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Mutant Fibroblast Growth Factor Receptor 3 Induces Intracellular Signaling and Cellular Transformation in a Cell Type- and Mutation-Specific Manner

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Abstract

Although activating mutations of FGFR3 are frequent in bladder tumors, little information is available on their specific effects in urothelial cells or the basis for the observed mutation spectrum. We investigated the phenotypic and signaling consequences of three *FGFR3* mutations (S249C, Y375C, and K652E) in immortalized normal human urothelial cells (TERT-NHUC) and mouse fibroblasts (NIH-3T3). In TERT-NHUC, all mutant forms of FGFR3 induced phosphorylation of FRS2 α and ERK1/2, but not AKT or SRC. PLC γ 1 phosphorylation was only observed in TERT-NHUC expressing the common S249C and Y375C mutations, and not the rare K652E mutation. Cells expressing S249C and Y375C FGFR3 displayed an increased saturation density, related to increased proliferation and viability. This effect was significantly dependent on PLC γ 1 signaling and undetectable in cells expressing K652E FGFR3, which failed to phosphorylate PLC γ 1. In contrast to TERT-NHUC, expression of mutant FGFR3 in NIH-3T3 resulted in phosphorylation of Src and Akt. Additionally, all forms of mutant FGFR3 were able to phosphorylate Plc γ 1 and induce morphological transformation, cell proliferation, and anchorage independent growth. Our results indicate that the effects of mutant FGFR3 are both cell type- and mutation-specific. Mutant FGFR3 may confer a selective advantage in the urothelium by overcoming normal contact inhibition of proliferation.

Keywords

fibroblast growth factor; FGFR3; bladder; urothelium; signaling

Introduction

Fibroblast growth factor receptor 3 (FGFR3) is one of four trans-membrane receptors that mediate the intracellular effects of fibroblast growth factors (FGFs) (Ornitz and Itoh, 2001). Structurally, FGFRs consist of an extracellular portion with three immunoglobulin-like domains, a trans-membrane segment, and an intracellular split tyrosine-kinase region. In normal physiological situations, signaling is triggered by the interaction of FGF with the extracellular domain of the receptor, which induces receptor dimerization and auto-phosphorylation. Activation of the receptor is followed by a phosphorylation cascade

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Conflict of interests

The authors declare no conflict of interest.

culminating in the activation of a number of signaling pathways, such as MAPK, PI3K and PKC (Dailey *et al.*, 2005; Eswarakumar *et al.*, 2005).

Germline missense mutations of *FGFR3* resulting in its constitutive ligand-independent activation have been described in number of skeletal dysplasia syndromes (Eswarakumar *et al.*, 2005). Functional and animal studies have shown that in chondrocytes, pathological activation of FGF signaling causes premature cell differentiation that halts limb growth and development. These effects are mediated by the activation of downstream targets with anti-proliferative and pro-apoptotic activity, such as the transcription factors STAT1, STAT3, and STAT5, and the cell cycle inhibitors p16, p18, p19, and p21, (Chen *et al.*, 1999; Legeai-Mallet *et al.*, 2004; Li *et al.*, 1999; Sahni *et al.*, 1999; Su *et al.*, 1997).

Interestingly, somatic activating mutations of *FGFR3* corresponding to the germline mutations found in skeletal dysplasia syndromes are observed in many urothelial carcinomas (UC) of the bladder (Cappellen *et al.*, 1999; Sibley *et al.*, 2001a; van Rhijn *et al.*, 2002), suggesting that in a different cellular context *FGFR3* may act as an oncogene. The *FGFR3* mutations observed in UC are clustered in exons 7 (codons 248 and 249), 10 (codons 372, 373, 375, 382, and 393) and 15 (codon 652). S249C is the most common mutation in UC, occurring in more than 70% of mutant tumors, while Y375C is the second most common mutation with a frequency of around 22% (Tomlinson *et al.*, 2007a). Both these mutations create unpaired cysteines in the proximal extracellular region, leading to the formation of disulfide bonds between adjacent receptors, thereby inducing ligand-independent dimerization and activation (Adar *et al.*, 2002; d'Avis *et al.*, 1998). Mutations within the kinase domain, such as K652E, are thought to induce a conformational change in the activation loop, resulting in constitutive auto-phosphorylation of the receptor (Webster *et al.*, 1996). These mutations are only observed in a small proportion of cases. Currently, the reason for the differences in frequency of specific *FGFR3* mutations in UC is unclear, although a role of smoking-related carcinogens in determining the occurrence or the type of *FGFR3* mutations has been excluded (Wallerand *et al.*, 2005).

