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An integrated genomic, transcriptional and protein investigation of *FGFRL1* as a putative 4p16.3 deletion target in bladder cancer

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Abbreviated title: *FGFRL1* as 4p16.3 deletion target

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ABSTRACT

Loss of heterozygosity (LOH) of chromosome 4p is a common event in bladder and other malignancies. At least three distinct regions of deletion have been identified, but the deletion targets have so far remained elusive. In this study, we have identified a novel region of deletion mapping to 4p16.3 spanning 0-2.1 Mb, in 15% of bladder tumours and 24% of bladder cancer cell lines. *FGFRL1*, which maps within this region, was investigated as putative deletion target. The retained *FGFRL1* allele was not mutated in cell lines and tumours with LOH, although in patients heterozygous for the rs4647930 functional polymorphism, the common allele was preferentially lost in tumour tissue. Epigenetic silencing of the retained allele was also excluded as levels of FGFRL1 mRNA and protein were similar in cell lines and tumours with and without 4p16.3 loss. However, while FGFRL1 protein was moderately expressed in all layers of the normal bladder epithelium, the majority of tumours showed areas of down-regulation. Overall, average FGFRL1 protein expression was significantly lower in bladder tumours compared to normal tissue, but down-regulation was independent from 4p16.3 LOH status, *FGFR3* mutation, and tumour grade and stage. In conclusion, although we found no evidence supporting a 'two-hit' inactivation of *FGFRL1* in bladder carcinogenesis, the effect of heterozygous deletion coupled with functional polymorphisms, and the role of post-transcriptional down-regulation deserves further investigation.

INTRODUCTION

Urothelial cell carcinomas (UC) arise through at least two distinct molecular pathways involving a sequence of genetic and epigenetic changes leading to the inactivation of tumour suppressor genes (*TP53*, *RB1*, *CDKN2A*, *PTEN*, *DBC1*, *TSC1*) and the activation of oncogenes (*FGFR3*, *RAS* genes, *PIK3CA*, *CCND1*, *MDM2*, *E2F3*)(Goebell and Knowles 2010). Some of these changes are almost exclusively found either in non-invasive or invasive tumours (e.g. *FGFR3* and *TP53* mutations), while others occur in both types (e.g. *CDKN2A* loss, *RAS* gene mutation). Inactivation of tumour suppressor genes can occur through deletion, mutation or silencing *via* methylation and usually requires a combination of two distinct events, each one targeting one of the two alleles, according to Knudson's 'two-hit' hypothesis (Knudson 1971). However, for haploinsufficient genes (e.g. *PTEN*), loss of one allele may be enough to confer a selective advantage and allow clonal expansion (Santarosa and Ashworth 2004).

Loss of heterozygosity (LOH) analysis has been employed widely to identify chromosomal regions deleted in UC, which may harbour potential tumour suppressor genes (Knowles 1999). An early report using two polymorphic markers on 4p showed that LOH of this region is a common event, occurring in around 22% of cases (Knowles, et al. 1994). Deletions involving the short arm of chromosome 4 are also common in a number of other malignancies, including lung (Girard, et al. 2000), colon (Shivapurkar, et al. 2001; Zheng, et al. 2008), head and neck (Beder, et al. 2003), esophageal (Hu, et al. 2000), gastric (Jiao, et al. 2006), and breast cancer (Shivapurkar, et al. 1999). These studies have identified several distinct regions of deletion on 4p, suggesting the existence of multiple tumour suppressor genes on this chromosome. This is in line with early microcell fusion experiments, showing that the introduction of a normal human chromosome 4 in immortalized cell lines, including the bladder-derived line J82, slowed proliferation and induced senescence (Ning, et al. 1991).

Later studies attempting to narrow down the region of 4p loss in bladder tumours identified three distinct minimal regions of LOH (Elder, et al. 1994; Polascik, et al. 1995), which overlap with

those reported in the other tumour types. A telomeric region on 4p16.3, between D4S43 and D4S127, was lost in 9% of cases, a region on 4p12-13 proximal to D4S174 was lost in 4% and a region on 4p15.2-3, between D4S404 and D4S1608, was lost in 12%. In addition, around 20% of cases displayed loss of the whole 4p arm or an entire chromosome 4. Subsequent studies sequenced candidate genes in the telomeric region, *SH3BP2* (Bell, et al. 1997), *ADD1* and *RES4-22* (Sibley, et al. unpublished data), in the attempt to find evidence of a 'second hit' but no mutations were found.

