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Original Paper

Expression of FGFR2 BEK and K-SAM mRNA Variants in Normal and Malignant Human Breast

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The expression of mRNA encoding alternative forms of fibroblast growth factor receptor 2 (FGFR2) differing in the carboxy terminal half of their third immunoglobulin-like domain, was investigated in 77 human breast cancer tissues, 12 non-malignant breast biopsies and 29 cell lines, using a reverse transcriptase (RT) polymerase chain reaction (PCR) method. RNA from the two tissue groups yielded PCR product corresponding to both the BEK and the K-SAM form; amounts normalised to glyceraldehyde phosphate dehydrogenase product were similar in both groups. The level of either variant or of the total FGFR2 product was essentially unrelated to prognosis or clinical status except that patients with advanced clinical T staging had a higher proportion of BEK to K-SAM ($P = 0.01$). RNA from 1/2 normal breast derived and 8/10 breast cancer cell lines expressed exclusively or predominantly the K-SAM form; 2/10 had significant amounts of both BEK and K-SAM mRNA. Of 12 other epithelial lines, seven expressed mainly K-SAM mRNA, four expressed BEK and one was negative. Of five non-epithelial lines, one was negative, two expressed only BEK mRNA and two had significant amounts of both variants. We conclude that tissue levels of FGFR2 mRNA are unaltered in breast cancer extracts and that the splicing mechanism for this exon selection appears not to be significantly disrupted.

Key words: human breast, fibroblast growth factor receptor, BEK, K-SAM, PCR
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INTRODUCTION

THE FIBROBLAST growth factors (FGFs) [1] constitute a family of several closely related heparin binding polypeptide growth factors whose pleiotropic range of action influences angiogenesis, mitogenesis, tissue regeneration, cellular differentiation, cell migration, neurite growth, cell senescence and embryonic development, emphasising a rather important role in almost every cellular function investigated. This remarkable diversity is enhanced by the existence of cell surface receptors encoded by at least five separate genes [2]. Adding yet further to this complexity, two of these receptors, FGFR1 and FGFR2, appear to have multiple forms. The longest transcript encodes a transmembrane protein with three extracellular Ig domains and an intracellular split tyrosine kinase domain but several shorter cDNA clones have been isolated. mRNA splicing events detected using the polymerase chain reaction (PCR), can lead to exon deletion or to alternative usage as well as alternative initiation and polyadenylation signals, resulting in four classes of predicted variants: those encoding secreted extracellular domains, those with deletions/insertions in the extracellular domain, and those with either deletions or alternative exons in the intracellular domain [2–6]. It is unclear how and when these different forms arise or indeed why there should be such diversity.

In previous studies, we have shown that breast malignancy is usually characterised by an overexpression of a variety of growth factors and hormones [7, 8], but we have not observed this to be the case for either basic FGF or for FGFR1 and FGFR2 [9]. Indeed, in our previous study, normal breast biopsies appeared to have higher mRNA levels of these factors than cancer tissue. This suggested to us that it would be more fruitful to examine spliced mRNA variants and, in particular, those which could lead to differences in ligand binding specificities. Both FGFR1 and FGFR2 have a similar arrangement of alternative exons encoding the carboxyl terminal half of the third Ig domain [10]. Several groups have cloned and identified two variants of *FGFR2* that encode receptors termed

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BEK (type IIIc) and K-SAM (type IIIb) [11–14]. This single exon exchange confers (high affinity) binding specificities for either acidic and basic FGF or acidic FGF and keratinocyte growth factor (KGF), respectively [14].

Given the obvious potential this presents for differential control of cellular response, we carried out a study on human breast biopsies and a number of breast and other tissue derived cell lines to determine (a) whether these alternatively spliced forms exist/co-exist and whether the processing differs between cancer and normal tissue, (b) whether the level of expression is related to clinical features of breast cancer and patient prognosis and (c) if the splicing is cell type related.

