receptors were excluded from any multivariate analysis because of the considerable numbers of missing values. Statistical analyses were considered significant if the p value was 0.05 or less. These analyses were performed using SPSS v11 for Windows (SPSS, Chicago, Ill., USA).

Results

Association of FGFR3 Expression with Clinicopathological Features or Molecular Markers of Breast Cancer

In our breast cancer cohort, weak FGFR3 immunoreactivity was found in the cytoplasm and/or cell membranes in non-neoplastic cells, including the vessel smooth muscle cells, myoepithelial cells of the mammary duct and/or lobule, and hair follicle cells (fig. 1, 2a). In the tumor cells, FGFR3 staining was also observed predominantly in the cytoplasm and/or cell membrane, but occasionally also in the nuclei (fig. 3). Nineteen (38.0%) of our cases exhibited positive staining for FGFR3, with 9 showing weak staining and 10 cases of intense staining (fig. 2). FGFR3 expression was observed predominantly in the cytoplasm and the cell membrane in tumor cells (fig. 3a) but was also found in the nuclei in 3 of 10 cases with intense staining (fig. 3b). Most of the non-neoplastic mammary duct epithelial cells showed little immunoreactivity to FGFR3. However, non-neoplastic duct epithelial cells and duct papillomatosis in 5 of 10 cases with intense staining also demonstrated immunoreactivity to the FGFR3 antibody (fig. 3c, d). Histologically, FGFR3 ex-