To our knowledge, no deletion studies have so far examined the most distal 4p16.3 portion, between D4S43 and the telomere. Interestingly, two members of the fibroblast growth factor receptor (FGFR) family, FGFR3 and FGFRL1, are encoded by genes mapping to 4p16.3, within the region not covered by the previous investigations and in close proximity to each other, approximately 1.8 Mb and 1.0 Mb from the telomere, respectively. FGFRs are trans-membrane tyrosine-kinase receptors mediating the cellular effects of fibroblast growth factors (FGFs) (Powers, et al. 2000). FGFRs1-4 are composed of an extracellular domain with specificity for binding FGFs and heparan sulphate proteoglycans, a transmembrane domain, and an intracellular split tyrosine-kinase domain (Powers, et al. 2000). FGF binding induces receptor dimerization and autophosphorylation of the kinase domain, which is followed by a phosphorylation cascade activating a number of downstream signalling pathways including RAS/MAPK, PLCγ1 and PI3K (Klint and Claesson-Welsh 1999). Activating mutation of *FGFR3* is a common and early oncogenic event in bladder cancer (Knowles 2008), and results in hyperproliferation of pre-malignant urothelial cells (di Martino, et al. 2009). Overexpression of wild type FGFR3 and FGFR1 has also been reported in UC (Knowles 2008). As FGFR3 acts as an oncogene in the urothelium, it is an unlikely target for 4p16.3 LOH. The most common mutant forms of FGFR3, S249C and Y375C, have unpaired cysteine residues in the extracellular domain and form constitutive dimers, leading to autophosphorylation in the absence of ligand (Adar, et al. 2002). Thus, loss of the wild type allele would not result in additional selective advantage in cells harbouring a heterozygous FGFR3 mutation.

FGFRL1, however, is an interesting candidate for a tumour suppressor role in bladder cancer for multiple reasons. Firstly, it is highly homologous to the other FGFRs in the extracellular region and can bind a range of FGFs, but it lacks the tyrosine-kinase domain, which is replaced by a short histidine-rich segment (Wiedemann and Trueb 2000). Consequently, it has been suggested to act as a decoy-receptor, scavenging FGFs and preventing activation of the full-length receptors (Steinberg, et al. 2010b). Secondly, it interacts with SPRED1 (Zhuang, et al. 2011), a negative regulator of the MAPK pathway, which is one of the major signalling pathways activated by mutant FGFR3 in urothelial cells (di Martino, et al. 2009). Furthermore, FGFRL1 has been shown to promote cell adhesion by forming dimers between adjacent cells (Rieckmann, et al. 2008), and could therefore prevent tumour development or spreading by enhancing cell-cell adhesion and inhibiting invasion and metastasis. Finally, FGFRL1 has been previously shown to be down-regulated in ovarian tumours (Schild and Trueb 2005) and to reduce cell proliferation in response to FGF2 when ectopically expressed in the osteosarcoma cell line, MG-63 (Trueb, et al. 2003).

Based on this evidence, we hypothesized that FGFRL1 may play a role as a tumour suppressor in the bladder by inhibiting FGF signalling through conventional FGF receptors, and may therefore be one of the targets of 4p LOH in bladder tumours. FGFRL1 loss may offer a selective advantage to urothelial cells, particularly if coupled with FGFR1 or FGFR3 overexpression or *FGFR3* mutation. The aims of this study were to assess whether the most telomeric 4p16 region, to which *FGFRL1* maps, is specifically deleted in bladder tumours, to test whether *FGFRL1* is a likely target of LOH in this region, and to investigate whether FGFRL1 is altered at the genomic, transcriptional or protein level in bladder tumours.

MATERIALS AND METHODS

Cell lines

Normal urothelium cell strains (NHUC) were derived from ureters of nephrectomy patients without bladder cancer collected with informed consent, as previously described (Hutton, et al.

1993; Southgate, et al. 1994). Telomerase-immortalized normal urothelial cells (TERT-NHUC) were derived from NHUC as previously described (Chapman, et al. 2006). Forty-one UC cell lines (5637, 253J, 639V, 647V, 92-1, 94-10, 96-1, 97-1, 97-18, 97-24, 97-7, BC3c, BFTC905, BFTC909, CAL 29, DSH1, HT-1197, HT-1376, J82, JMSU-1, JO'N, KU-19-19, LUCC1, LUCC2, LUCC3, LUCC4, LUCC5, MGH-U3, RT112M, RT4, SCaBER, SD, SW-1710, SW-780, T24, TCCSUP, U-BLC1, UMUC3, VM-CUB-1, VM-UCB-2, VM-UCB-3) were cultured in standard growth media at 37°C in 5% CO_2. LUCC1-5 cell lines were established in our laboratory from surgical UC specimens. All other cell lines were obtained either from the laboratory of origin or a recognized cell repository and were authenticated by DNA profiling using the PowerPlex® 16 kit (Promega UK, Southampton, UK).