MATERIALS AND METHODS

Chemicals

Reverse transcriptase (MMLV RT) was purchased from GIBCO-BRL (Paisley, U.K.), Taq polymerase from Peninsula Laboratories (U.K.), Polynucleotide kinase, DNA polymerase klenow fragment and dNTPs from Pharmacia (Uppsala, Sweden). RNAzol was supplied by Biogenesis (Bournemouth, U.K.). Alpha ^{32}P-dCTP (3000 Ci/mmol) and gamma ^{32}P-dATP were purchased from Amersham (U.K.), as were Hybond N$^+$ membrane and Hyperfilm.

Oligonucleotides

Primers for *GAP* amplification have been described previously [9]. For *FGFR2*, we used two primers which were designed to span the region from amino acids 245–369: forward sequence, CTGGATGTTGTGGAGCGAT (primer 3); reverse sequence, TGTAATCTCCTTTTCTCTTCCA (primer 4). The BEK and K-SAM specific oligonucleotides used for hybridisation were CTCTTTGTCCGTGGTGTT (primer 5) and TGGGAACTATTTATCCCCG (primer 6) respectively (5′ to 3′, reverse sequences) (Figure 1).

All oligonucleotides were synthesised using phosphoramidite chemistry on an ABI DNA Synthesiser, deprotected by treatment with NH_4OH for 5–6 h at 55°C, vacuum dried and used without further purification.

Tissue samples

Breast biopsies were obtained from patients undergoing surgery (without prior treatment) at Breast Clinics principally in the Royal Marsden and St George's Hospitals in London.

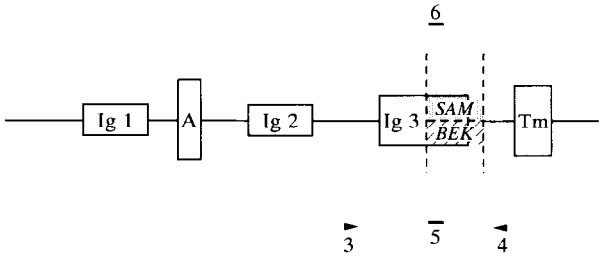

Figure 1. Schematic representation of the extracellular portion of FGFR2 showing the position of the PCR primers 3 and 4 and the specific oligonucleotides BEK (5) and K-SAM (6) used as probes. Ig 1, first Ig-like domain; A, acidic box; Ig 2, second Ig-like domain; Ig 3; third Ig-like domain; Tm, transmembrane domain; (shown in proportionate scale). The position of the alternately selected BEK and K-SAM exons are delineated by the dashed lines and incorporate the last 29 amino acids of Ig 3 and the first 20 amino acids of the region preceeding the Tm domain.

All diagnoses were histologically confirmed, and tissue samples were snap frozen and stored in liquid nitrogen until required. Tissue was obtained from 77 carcinomas from patients aged between 29 and 67 years, details of whom are presented in Table 1. The normal samples used in this study were either tissue taken from outside the tumour margins or were from benign conditions.

Cell lines

The growth conditions used for the cell lines used in this study as well as their origins have been described previously [15,16].

RNA extraction

Total cellular RNA was extracted from pulverised frozen breast tissue by the guanidinium isothiocyanate method, and from cell lines by the modified procedure utilising RNAzol [17]. Assessment of quantity and quality was carried out by spectrophotometry and agarose gel electrophoresis, respectively.

RT and PCR amplification

First strand synthesis was carried out as described previously [9] using MMLV RT and 2 μg total RNA in 20 μl. For PCR amplification, 1 μl of RT product was added to a 98 μl mixture containing (in final concentrations), 67 mM Tris–HCL pH 8.8, 1.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 200 μg/ml gelatine, 200 μM each of dATP, dCTP, dGTP and dTTP and 1 unit taq polymerase, and then divided equally into two Eppendorf tubes.