Although *FGFR3* mutations have also been found in multiple myeloma (Chesi *et al.*, 2001) and carcinoma of the cervix (Cappellen *et al.*, 1999), their frequency in these malignancies is much lower than in UC. Furthermore, except for benign skin tumors (Hafner *et al.*, 2006), other solid epithelial tumors have been found negative for *FGFR3* mutation (Karoui *et al.*, 2001; Sibley *et al.*, 2001b), pointing to a specific role in bladder carcinogenesis. Notably, *FGFR3* mutations in UC are also found in flat urothelial hyperplasias (van Oers *et al.*, 2006), implying that they may represent an early event during malignant transformation. The importance of *FGFR3* signaling in bladder transformation is further emphasized by the effects of *FGFR3* silencing in the bladder cancer cell lines MGHU3 and TCC97-7, which harbor the Y375C and S249C mutations respectively (Bernard-Pierrot *et al.*, 2006; Tomlinson *et al.*, 2007b). In both cases, decreased expression of mutant *FGFR3* inhibited cell proliferation and anchorage-independent growth. Furthermore, a specific anti-*FGFR3* antibody has been shown to inhibit proliferation of RT112, a bladder carcinoma cell line known to over-express wildtype *FGFR3* (Martinez-Torrecuadrada *et al.*, 2005).

Despite the overwhelming evidence linking *FGFR3* mutation to bladder carcinogenesis, and its potential as a therapeutic target, most functional studies on *FGFR3* have been performed either in chondrocytes or mouse fibroblasts. As *FGFR3* activation appears to have diverse outcomes in different cellular contexts, it is crucial to investigate the signaling pathways mediated by mutant *FGFR3* and their cellular consequences specifically in urothelial cells. In this study, we elucidate *FGFR3* signaling in normal urothelial cells and show that mutant *FGFR3* may confer a selective advantage by promoting proliferation and survival, in a partially PLC γ 1-dependent manner. We also demonstrate a clear correlation between the

frequency of different *FGFR3* mutations in bladder tumors and the level of ligand-independence, signaling activation, and phenotypic consequences.

Results

Mutant forms of FGFR3 induce transformation of mouse fibroblasts

Initially, we assessed the ability of three mutant forms of FGFR3 that are found in UC (S249C, Y375C, and K652E) to transform NIH-3T3 cells. In these cells, expression of all *FGFR3* mutations resulted in a transformed spindle-like morphology (Figure 1a), higher proliferation rate ($p=0.004$) (Figure 1b) and colony formation in soft agar ($p=0.05$) (Figure 1c). No effect on morphology, proliferation or anchorage-independence was observed for the 'kinase dead' control (S249C KD) (data not shown). All mutant forms of FGFR3 showed high levels of constitutive receptor phosphorylation (Figure 1d). Both the 130 and 120 kDa glycosylated forms of K652E FGFR3 were phosphorylated, while S249C and Y375C mutants showed activation only of the 130 kDa protein. Expression of all forms of mutant FGFR3 in NIH-3T3 increased phosphorylation of Frs2 α , Plc γ 1, and Src, and activated Erk1/2 and Akt (Figure 1d, Supplementary Figure 1).