Matched blood and tumour DNA samples

A total of 97 bladder tumour samples classified according to the 1973 WHO and TNM guidelines (Mostofi, et al. 1999; Sobin, et al. 2010) were collected between 2000 and 2009 with informed consent. Cold cup biopsies were snap-frozen and stored in liquid nitrogen until processed for RNA and DNA extraction. The remaining tumour tissue was formalin-fixed and paraffin-embedded and used for diagnostic purposes and immunohistochemistry. Only samples containing more than 70% tumour tissue were included in the study. A venous blood sample was collected from each patient as source of control genomic DNA. Information regarding grade, stage, and FGFR3 status of the tumours is summarized in Supplementary Table 1.

Microsatellite analysis

DNA was obtained from tumour cell lines or from snap frozen tumour tissue using the QIAamp® DNA mini kit (Qiagen, Crawley, UK) and from matched venous blood by salt-precipitation. DNA (5-10 ng) was amplified using primers specific for nine polymorphic short tandem repeats on 4p16.3-2 (Supplementary Table 2). PCR reactions contained 400 nM FAM-labelled forward primer and 400 nM reverse primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, and 1 unit of AmpliTaq Gold® DNA

polymerase (Life Technologies Ltd., Paisley, UK). PCR conditions were: 95 °C for 5 min, 25-27 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, followed by a final elongation of 5 min at 72°C and 30 min at 60°C. One µl of PCR product was denatured and run along with Genescan™ ROX™-500 Size standard (Life Technologies Ltd.) on a ABI 3130 Genetic Analyzer (Life Technologies Ltd.). Analysis was performed using ABI PRISM® GeneMapper® Software v3.7 (Life Technologies Ltd.). LOH was determined by comparison of the peak heights in matched normal and tumour tissue. A decrease ≥75% of one allele was considered as LOH. Loci with a decrease ≤75% were considered 'imbalanced'. Loci that were homozygous in the normal DNA were scored as 'not informative'.

Mutation screening by high resolution melting (HRM) and sequencing

FGFRL1 genomic sequence was obtained from publically available databases (www.ensembl.org, www.ncbi.nlm.nih.gov), and exons were annotated and numbered consistently with Lopez-Jiminez *et al* (LopezJimenez, et al. 2010). Of the six coding exons, five (exons 2, 3, 4, 6, and 7) were screened for mutations using high resolution melting (HRM) followed by bidirectional dye-termination sequencing of samples with altered melting profile. Exon 5 was analysed by bidirectional sequencing only, as described previously (Rieckmann, et al. 2009). For HRM, 1 ng genomic DNA was amplified by PCR using 1x HotShot MasterMix (Clent Life Science, Stourbridge, UK), 1x LC Green Plus (Clent Life Science, Stourbridge, UK), 10% Dimethyl sulfoxide (Sigma-Aldrich Company Ltd., Dorset, UK) and 400 nM forward and reverse primers (Supplementary Table 3). Primers were designed to amplify regions between 100 and 300 bases long. Longer exons were divided into two or three amplicons. PCR conditions were: 5 min at 95°C, 45 cycles of 20 sec at 95°C, 10 sec at 57-61°C (Supplementary Table 3), 15 sec at 72°C, and a final denaturation of 30 sec at 94°C followed by cooling to 25°C (at 0.1°C/sec) to promote heteroduplex formation. Melting profiles were generated using a Lightscanner® system (Idaho Technology Inc.) and analysed using LightScanner® Software with Call-IT 2.0 (Idaho Technology Inc.). For bidirectional sequencing of HRM products, reactions contained 1.25 µl PCR product and either forward or reverse primer and were carried out

using the BigDye Terminator V1.1 Cycle Sequencing Kit (Life Technologies Ltd.). Products were run on an ABI 3130xl Genetic Analyzer (Life Technologies Ltd.) and electropherograms were visually analysed using 4Peak Software (Mekentosj, The Netherlands).

mRNA expression analysis

RNA was extracted from snap frozen tumour tissue or from cell lines using RNeasy mini kit (Qiagen), including the optional DNAse treatment step. cDNA was synthesized from 1-5 µg of RNA using oligo-d(T) primers (cell lines) or random hexamers (tumours) (Life Technologies Ltd.). Relative mRNA expression of FGFRL1 was determined by Taqman® Real Time PCR. Samples were amplified using TaqMan® Gene Expression Assays Hs01113161_g1, Hs00222484_m1 (*FGFRL1*) and Hs00188166_m1 (*SDHA*, internal control gene) (Life Technologies Ltd.). Reactions were performed in triplicate using an ABI 7500 RealTime PCR System (Life Technologies Ltd.). Non-template negative controls were included in each plate. FGFRL1 expression was normalized to SDHA using the ΔCt method and semi-quantified relative to a positive control sample.