Table 1. Clinical details of breast cancer patients

Parameter	Number	%
Total	77	
Age range	29–89	
Age median	55	
Menopausal status		
Pre	23	30
Peri	8	11
Post	45	59
Unknown	1	
Clinical T stage		
T1/T2	53	83
T3/T4	11	17
Unknown	13	
Pathological stage		
T1/T2	44	69
T3/T4	20	31
Unknown	13	
Node status		
Positive	26	41
Negative	37	59
Unknown	14	
Oestrogen receptor*		
Positive	19	56
Negative	15	44
Unknown	43	
Histological type		
Infiltrating ductal	69	95
Infiltrating lobular	4	5
Unknown	4	

* Oestrogen receptor status was determined as described previously [8].

To one tube was added 1 μl of a mixture of *FGFR2* primers 3 and 4 to give 200 ng of each one, and to the other tube, 1 μl of *GAP* primers 1 and 2 at the same amounts. Samples were overlaid with mineral oil. The *FGFR2* primer-containing tubes were placed in a Hybaid thermocycler programmed for 28 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 45°C and extension for 1 min at 72°C, with a final extension of 10 min. The tubes containing *GAP* primers were placed in the thermocycler on completion of the tenth cycle so that they received only the last 18 cycles. In preliminary experiments, cycle number was varied to determine, using varying amounts of input RT product, the upper and lower limits of linear amplification. In some cases, amplification was performed for 35 cycles in order to visualise products with ethidium bromide.

Electrophoresis, blotting and hybridisation

Electrophoresis and alkali transfer to Hybond N^+ membrane was carried out essentially as described previously [9] using 10 μl of chloroform-extracted PCR product. For *FGFR2*, samples were electrophoresed and blotted in duplicate for subsequent hybridisation to ^{32}P-labelled oligo-probe 5 (BEK specific) or to oligo-probe 6 (K-SAM specific). These oligonucleotides were end-labelled by incubation at 37°C for 30 min, of 20 pmol in 20 μl containing 50 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 20 μCi gamma ^{32}P-dATP and 2 units of polynucleotide kinase, and used for hybridisation without purification. The *GAP* samples were hybridised to random primer labelled *GAP* cDNA [9].

Hybridisation was performed in a Hybaid rotisserie oven using the manufacturer's bottles. Filters hybridised with GAP probe were subsequently washed in 2 × SSC, 0.5% sodium dodecyl sulphate (SDS) at 42°C for 15 min with three buffer changes, then in 0.1% SSC, 0.1% SDS at 65°C for up to 60 min. Filters hybridised with *FGFR2* oligonucleotides were washed in 4 × SSPE at room temperature for 15 min, then at 42°C for 15–30 min and finally in 2 × SSPE at 42°C for 15–30 min. Autoradiography was performed using Amersham Hyperfilm at −70°C with intensifying screens. Bands were quantified by laser densitometry.

Calculation of results and statistical tests

Band intensities on individual autoradiographs were normalised by reference to an arbitrary sample which was included in each run and was present on each blot. Results are expressed as a ratio of the autoradiographic signals obtained with oligo-probes 5 and 6 representing the relative amount of PCR product of BEK and K-SAM, respectively. The relative levels of each variant were also assessed separately by reference to the corresponding *GAP* values and are expressed as a ratio of the densitometric signal of the variant to that of the GAP PCR products. Clinical correlations were performed using the non-parametric Mann–Whitney test, and the log rank test for survival analyses.

RESULTS

Expression of BEK and K-SAM variants in tissues

In preliminary experiments, we established the optimum conditions for linear PCR amplification using the procedures described before [9]. For *GAP*, we chose 18 cycles, and for *FGFR2* we found that 28 cycles gave the most consistent results, being within the linear phase of amplification (data not shown) over a sufficient template input range. This was important in order to quantify the relative amounts of PCR product, and the rationale for this has been discussed in detail previously [9]. The *GAP* and *FGFR2* primers interfered with each other and amplification had to be performed separately for each gene. Our procedure, however, minimised tube differences, and we observed no more than 25–30% variation between random duplicate measurements.