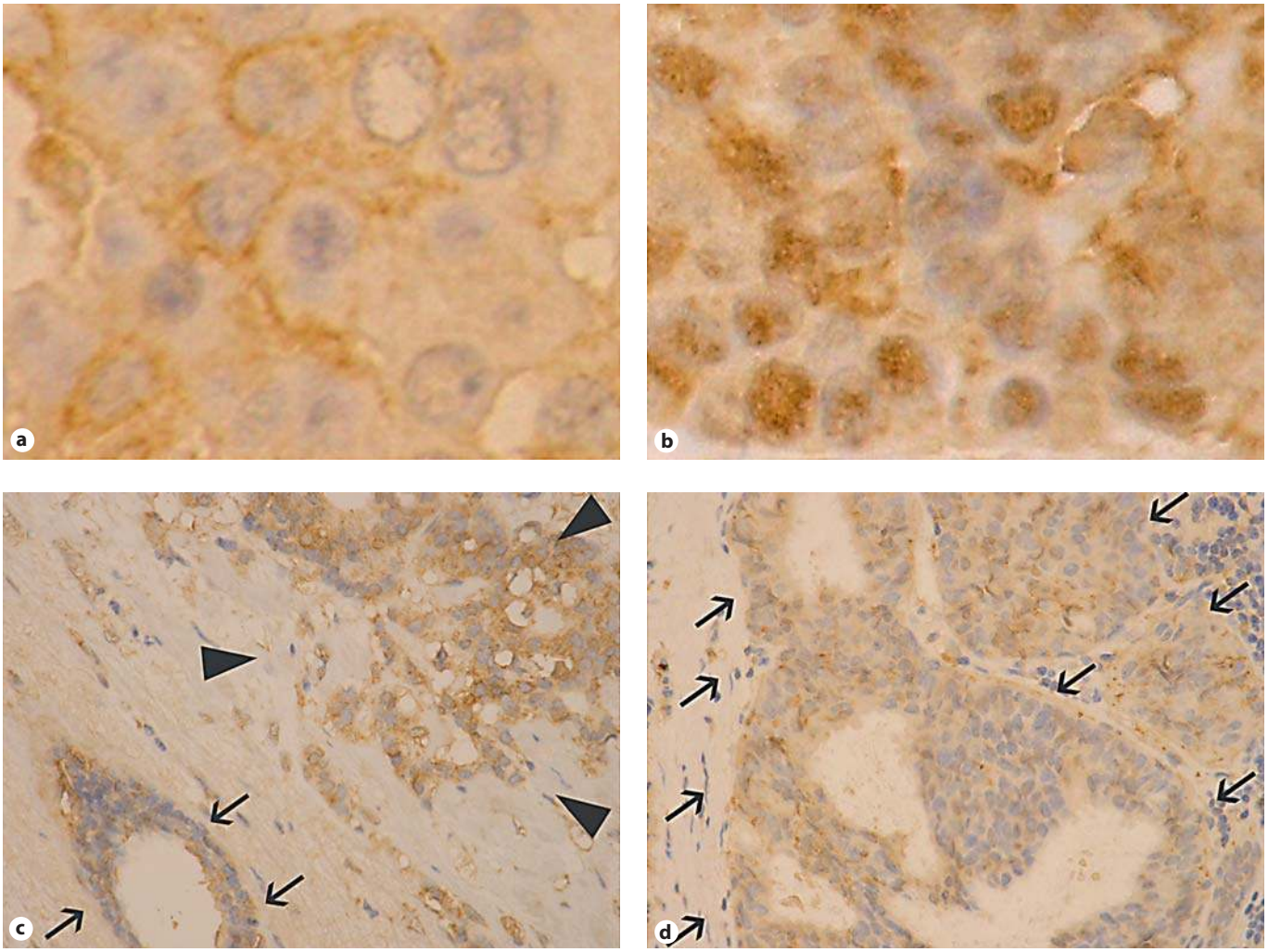

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Fig. 3. Altered FGFR3 protein expression in invasive breast cancer. Formalin-fixed, paraffin-embedded tissue samples were analyzed by IHC. **a** FGFR3 expression in the cytoplasm and/or cell membrane of breast cancer cells (original magnification, ×40). **b** FGFR3 expression in the nuclei as well as in the cytoplasm and/or cell membrane of breast cancer cells (original magnification, ×40). **c** FGFR3 expression in non-malignant duct epithelial cells (arrows) as well as in myoepithelial cells in invasive breast cancer cases with intense FGFR3 staining (arrowhead; original magnification, ×10). **d** FGFR3 expression in non-malignant duct papillomatosis (arrows) in invasive breast cancer cases with intense FGFR3 staining (original magnification, ×10).

pression was observed in 5 (35.7%) out of 14 papillotubular carcinomas, 4 (33.3%) of 12 solid-tubular carcinomas, and 10 (41.7%) of 24 cases of scirrhous carcinomas. There was no significant correlation found between FGFR3 expression and papillotubular histological subtype (p = 1.000). FGFR3 expression was noted in 15 (36.6%) of 41 cases with a pT1/pT2 tumor stage and 4 (44.4%) of 9 cases with a pT3/pT4 tumor stage (p = 0.715). In addition, FGFR3 expression was observed in 8 (36.4%) of 22 cases without nodal involvement and 11 (39.3%) of 28 cases with nodal involvement (p = 1.000).

Seventeen (34.0%) of our breast cancer cases were positive for p53 overexpression. FGFR3 expression was noted in 10 (30.3%) of 33 cases without p53 overexpression and 9 (52.9%) of 17 cases with p53 overexpression (p = 0.210). HER-2 amplification was observed in 9 (18.0%) of 50 cases. FGFR3 expression was further noted in 15 (36.6%) of 41 cases without HER-2 amplification and 4 (44.4%) of 9 cases with HER-2 amplification (p = 0.715). Hormone receptors are well-known prognostic factors in breast cancer. ER and PR expression in the tumors among our cohort had been analyzed for clinical reasons in 36 and 34

Table 1. Association of FGFR3 expression with clinicopathological features and/or molecular markers of breast cancer

Variables	FGFR3		p value
	negative (n = 31)	positive (n = 19)	
Histological type			1.000[a]
Papillotubular carcinoma	9	5	
Solid-tubular carcinoma	8	4	
Scirrhous carcinoma	14	10	
Stage			0.715[b]
pT1/pT2	26	15	
pT3/pT4	5	4	
Lymph node involvement			1.000[c]
Negative	14	8	
Positive	17	11	
Chemotherapy (non-hormonal)			0.722[b]
Not done	7	3	
Done	24	16	
Hormonal therapy			0.404[b]
Not done	3	4	
Done	28	15	
Irradiation			1.000[b]
Not done	7	4	
Done	24	15	
p53 protein			0.210[c]
Negative	23	10	
Positive	8	9	
HER-2			0.715[b]
<2	26	15	
>2	5	4	
	negative (n = 21)	positive (n = 15)	
ER			0.499[c]
Negative	9	9	
Positive	12	6	
	negative (n = 21)	positive (n = 13)	
PR			0.296[b]
Negative	10	9	
Positive	11	4	

[a] Comparison between papillotubular subtype and non-papillotubular subtype by Fisher's exact probability test.
[b] Fisher's exact probability test.
[c] χ^2 test with Yates' continuity correction.