FGFRL1 cloning and overexpression in TERT-NHUC

A plasmid containing the coding sequence for human FGFRL1 (pDONR223-FGFRL1) was obtained from Addgene (Cambridge, US). FGFRL1 sequence was cloned into a retroviral expression vector (pFB; Stratagene, La Jolla, CA) containing a hygromycin resistance cassette using In-Fusion® HD Cloning Plus (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). The expression vector was transfected into Phoenix A cells using TransIT®-293 transfection reagent (Cambridge BioScience Ltd., Cambridge, UK). Retroviral supernatants supplemented with 8 mg/ml polybrene were used to transduce TERT-NHUC, followed by selection with hygromycin.

Western blotting

Heat-denatured proteins (40 µg) were separated by SDS-PAGE using pre-cast 10% polyacrylamide mini-protean® TGX gels™ (Bio-Rad Laboratories Ltd., Hertfordshire, UK), transferred to 0.2 µm nitrocellulose Trans-Blot® Turbo™ membranes (Bio-Rad Laboratories Ltd.), and incubated for 1 hr at room temperature with 1:750 anti-FGFRL1 rabbit polyclonal antibody (ab95940) (Abcam plc., Cambridge, UK) or 1:2000 anti-α-tubulin rat monoclonal antibody (MCA77G) (AbD Serotec, oxford, UK) in 5% bovine serum albumin (Sigma-Aldrich Company Ltd., Dorset, UK). Bound primary antibodies were detected using HRP-conjugated anti-rabbit (Cambridge BioScience Ltd.) or anti-mouse (Bio-Rad laboratories Ltd.) secondary antibodies and the Luminata ™ Forte Western HRP Substrate (Millipore Ltd., Watford, UK). Protein deglycosylation was performed using a commercial kit (Merck Ltd., Feltham, Middlesex, UK), according to manufacturer's instructions.

Immunohistochemistry

Three µm sections of formalin-fixed paraffin-embedded tissue were dewaxed in xylene, rehydrated in descending grades of ethanol and endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. Antigen retrieval was performed by heating in a pressure cooker for 2 min in 0.01 M citric acid buffer pH 6.0 (Sigma-Aldrich Company Ltd). Non-specific binding was blocked by incubation in 10% casein (Vector Laboratories Ltd, Peterborough, UK) for 20 min. Samples were incubated with 1:250 rabbit anti-FGFRL1 antibody (ab95940; Abcam plc, Cambridge, UK) for 1 hr at room temperature. Antigen detection was performed with X-Cell-Plus Polymer HRP Detection Kit (Menarini Diagnostics Ltd, Wokingham, Berkshire, UK) and included incubation with anti-rabbit HRP-polymer antibody for 30 min, followed by visualization using 3,3'-Diaminobenzidine (DAB). Slides were scored independently by three investigators (J.R., E.d.M, and M.A.K.) blinded to 4p LOH information and *FGFR3* mutation status. Samples were scored based on average FGFRL1 cytoplasmic staining intensity across the whole tumour, which was quantified using the following

arbitrary units: 0=negative, 1=weak, 1.5=weak/moderate, 2=moderate, 2.5=moderate/strong, 3=strong. Subcellular localization (cytoplasmic, membraneous, nuclear) was also noted.

Statistical analysis

Significant differences were assessed using the Mann-Whitney test (ordinal variables) or Pearson's Chi-Square test (categorical variables), with the SPSS© Statistics version 19 analysis software (SPSS Inc., Chicago, US). A $p \leq 0.05$ was accepted as significant.

RESULTS

LOH analysis in cell lines

Forty-one UC cell lines were genotyped at nine polymorphic loci on chromosome 4p16.3-2. The loci analysed covered a region spanning 4.5 Mb, starting 0.1 Mb from the 4p telomere. Because no matched normal DNA was available for most cell lines, LOH was predicted based on long stretches of contiguous homozygosity. Based on heterozygosity frequencies and after Bonferroni correction for multiple testing, the probability of homozygosity of 6, 7, 8 or 9 contiguous markers was estimated as p=0.057, p=0.012, p=0.002, and p=0.0004, respectively. Therefore, homozygosity of 6 contiguous markers was considered strongly suggestive of LOH, while homozygosity of 7 or more contiguous markers was considered as conclusive evidence of LOH. A region of continuous homozygosity (RCH) of 6 or more markers was identified in ten (24%) cell lines (639V, 97-1, BFTC905, TCCSUP, LUCC1, LUCC3, VM-CUB-1, MGH-U3, 97-24 and SCaBER) (Fig. 1). Definitive LOH was confirmed for two cell lines (LUCC1 and LUCC3), for which matched DNA was available. One of the cell lines, MGH-U3, retained the D4S127 locus, while 97-24 and SCaBER retained the more telomeric markers. LOH encompassed both *FGFRL1* and *FGFR3* in all cell lines, although one of the markers flanking *FGFRL1* was retained in 97-24. No association was found between 4p16 LOH and *FGFR3*

mutation status. Of the 41 cell lines in the panel, five were *FGFR3* mutant (J82, 97-7, 94-10, MGH-U3, 639V), and of these 639V and MGH-U3 displayed 4p16.3 loss and the other three did not.