Amplification of a breast tumour RNA sample with *FGFR2* primers 3 and 4 resulted in a single 342 bp product detectable by ethidium staining after 35 cycles of PCR. On Southern blotting, this was found to hybridise to both oligonucleotides 5 and 6, representing a sequence unique to either the BEK or K-SAM exons, respectively. Using an *FGFR2* plasmid construct containing the BEK exon as template, we found that the resulting 342 bp OCR product hybridised only to oligonucleotide 5, and not to 6. We examined RNA extracted from 12 biopsies taken either from benign breast conditions or from histologically confirmed normal areas adjacent to cancer and from 77 cases of malignant tumour. In all instances, we found that both BEK and K-SAM sequences were present although in widely varying amounts. Examples are shown in Figure 2. There was no significant difference in the relative levels of either variant between the non-malignant and cancer samples. For BEK, the median value for the non-malignant samples was 2.6 (range 0.5–229) and for the cancers, 1.25 (range 0.003–59). For K-SAM, the median value for the non-malignant tissues was 1 (range 0.3–162) and for the cancers, 0.85 (range 0.002–30). The ratio of the PCR products for the two variants was also similar for both non-malignant (median 1.65; range 0.5–31) and cancer tissues (median 2.35; range 0.03–261). However, there was a direct linear relationship between the amounts of product for the two forms in the cancer samples ($r = 0.7$) (Figure 3).

Relationship to clinical features

The BEK to K-SAM ratio obtained for the cancer samples was examined with respect to the common clinical parameters. We found a significant correlation with clinical T stage. Patients with T3/T4 staging had a greater proportion of tumour FGFR2 in the BEK form, than those staged as T1/T2 ($P = 0.01$).

There was no difference with regard to pathological T stage, menopausal status or incidence of nodal metastases (Table 2). When the tumour samples were divided into two groups using the median value, we observed no difference in either overall or relapse-free survival between such groups, taking either the amounts of the BEK or K-SAM variants separately, or as a ratio (data not shown).

Expression of BEK and K-SAM in cell lines

We analysed RNA from 29 cell lines, mostly of epithelial origin. All the breast lines examined except for HBL100 expressed FGFR2 (Table 3). In MDA-MB-361 and MDA-MB-453 this was exclusively as the K-SAM variant; in MCF7, T475D, ZR75, MDA-MB-415, SKBR111, BT20 and (the normal breast derived) HBRSV1.6.1, the K-SAM/BEK ratio varied from 16 to 50 suggesting a predominance of the K-SAM variant, but with a small amount of hybridisation observed with the BEK probe. For MDA-MB-157 and PMC42, we obtained strong signals with both probes suggesting significant levels of expression of both variants in these lines. Results with non-breast tissue derived cell lines are also shown in Table 3. Two of the gastric lines (MKN-1, MKN-