Table 2. Univariate analysis of clinical outcomes in patients with invasive breast cancer

Variables	log-rank test (p value)	
	recurrence-free survival	overall survival
Histological type		
Papillotubular vs. non-papillotubular (n = 14 vs. n = 36)	0.0583	0.8542
Stage		
pT1/pT2 vs. pT3/pT4 (n = 9 vs. n = 41)	0.0003	0.0046
Lymph node involvement		
Negative vs. positive (n = 22 vs. n = 28)	0.0002	0.0102
ER		
Negative vs. positive (n = 18 vs. n = 18)	0.6938	0.1743
PR		
Negative vs. positive (n = 19 vs. n = 15)	0.0536	0.1041
p53 protein		
Negative vs. positive (n = 33 vs. n = 17)	0.3310	0.0214
HER-2		
<2 vs. >2 (n = 41 vs. n = 9)	0.1675	0.3996
FGFR3		
Negative vs. positive (n = 31 vs. n = 19)	0.1203	0.0018

patients, respectively. An association of FGFR3 expression with these molecular markers was found not to be statistically significant (p = 0.499 for ER and p = 0.296 for PR; table 1). Since it is known that breast cancers with interaction between carcinoma cells and stromal elements frequently belong to triple-negative breast cancer (negative for ER, PR, and HER-2), association of FGFR3 with this category was investigated. However, FGFR3 expression was not associated with this category in our small cohort (4 of 8 triple-negative cases vs. 9 of 26 non-triple-negative cases; p = 0.679 by two-sided Fisher's exact test).

Survival Analysis in Association with the Clinicopathological Features and/or Molecular Markers of Breast Cancer

The possible association of recurrence-free survival/overall survival with the clinicopathological features and molecular markers of breast cancer in our patient cohort was analyzed using Kaplan-Meier curves with log-rank test and by Cox regression analysis. Patients were followed up for up to 10 years after surgery. The Kaplan-Meier method linked with the log-rank test revealed a significant association of recurrence-free survival with tumor stage (p = 0.0003) and/or nodal involvement (p = 0.0002), and a marginal association of recurrence-free