LOH analysis in tumours

DNA from ninety-seven UC tumours and matched normal blood was tested for five of the microsatellite loci on 4p16.3-2 (D4S2936, D4S3038, D4S43, D4S127 and HOX7). Seventeen samples (17%) displayed LOH of one or more markers. These samples were genotyped for additional markers in order to accurately define the deleted region (Fig. 1). Overall, two distinct regions of LOH were identified, a novel region distal to D4S1182 (Region 1) and a region proximal to D4S43 (Region 2), as previously described (Elder, et al. 1994). Nine samples (tumours 454, 1427, 1230, 1207, 675, 1350, 1049, 1145, 417) had extensive LOH covering the whole 4p16.3-2 region. For five samples (tumours 228, 385, 996, 1352, 540) LOH was confined to the telomeric region (Region 1). Two samples (tumours 94 and 1082) retained the telomeric markers but had LOH of the proximal region (Region 2), although for tumour 1082 a small deletion overlapping Region 1 proximal to marker D4S1.36 could not be excluded. One sample (tumour 1006) had LOH of both the distal and proximal regions but retained a small region in between. In at least 14 of the 17 tumours (82%) with 4p16.3-2 loss, LOH encompassed *FGFRL1*. For sample 228, LOH of *FGFRL1* could not be confirmed as the three markers distal to this gene were uninformative. In 13 out of the 17 tumours with 4p16 loss, LOH encompassed *FGFR3*. In a further two tumours with 4p16 loss (samples 1082 and 1352), one of the markers flanking *FGFR3* was lost but the other was either retained or not informative so no conclusions could be drawn regarding *FGFR3* deletion in these samples. Overall, in the vast majority of tumours with 4p16.3-2 LOH, both *FGFRL1* and *FGFR3* were lost. In two tumours (540 and 1006) retention of the proximal *FGFR3* flanking marker suggested exclusive loss of *FGFRL1* but this was not conclusive due to the distal *FGFR3* flanking marker being not informative. One tumour with retention of *FGFRL1* (1082) displayed loss of the proximal *FGFR3* flanking marker but this case was also inconclusive because the distal *FGFR3* flanking marker was retained.

When all the 97 tumours genotyped were considered, LOH was almost twice as common in *FGFR3* mutant than in *FGFR3* wild type tumours (9/37, 24% vs 8/60, 13%, respectively) but this difference was not significant (p=0.181). When only tumours with distal 4p16.3 loss were considered, with the rationale that extensive LOH may be targeting genes in the other previously described 4p minimal regions, LOH was significantly associated with *FGFR3* mutation, as 80% (4 out of 5) tumours with distal LOH were *FGFR3*-mutant compared with only 31% of tumours without 4p16.3-2 LOH (p=0.05). These numbers, however, are extremely small and do not allow any definitive conclusion to be drawn regarding association between 4p16.3 LOH and FGFR3 mutation status. No significant association was found between 4p16.3-2 LOH and tumour stage or grade.

***FGFRL1* mutation analysis**

To find evidence for a 'second hit', the 10 cell lines and 14 tumours with LOH encompassing *FGFRL1* were tested for mutation in the retained allele using HRM and bidirectional dye-termination sequencing but no missense mutations were found. The cell line 639V had a synonymous GAG>GAA change at codon 240 (*not shown*). The change was heterozygous, suggesting that it may have arisen in culture in the retained allele in a subpopulation of cells. Previously described polymorphisms of *FGFRL1* were identified in some samples, including the rs4647930 C/A polymorphism in exon 7, which results in a proline to glutamine change in the membrane-proximal region of the protein (P362Q). Interestingly, in the tumour tissue, the frequency of the rare A allele was higher than expected according to the previously reported frequencies (0.500 vs 0.275) (LopezJimenez, et al. 2010). Analysis of matched blood samples suggested that in heterozygous patients with 4p16.3 LOH, the common C allele was preferentially lost in the tumour tissue (6/8, 75%) (Table 1) but numbers are too small to draw any definitive conclusion.