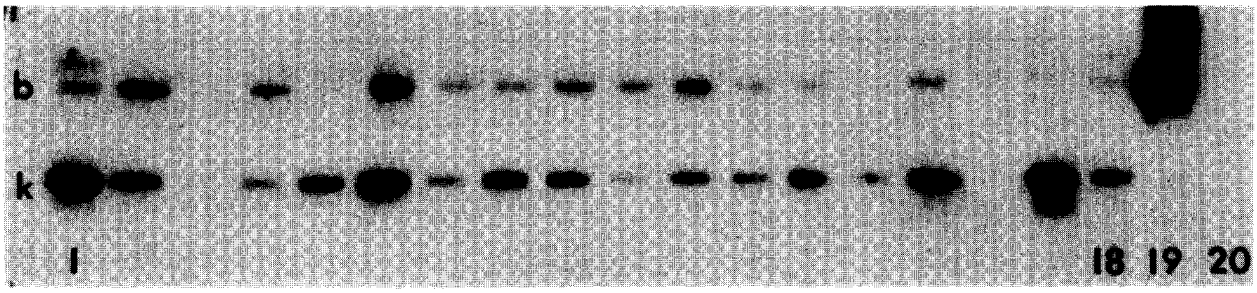

Figure 2. FGFR2 PCR products were obtained by 28 cycles of amplification with primers 3 and 4, of RT products from breast tumour RNA samples, as described under Materials and Methods. Products (10 μl) were electrophoresed in duplicate through 1.5% agarose, blotted onto Hybond N+ membrane and hybridised with ^{32}P end-labelled oligo probe 5 (BEK (b)) or 6 (K-SAM (k)). Lanes 1–18 are various random tumour samples showing differing amounts of the two FGFR2 variants. Lane 19 contains PCR product with FGFR2 (BEK form) plasmid as template illustrating the absence of crosshybridisation between the two probes. Lane 20 is a negative control. Autoradiography was performed using Amersham Hyperfilm with exposure at −70°C using intensifying screens.

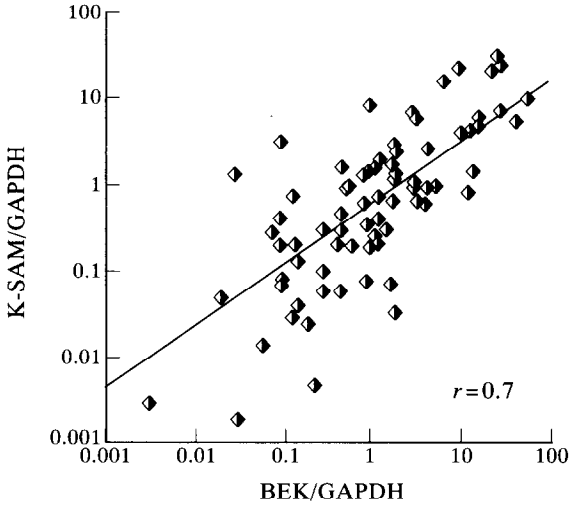

Figure 3. Relationship between the amount of PCR product for BEK and K-SAM normalised to the amount of *GAP*, in breast cancer biopsies, determined as described under Materials and Methods ($r = 0.7$).

28) had predominantly K-SAM with a small amount of BEK, while KATO 111 expressed K-SAM exclusively. The keratinocyte lines (SK+ and SK− were derivatives of the parental line) also expressed K-SAM with only a faint BEK signal detectable in them. Of two squamous carcinomas, SMN was negative and GEE was weakly positive for BEK, as was the colonic apodoma Colo320. Of the three cervical carcinoma lines, Hela and Siha had exclusively BEK, whereas Caski had predominantly K-SAM with a smaller amount of BEK signal. We examined five other non-epithelial lines: the A204 rhabdomyosarcoma was negative for FGFR2, the MRC-5 fetal lung fibroblasts and the myoblast line expressed BEK alone, the neuroblastoma Kelly had predominantly BEK but also some K-SAM signal and the Jar choriocarcinoma had significant amounts of both variants.

DISCUSSION

In this study we used a PCR based method to analyse a large number of human breast biopsies and cell lines to determine the relative levels of expression of two transcripts encoding variant forms of FGFR2 which differ in a stretch of 49 amino acids in their extracellular domains. Genomic analysis has shown [10,14] the presence in the *FGFR2* gene of two

Table 2. Relationship between BEK to K-SAM PCR product ratio and clinical parameters

Parameter	No.	BEK/K-SAM range	Ratio median	*P**
Oestrogen receptor				
Positive	19	0.04–205	1.8	0.09
Negative	15	0.9–15		
Clinical stage				
T1/T2	53	0.04–261	1.9	0.01
T3/T4	11	0.4–494	5.8	
Pathological stage				
T1/T2	44	0.03–261	2.9	0.98
T3/T4	20	0.04–43	2.1	
Nodal status				
Positive	26	0.2–16	2.9	0.66
Negative	37	0.03–261	3.0	
Menopausal status				
Pre–peri	31	0.5–28	2.2	0.50
Post	45	0.03–261	2.9	

* Probability determined by Mann–Whitney test.