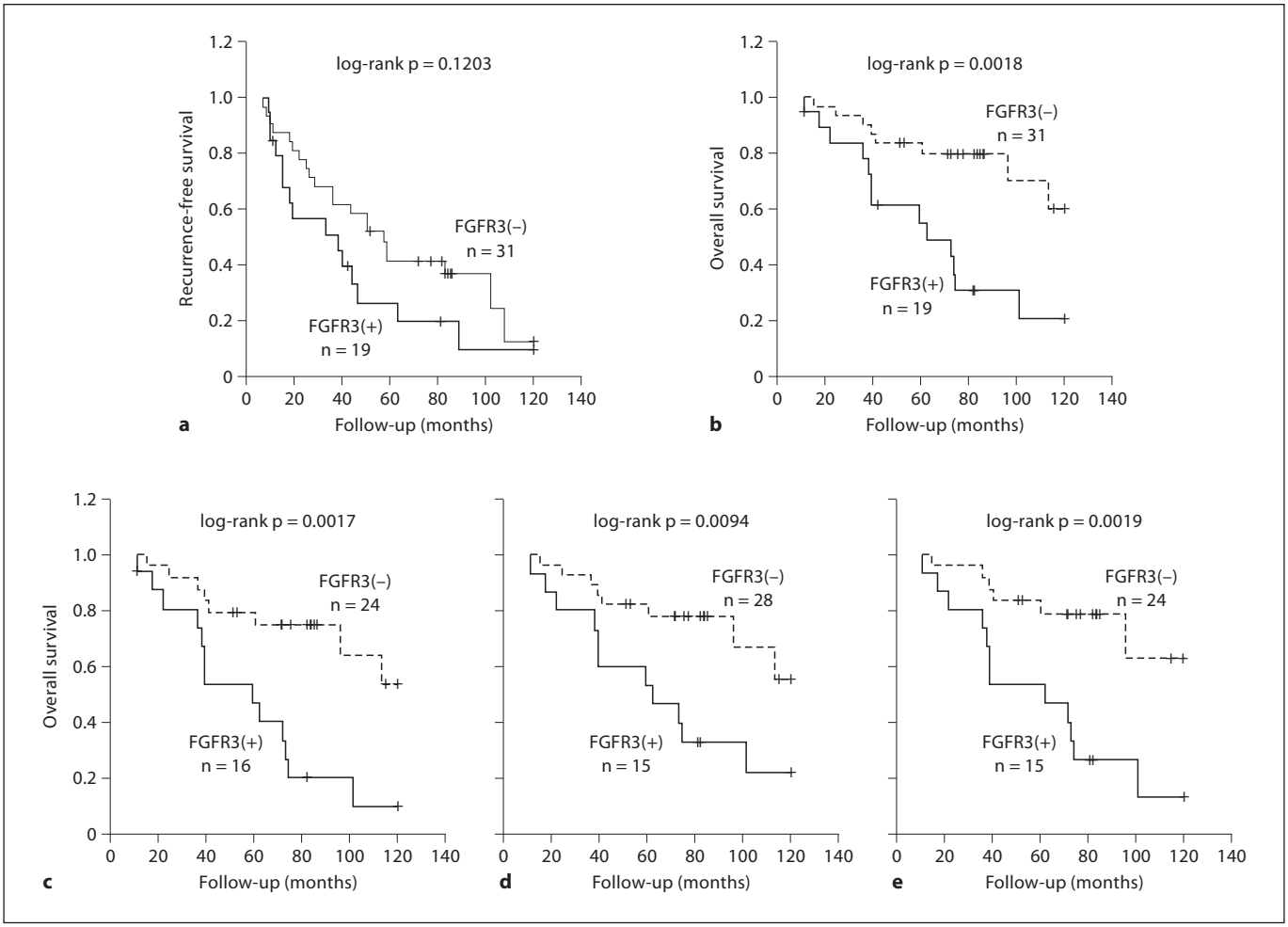

Fig. 4. Univariate analysis of prognosis according to FGFR3 expression in invasive breast cancer cases. **a** Recurrence-free survival. **b** Overall survival. **c** Overall survival in breast cancer cases that had received additional chemotherapy. **d** Overall survival in cases that received additional hormonal therapy. **e** Overall survival in cases that received additional irradiation.

survival with histological subtype (p = 0.0583), PR expression (p = 0.0536), HER-2 expression (p = 0.1675), and/or FGFR3 expression (p = 0.1203; fig. 4a). This method also revealed a significant association of overall survival with tumor stage (p = 0.0046), nodal involvement (p = 0.0102), p53 expression (p = 0.0214), and/or FGFR3 expression (p = 0.0018; fig. 4b), and marginal association of overall survival with ER expression (p = 0.1734), and/or PR expression (p = 0.1041; table 2).

Additional treatments, such as chemotherapy, hormonal therapy, and irradiation, may well affect the prognosis in cancer patients. To further validate the prognostic significance of FGFR3 expression, univariate analysis of this protein was performed in cases that received these additional therapies. FGFR3 was found to be marginally associated with recurrence-free survival in cases who had received chemotherapy, hormonal therapy, or irradiation (p = 0.0686, 0.1229, and 0.0825, respectively; data not shown). FGFR3 was also found to be significantly associated with overall survival in cases who had received chemotherapy, hormonal therapy, or irradiation treatments (p = 0.0017, 0.0094, and 0.0019, respectively; fig. 4c–e).