FGFRL1 mRNA and protein expression in normal urothelium

As FGFRL1 expression has been shown only in a limited number of human adult tissue types, we investigated whether the gene is expressed in normal urothelial cells. Firstly, we tested mRNA expression by Taqman Real Time PCR and found heterogeneous expression in both uncultured and cultured normal urothelial cells (Supplementary Fig. 1A), with Ct values suggestive of a medium-to-low level (Supplementary Fig. 1B). Comparable results were obtained using two different Taqman assays. We also confirmed FGFRL1 mRNA expression in these cells by standard RT-PCR using specific primers, followed by sequencing of the PCR product (*data not shown*). We then tested levels of FGFRL1 protein expression in normal bladder and detected a weak-to-moderate expression in all layers of the epithelium (Supplementary Fig. 1C). A range of human tissues was also tested. Consistent with previous reports, expression was negative in most, including colon, lung, stomach, tonsil, esophagus, brain and smooth muscle. A weak positivity, barely above background, was found in skin, liver and spleen, while a moderate staining was exclusively detected in pancreas, skeletal muscle, and bladder epithelium (Supplementary Fig. 1C).

To further confirm FGFRL1 expression in urothelial cells, we analysed protein lysates by Western blotting (Supplementary Fig. 1D). As a positive control, FGFRL1 was overexpressed in telomerase-immortalized normal urothelial cells (TERT-NHUC). Three clear bands were detected in overexpressing cells, compatible with the molecular weight of fully gycosylated (64 KDa), partially glycosylated and unglycosylated (54 KDa) FGFRL1 (Supplementary Fig. 1D). A similar pattern has previously been seen in the bladder for other FGFRs (Tomlinson, et al. 2007). Indeed after deglycosylation, a single band of around 54 KDa was observed in these cells. FGFRL1 protein expression was also confirmed in a range of normal urothelial cells from different donors and it was comparable to levels in HEK293 cells, which have been previously reported to express FGFRL1 mRNA (Schild and Trueb 2005). Interestingly, only bands of a size corresponding to unglycosiyated or partially glycosylated protein were detected in these cells.

Overall these results indicate that normal urothelium express both FGFRL1 mRNA and protein, at a low-to-moderate level.

FGFRL1 mRNA and protein expression in UC cell lines and tumours

Having excluded mutation as a mechanism for a 'second hit', and having confirmed FGFRL1 expression in normal urothelial tissue, we tested whether the expression of the retained *FGFRL1* allele in tumours with LOH was silenced by epigenetic or other mechanisms. FGFRL1 mRNA expression levels were compared using Taqman Real Time RT-PCR in UC lines with (N=10) and without (N=18) 4p16.3 RCH (Fig. 2A). Average expression levels were lower in RCH positive cell lines but the difference did not reach statistical significance. RNA was available for 3 of the tumours with 4p16.3 LOH. FGFRL1 mRNA expression was compared between these tumours and 8 tumours without 4p16 LOH (Fig. 2A). Surprisingly, average mRNA expression levels were higher in tumours with 4p16.3 LOH, although the difference was not significant. Formalin-fixed paraffin-embedded tissue was available for 9 of the tumours with 4p16.3 LOH. FGFRL1 protein was detected by immunohistochemistry and expression levels in these tumours were compared with levels in 48 tumours without 4p16.3 LOH. Consistent with mRNA results, no differences in FGFRL1 protein expression levels were observed between groups (Fig. 2B). Overall this data excludes silencing of the retained *FGFRL1* allele as a common event in bladder tumours with 4p16.3 LOH.

However, when FGFRL1 protein levels were compared between normal and tumour tissue, average protein staining in bladder tumours was significantly lower than in normal bladder epithelium ($p=0.017$), irrespective of LOH status (Fig. 2C). In normal bladder and normal ureter, FGFRL1 protein was moderately expressed in all layers of the epithelium (Fig. 3A-B) and in the endothelial cells, while muscle and connective tissue were negative. In UC tissue, FGFRL1 protein expression levels were variable but most samples exhibited extensive areas of weak staining although smaller areas of moderate staining were also observed (Fig. 3C-D).

In both normal and tumour bladder tissue, protein localization was mainly cytoplasmic with small areas of strong membranous staining, but occasional moderate nuclear staining was also detected. The significance of the nuclear staining is unclear, as this has not previously reported for FGFRL1 although it has been shown for splice variants of other FGFRs (Zammit, et al. 2001). Cytoplasmic localization however is in line with a previous report, showing that after overexpression in other cell types FGFRL1 is rapidly internalized from the cell surface and localizes mainly in the Golgi complex and endoplasmic reticulum (Rieckmann, et al. 2009).

No significant differences were observed between grades or stages, or between tumours with or without *FGFR3* mutation. No major differences in protein localization or expression were observed between tumours with different genotypes for the rs4647930 C/A polymorphism, but the number of samples with known genotype was extremely small (N=4 AA and N=4 CC).