Table 3. The relative expression of K-SAM and BEK in cell lines

Cell line	FGFR2 expression	K-SAM	BEK	K-SAM: BEK ratio when both present
Breast				
MCF7	+	+	+	50
MDA-MB-453	+	+	−	
MDA-MB-361	+	+	−	
MDA-MB-415	+	+	+	35
MDA-MB-157	+	+	+	1.6
BT20	+	+	+	16
PMC42	+	+	+	0.4
SKBR111	+	+	+	22
ZR75-1	+	+	+	22
T475D	+	+	+	12.5
HBL 100	−			
HBR.SV1.6.1	+	+	+	15
Non-breast				
Keratinocyte	+	+	+	23
SK+	+	+	+	32
SK−	+	+	+	19
MKN-28	+	+	+	34
MKN-1	+	+	+	38
KATO111	+	+	−	
Colo320	+	−	+	
SMN	−			
GEE	+	−	+	
Caski	+	+	+	5.8
Siha	+	−	+	
Hela	+	−	+	
Jar	+	+	+	1.6
Kelly	+	+	+	0.15
Myoblast	+	−	+	
MRC-5	+	−	+	
A204	−			

K-SAM and BEK variants in PCR products of RT products of RNA extracted from a variety of breast and non-breast cell lines; the latter derived from both epithelial and non-epithelial tissue: skin (Kerat, SK+, SK−); stomach (MKN-28, MKN-1, KAT0111); colonic apodoma (Colo320); cervical (Caski, Siha, Hela); choriocarcinoma (Jar); neuroblastoma (Kelly); muscle (Myoblast); fetal lung fibroblast (MRC-5); rhabdomyosarcoma (A204). +, PCR product detectable; −, not detectable.

tandemly arranged exons whose alternative usage is responsible for this divergence. The importance of this region is that it appears to determine ligand binding specificity. BEK receptor constructs encoding the full three Ig-like domains, transfected and expressed in NIH3T3 cells, have been reported to bind both acidic and basic FGF with high affinity [18]. Forms with deletions of the first Ig-like domain and the acidic box [19] are equally effective in binding these two FGFs, suggesting that those regions are dispensable in this respect. K-SAM constructs with either two or three Ig-like domains bound KGF and acidic FGF with high affinity [14], but did not bind basic FGF. The BEK containing transfectants lacked binding affinity for KGF. Studies utilising synthetic peptides [20], made using the predicted K-SAM sequence, showed that these could block KGF induced mitogenic activity, supporting the notion that this exon encodes the KGF binding site. However, other binding and crosslinking studies involving chimeric constructs [21] of FGFR1, into which the K-SAM exon from KGFR has been substituted in place of the homologous exon, indicate that while high affinity for aFGF and also FGF4 are retained, affinity for KGF is greatly reduced. The introduction of sequence encoding Ig domain 2 from KGFR into the FGFR1 chimera restored full binding of KGF, suggesting that the variable domain encoded by K-SAM is not the complete binding site for KGF, but also requires the presence of the adjacent loop.

Our present data shows that both BEK and K-SAM variants are expressed together in breast tissues, although we cannot say whether this is in the same or different cell types. Localisation studies using *in situ* hybridisation are needed to address this question, but may be severely hampered by both sensitivity and selectivity problems. We did not find any difference in the levels of amplified product for either form between cancerous and non-malignant tissues, suggesting that the mechanisms for selecting the BEK/K-SAM exons are unaltered in cancer cells. Thus we saw no relationship between the expression of these forms and clinical parameters, except that patients with advanced clinical staging had a greater proportion of the BEK form. Neither the total amount of FGFR2 nor the relative amounts of these two variants influenced patient survival.