Multivariate Cox regression analysis via the forced entry method revealed that the pT3/pT4 tumor stage (p = 0.032), nodal involvement (p = 0.008), HER-2 expression (p = 0.036), and FGFR3 expression (p = 0.031) may be independent predictive factors for the recurrence of invasive breast cancer (table 3). This analysis also showed with

Table 3. Multivariate analysis of clinical outcomes in patients with invasive breast cancer (Cox regression analysis)

Variables	Recurrence-free survival		Overall survival	
	risk ratio (95% CI)	p value	risk ratio (95% CI)	p value
Histological type non-papillotubular	2.096 (0.814–5.397)	0.125	ND	
Stage pT3/pT4	2.609 (1.088–6.257)	0.032	2.667 (0.921–7.727)	0.071
Lymph node involvement positive	2.999 (1.328–6.773)	0.008	3.598 (1.128–11.476)	0.031
p53 protein positive	ND		2.779 (0.962–8.024)	0.059
HER-2 >2	2.671 (1.066–6.693)	0.036	ND	
FGFR3 positive	2.221 (1.076–4.584)	0.031	3.514 (1.374–8.983)	0.009

CI = Confidence interval; ND = not determined.

statistical significance that nodal involvement (p = 0.031) and FGFR3 expression (p = 0.009) may be independent predictive factors for poorer overall survival outcomes for breast cancer patients (table 3).

Discussion

Fibroblast growth factors (FGFs) and their signaling pathways appear to play significant roles not only in normal development and wound healing, but also in tumor development and progression [30]. More than 20 distinct FGFs have been identified to date, and this number is still increasing. The two best studied and most widely expressed are the so-called acidic FGF (FGF1) and basic FGF (FGF2), which are approximately 17-kDa proteins that share a 55% sequence homology. FGF1 has been found to be expressed to a much greater extent in breast cancer cells than in benign tumors such as mastopathy and fibroadenoma. In addition, FGFR1 has also been found to be overexpressed in breast cancer cells compared with benign tumor cells or interstitial cells [31]. FGFR3 is known as one of the receptors for both FGF1 and FGF2. Mutational analysis and expression analysis of FGFR3 with reference to the clinicopathological features in breast cancer has not been extensive thus far. In addition, the effects of FGFR3 expression upon clinical outcomes have not been well investigated.

Zammit et al. [26] previously reported positive FGFR3 expression in 80 breast cancer specimens and 32 non-malignant tissue samples. They detected FGFR3 expression in all of the tissues they examined and observed no differences in the level of staining between malignant and non-malignant samples. However, FGFR3 was predominantly detected in the nuclei of malignant epithelial cells but in the cytoplasm in non-malignant epithelial cells. Possible explanations for the nuclear FGFR3 accumulation in malignant cells, such as the presence of an exon-deleted form that lacks the appropriate signal peptides and the transmembrane domain (amino acids 312–422) [32], *FGFR3* gene mutations, and ligand activation, have been discussed, and further investigation of FGF1 expression in normal and malignant breast tissues has been performed [26]. FGF1 was found to be expressed in the epithelial cells but not in the stroma of the normal human breast, whilst FGF1 was shown to be highly expressed in the stroma surrounding malignant epithelial cells. Accordingly, the authors attributed the altered FGFR3 distribution to the ligand activation of FGFR3 by FGF1 [26].

In our present study, FGFR3 protein expression was observed in 19 (38%) of 50 breast cancer tissues. However, the FGFR3 protein was found to be expressed predominantly in the cytoplasm and the cell membrane, with nuclear staining noted in only 3 (6.0%) cases. In non-malignant cells, FGFR3 expression was detected in luminal epithelial cells but not in the myoepithelial cell layer in the previous study [26]. However, we found in our current experiments that FGFR3 was generally detectable in myoepithelial cells with the exception of a few cases in which duct epithelial cells showed FGFR3-positive staining along with intense immunoreactivity for this receptor in breast cancer cells. In relation to this discrepancy, we speculate that ethnic differences between the study subjects and/or distinct experimental procedures may have affected the results. Frozen samples were used for IHC in the previous study, whereas we routinely used formalin-fixed paraffin-embedded samples in our current analyses. In addition, the primary antibody against FGFR3 used in the previous study was a rabbit polyclonal antibody raised against amino acids 792–806 of FGFR3 pur-

chased from Santa Cruz, whereas our primary antibody was a rabbit polyclonal antibody raised against amino acids 359–372, which are located in the juxta-transmembrane domain. Hence, the FGFR3 detected in our present study must not be an exon-deleted form that lacks the transmembrane domain.