DISCUSSION

FGFRL1 is an atypical member of the FGFR family, with incompletely characterized cellular functions. It is thought to act as a decoy receptor preventing activation of conventional FGFRs (Steinberg, et al. 2010b), favour cell-cell adhesion through formation of inter-cellular dimers (Rieckmann, et al. 2008), be involved in cell-cell fusion of heterologous cells (Steinberg, et al. 2010a), and interact with SPRED1 (Zhuang, et al. 2011), a negative regulator of the MAPK signalling pathway. While other FGFRs are known to play a key part in the development of bladder and other cancers (Ahmad, et al. 2012), so far only a limited number of studies have investigated the role of FGFRL1 in malignant transformation, with conflicting results. Earlier reports suggested a tumour suppressor role (Schild and Trueb 2005; Trueb, et al. 2003), but in a more recent study FGFRL1 protein expression was found to be up-regulated in esophageal tumours compared to matched normal tissue, and knock-down of *FGFRL1* in esophageal cancer cells induced cell cycle arrest (Tsuchiya, et al. 2011).

This is the first comprehensive study to investigate the expression and role of FGFRL1 in healthy and malignant human urothelium. Previous studies have shown that expression of FGFRL1 is highly tissue-specific. Although no comprehensive protein expression studies have been carried out so far, two investigations have looked at mRNA expression in a range of mouse and human tissues by Northern blotting, but none of them included bladder. In mouse, FGFRL1 mRNA was detected at high level in the tongue, vertebrae and sternum, and at lower levels in the heart, aorta, lung, kidney brain and liver (Sleeman, et al. 2001; Trueb and Taeschler 2006). Similarly, expression of FGFRL1 mRNA has been shown only in a few human adult tissues such as pancreas, kidney, brain, liver, heart and skeletal muscle, while spleen, colon, lung, placenta and stomach are negative (Kim, et al. 2001; Sleeman, et al. 2001). In this study, we found moderate levels of FGFRL1 protein expression in pancreas, skeletal muscle, and urothelium. Liver, skin and spleen had levels barely above background, while the others were negative. Our results are therefore consistent with previous mRNA studies. However, as this is the first report of FGFRL1 expression in the bladder, we also tested protein lysates from normal urothelial cells from different donors by Western blotting to confirm protein expression in urothelial cells. A band of the expected size for *FGFRL1* was detected. We also confirmed expression of FGFRL1 mRNA in uncultured and cultured normal urothelial cells by Real Time Taqman PCR and standard RT-PCR followed by sequencing. Therefore, overall our mRNA and protein results suggest that, in contrast with other tissues, FGFRL1 mRNA and protein are expressed at detectable level in the human urothelium.

To investigate whether FGFRL1 is a likely tumour suppressor gene in the bladder, we carried out a detailed analysis of 4p16.3-2 in a panel of bladder tumours and identified a novel region of deletion on 4p16.3 (0-2.1Mb), spanning *FGFRL1*. This region was specifically lost in 6 out of 97 (6%) of bladder tumours examined, while an additional 9 (9%) displayed extensive 4p16.3-2 LOH. Interestingly, we found that *FGFR3* mutation was more common in tumours with 4p16 LOH, particularly those with specific loss of the telomeric region, consistent with the hypothesis that 4p16.3 LOH may be targeting a negative regulator of FGF signalling, therefore cooperating with

FGFR3 mutation during bladder carcinogenesis. Although one *FGFRL1* allele was lost due to LOH in 15% bladder cancer cases examined, we did not find any evidence of a 'second hit' involving the retained allele. No mutations were found in cell lines and tumours with 4p16.3-2 loss. Epigenetic silencing through methylation was also excluded as mRNA and protein levels in cell lines and tumours with and without 4p16.3-2 LOH were similar. Thus, our results do not appear to support *FGFRL1* as a target that requires biallelic intactivation. However, it is possible that it is haploinsufficient, and that heterozygous deletion leading to partial reduction may be sufficient to confer a selective advantage, particularly when coupled with other genetic and/or epigenetic events. Indeed, accumulating evidence suggest that chromosomal deletions may favour tumour development in a 'two-hit' independent manner, by inducing subtle changes in expression of several dosage-dependent tumour suppressor genes mapping within the same region (Henrich, et al. 2012; Xue, et al. 2012). Therefore, although other candidate genes in the 4p critical regions should be examined, we cannot exclude the possiblity that monoallelic loss of *FGFRL1* may have detrimental effects even in the absence of mutation or silencing of the retained allele.

Heterozygous loss may, for example, have cellular consequences when the retained allele carries rare polymorphisms that impact on protein function. Interestingly, we noticed that in patients who were heterozygous for the *FGFRL1* rs4647930 polymorphism, the common allele was preferentially lost in their tumours. The rare allele results in a proline to glutamine change in the membrane-proximal region of the protein and has been reported to modulate protein cleavage and shedding (Steinberg, et al. 2010b). In addition, one of the cell lines with heterozygous loss of *FGFRL1*, 639V, harboured two rare polymorphisms in exon 7 of the retained allele, resulting in two amino acid changes in the intracellular domain (rs4647930, P362Q; rs4647932, P464L). Therefore, future investigations should examine whether these polymorphisms impair the function of FGFRL1 in 639V cells.