From the initial report by Miki and colleagues [14], it appeared that epithelial and non-epithelial cells expressed K-SAM and BEK in a mutually exclusive manner, and this cell

type specificity was also assumed as the reason for appearance of only BEK forms on PCR amplification from a neonatal human brainstem cDNA library [19]. However, our results using cell lines show that while it appears to be the general trend that epithelial cells express only (or predominantly) the K-SAM variant, this is not at all clear cut. Two breast cell lines did contain significant amounts of both variants and this was also observed in a choriocarcinoma. The avian equivalent (cek3) of the human BEK was found primarily in brain with low expression in other tissues, while the K-SAM equivalent (bek), was predominantly in lung and less in brain [22]. The genomic organisation of FGFR1 is similar to FGFR2 in that there are also two alternative exons for Ig loop 3, which are highly homologous to those of FGFR2 [10]. Their pattern of expression indicates that while the BEK homologue is highly expressed in several non-epithelial derived cell lines, there is also some co-expression of the K-SAM homologue in all except the fibroblast line. This group also reported a high level of the K-SAM homologue in skin tissue and lower levels in several other tissues in which the BEK homologue was more abundant. Ishii and colleagues [23] found that six of the 12 epithelial lines they examined expressed both isoforms, while three had BEK only and three K-SAM. Their sarcoma lines were either FGFR2 negative or had low levels of BEK only. Thus, although there does appear to be some partial tissue specificity, clearly both forms can be expressed simultaneously in the same culture.

Nothing is known about the mechanism for the synthesis of these two variant transcripts. There is growing evidence for the involvement of specific small nuclear RNAs, and several SR proteins have been identified [24] which can determine exon usage in pre-mRNA splicing *in vitro*. We intend to investigate some of these factors using the cell lines expressing exclusively one form in comparison with those which are able to produce both variants, to determine whether cellular factors can regulate alternative expression. It will be interesting to see if we can identify specific factors that are responsible for other exon deleted FGFR isoforms [2]. However, unlike some of these other variants, whose predicted protein forms would generate receptors with some functional impairment compared to the full length receptor (and for which it is difficult to see a physiological role in most cases), the BEK/K-SAM selection can be envisaged as a way in which normal cells can modulate their response to different FGFs even if continuously exposed to a particular ligand. KGF has been found to be expressed in stromal fibroblast lines derived from several epithelial tissues, but was not detectable in epithelial lines [25]. Its presence in breast tissues [26] indicates a potential paracrine loop in which fibroblast derived KGF could stimulate a selective sub-population of K-SAM expressing cells, which themselves may be able to control their response by switching to BEK and thereby become sensitive to basic FGF and insensitive to KGF. The corresponding exon selection in FGFR1 can be similarly modulated, as the type 111b (equivalent to the FGFR2 K-SAM) binds KGF and acidic FGF, while the BEK equivalent binds acidic FGF and basic FGF but not KGF. Alternatively, acidic FGF binds to both the corresponding homologues of FGFR2 and FGFR1, and also to FGFR4 [27] and FGFR3 [28] (data is unavailable on flg2), providing a non-selective stimulus. Such a role may be important in recovery from cellular injury, consistent with its lack of signal peptide that may in healthy cells restrict it to an intracellular location preventing receptor interaction.

In summary, we have shown that human breast tissues express both the BEK and K-SAM forms of FGFR2, but that neither the amount nor the selection of these is altered in cancers. Preponderance of one form or the other is essentially unrelated to clinical status. In most breast cell lines, K-SAM predominated, but two lines did express both forms, suggesting that cells may be able to regulate production of these isoforms and thereby dynamically modify their response to FGFs.

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