FGFR3 is frequently expressed in urothelial carcinoma of the urinary bladder. In a previous analysis of 126 cases, cytoplasmic and/or membrane immunostaining for this receptor was observed in 62 (49.2%) cases, comprising 34 (48.6%) of 70 non-invasive cases and 28 (50.0%) of 56 invasive cases [22]. It has also been reported that there is no statistically significant relationship between FGFR3 expression and the stage, invasiveness, p53 status, or Ki-67 labeling index of the tumors. In our present study, no statistically significant relationship between FGFR3 expression and the clinicopathological/molecular parameters of breast cancer was observed. An association of the *FGFR3* gene mutation with papillary non-invasive tumors has been reported previously in bladder cancer [19, 20], whereas no mutation has been reported in breast cancer thus far [25]. Although we did not perform mutational analysis of the *FGFR3* gene in our current experiments, we found no significant relationship between FGFR3 expression and papillotubular histological subtype.

Many clinicopathological features and molecular markers of breast cancer have been introduced as prognostic factors for this disease [33]. Clinicopathological features may include histological subtype, grade of nuclear atypia, and tumor stage (consisting of tumor size, lymph node involvement, and hematogenous metastasis). With regard to molecular markers, the expression of hormone receptors, the p53 status, Ki-67 labeling index, and the HER-2 amplification status might be included. In our present study, we confirmed that tumor stage and nodal involvement significantly affect both the recurrence-free and overall survival. In addition, histological subtype and molecular markers, such as hormone receptors and HER-2, were found to be marginally associated with recurrence-free survival, in accordance with a number of previous studies. Tumor stage and nodal involvement were also found to be significantly associated with overall survival outcomes. Molecular markers, such as p53 and the hormone receptor status, were significantly and marginally associated with overall survival, respectively. Unexpectedly however, FGFR3 expression was marginally and significantly associated with poorer outcomes in terms of recurrence-free survival and overall survival, respectively. FGFR3 is a growth factor receptor that transmits FGF1 and FGF2 signals through the cell membrane. Zammit et al. [26] have reported previously that FGF1 is highly expressed not only in epithelial cells but in the stromal cells surrounding tumor cells. It may well be the case that FGFR3 expression is the result of the upregulated production of growth factors implicated in neoplastic processes in breast tissue. The detection of FGFR3 expression in non-neoplastic duct epithelial cells and/or duct papillomatosis may support this idea. Production of growth factors, such as FGF1, in the breast cancer field may upregulate FGFR3 expression in breast cancer cells. The upregulated FGFR3, in turn, may cause progression and/or dedifferentiation of breast cancer, further production of tumor growth factors, and finally kill the patients. In short, FGFR3 expression may be implicated in tumor progression via autocrine or paracrine mechanisms, resulting in poorer outcomes for breast cancer patients. FGFR3 expression was not found to be significantly associated with other prognostic factors such as tumor stage (or tumor size), lymph node involvement, and p53 overexpression by contingency table analysis, but it was significantly associated with worse prognosis of the patients. We therefore reasoned that this result might suggest FGFR3 expression as an independent prognostic factor of breast cancer. FGFR3 expression in breast cancer might affect grade of malignancy in the manner other than histology, metastasis or speed of tumor growth. Although chemotherapy, hormonal therapy, and irradiation may affect the prognosis in breast cancer patients, FGFR3 was found to be associated with worse prognosis in patients that had received those therapies. Poor prognosis of patients with FGFR3 expression might also be attributable to resistance to therapy. Further studies of a greater number of cases will be necessary to properly verify these speculations.

In conclusion, we observed FGFR3 expression in one third of the patients we examined with invasive ductal carcinoma of the breast. Although FGFR3 expression was not found to be associated with known clinicopathological/molecular parameters, our survival analysis data suggest that FGFR3 expression might have utility as a prognostic predictor of invasive breast cancer.

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