Furthermore, while there was no difference in FGFRL1 mRNA levels between tumour tissue and normal urothelial cells, average FGFRL1 protein expression in most bladder tumours was lower

than in normal bladder or ureter, suggesting that *FGFRL1* may be down-regulated irrespective of 4p16.3 LOH through post-transcriptional or post-translational mechanisms. Further studies on the mechanisms of *FGFRL1* regulation and its functional role in urothelial cells are needed to clarify whether loss of FGFRL1 protein expression may favour bladder malignant transformation.

A limitation of all LOH studies is that deletions occurring between polymorphic markers are missed and therefore tumours classified as without LOH may harbour small inter-marker deletions. The number and position of the markers used in this study were carefully selected in order to minimize average inter-marker distances and reduce the risk of underestimating the frequency of 4p16.3. In particular, two markers flanking *FGFRL1* were in close proximity with the gene, as they were located only 0.3 Mb distal and 0.1 Mb proximal to it. Another limitation is that although we had information about the *FGFR3* mutation status of the tumours examined, we did not know the level of expression of FGFR3 and FGFR1. Thus, we could not test whether reduced FGFRL1 expression correlated with FGFR3 and/or FGFR1 overexpression.

In conclusion, we have carried out a detailed investigation of the most distal region of 4p. We have identified a novel minimal region of deletion spanning *FGFRL1*, and confirmed that this gene is deleted in the majority of tumours with 4p16.3-2 LOH. No evidence of epigenetic silencing or mutation of the retained *FGFRL1* allele was found, suggesting that *FGFRL1* is not targeted by biallelic inactivation. However, heterozygous deletion combined with preferential retention of the rs4647930 rare allele in the tumour tissue could potentially result in altered protein function in LOH cases. Furthermore, *FGFRL1* was found to be down-regulated at the protein level in the majority of bladder tumours, independent of 4p16.3-2 LOH status. Functional studies into the role of FGFRL1 in the urothelial cells are required.

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TABLE 1. Genotypes for the rs4647930 polymorphism in tumour tissue of LOH patients and in matched blood

Tumour sample	Blood genotype	Tumour genotype
1207	AA	AA
1006	AC	CC
1427	AC	CC
1049	AC	AA
454	AC	AA
1350	AC	AA
675	AC	AA
417	AC	AA
1230	AC	AA
385	CC	CC
540	CC	CC
996	CC	CC
1145	CC	CC
1352	CC	CC

Figure legends

Figure 1. Bladder tumours and cell lines with LOH or RCH on 4p16.3-2. Based on loss or retention of the polymorphic loci, two regions of deletion were identified, one distal to D4S1182 (Region 1) and one proximal to D4S43 (Region 2). LOH encompassed FGFRL1 in all cell lines and 83% of tumours with 4p16 LOH.

Figure 2. (A) Relative expression of *FGFRL1* mRNA in UC lines with and without 4p16 RCH, and in UC tumours with and without 4p16 LOH; (B) FGFRL1 protein level in UC tumours with and without 4p16 LOH. (C) FGFRL1 protein levels in UC tumours compared to levels in normal bladder (NB) and normal ureter (NU). Samples were scored based on average staining intensity across the whole tumour, using the following arbitrary units: 0=negative, 1=weak, 1.5=weak/moderate, 2=moderate, 2.5= moderate/strong, and 3=strong.

Figure 3. FGFRL1 protein expression in (A) normal ureter, (B) normal bladder, (C) one papillary pTaG2 tumour, and (D) one invasive pT3G3 tumour. Size bar = 20 µm

Supplementary Figure Legends

Supplementary Figure 1. (A) FGFRL1 mRNA relative expression in normal bladder urothelium, normal urothelium from the urether, and in cultured normal urothelial cells (NHUC) from different donors. RNA extracted from normal bladder urothelium was kindly donated by Dr Jim Catto (University of Sheffield, UK). (B) FGFRL1 mRNA expression in normal ureter and NHUC as detected by Taqman Real Time PCR. Ct values are indicative of a medium level of expression. (C) FGFRL1 protein expression detected by immunohistochemistry in a range of normal human tissues. Size bar = 20 µm (40x magnification). (D) FGFRL1 protein expression detected by Western Blotting in control TERT-NHUC, TERT-NHUC with ectopic FGFRL1 overexpression (+FGFRL1), a range of NHUC from different donors and HEK293 cells.