Mutant FGFR3 increases saturation density in normal urothelial cells

Next we studied the effects of mutant FGFR3 in normal urothelial cells by expressing S249C, Y375C and K652E FGFR3 in TERT-NHUC. Expression levels were comparable to those observed in the bladder cancer cell line TCC97-7, derived from a low stage UC (data not shown). In contrast to the effects in NIH-3T3, expression of mutant FGFR3 in normal urothelial cells did not induce obvious morphological changes or anchorage-independent growth (data not shown). Furthermore, at subconfluence there was no difference in proliferation between cells expressing mutant FGFR3 and controls. However, TERT-NHUC expressing S249C and Y375C FGFR3 were characterized by an increased saturation density, and at confluence reached mean cell numbers 24% and 20% higher than controls ($p < 0.05$) (Figure 2a). Consistent with their increased saturation density, cells expressing mutant FGFR3 also acquired a different morphology at confluence. While controls produced a compact monolayer of flat cells, mutants displayed reduced cell-cell adhesion, formed ridges and acquired a multilayered appearance (Supplementary Figure 2).

The saturation density of cells expressing K652E FGFR3 was similar to controls and cells expressing wildtype FGFR3 or S249C KD. Therefore, the magnitude of the phenotypic effect was of the order S249C>Y375C>K652E=Wildtype=Control, correlating with the relative frequencies at which these mutations are observed in bladder tumors.

Mutant FGFR3 promotes proliferation and viability of confluent TERT-NHUC

To investigate the cellular mechanisms that determine the higher saturation density associated with expression of mutant FGFR3, we analyzed both cell cycle profile and viability in subconfluent and confluent cells.

In controls, the number of cells in the G0/G1 phase of the cell cycle progressively increased at confluence, while the number of viable cells decreased. No significant differences in cell cycle profile or viability were observed between mutant and control cells in subconfluent cultures. However at confluence on day 12, only 59% of S249C were in G0/G1, compared with 68% of control and wildtype cells ($p=0.004$) (Figure 2b). A corresponding increase was detected in the proportion of S249C in S (8% vs 7%) ($p=0.035$) and G2/M phase (33% vs 25%) ($p=0.004$). Similar results were observed on days 8 and 10 (data not shown). The cell cycle profile of Y375C- and K652E-expressing cells was intermediate between S249C

mutants and control cells (Figure 2b). No significant differences in cell cycle profile were measured between control cells and cells expressing S249C KD FGFR3 (data not shown).

Cells expressing S249C and Y375C FGFR3 were significantly more viable at confluence compared to controls or cells expressing wildtype FGFR3 ($p < 0.001$). On days 12 (Figure 2c) and 15 (data not shown), on average 45% of S249C cells were viable, compared with only 35% of control cells. Y375C showed an increase in viable cells on day 12 (41% vs. 35%) (Figure 2c) but no difference on day 15 (data not shown). In contrast to the other two mutants, the viability of confluent cells expressing K652E FGFR3 was similar to controls ($p > 0.05$).

Mutant FGFR3 alters the expression of cell cycle and survival proteins in normal urothelial cells

Consistent with their altered cell cycle profile, confluent TERT-NHUC expressing mutant FGFR3 exhibited changes in cell cycle-related proteins (Figure 2d, Supplementary Figure 3a). Whilst in control cells the expression of the retinoblastoma protein (RB) was switched off upon reaching confluence, RB was expressed and phosphorylated in confluent cells expressing S249C FGFR3 and, to a lesser extent, in Y375C mutants. Furthermore, expression of CDK1 was increased in S249C and Y375C cells, and the difference was particularly pronounced when the cells were starved in depleted medium to block stimulation by other growth factors. The protein profile of cells expressing K652E FGFR3 was different from the other mutants, with low RB and intermediate CDK1 levels, consistent with their lower saturation density.

Three proteins involved in cell survival, MCL1, BCL-X_L, and BCL2, were up-regulated in confluent cells expressing all types of mutant FGFR3 (Figure 2d, Supplementary Figure 3b). Surprisingly, there was no difference in the expression of these proteins between K652E and the other mutants, suggesting that differential expression of some other regulators of cell survival may be responsible for the lower viability of cells expressing K652E FGFR3.

Differential intensity of signaling is associated with distinct FGFR3 mutations

To investigate the reason for the differential behavior of cells expressing K652E FGFR3, we assessed the phosphorylation levels of FGFR3 mutant proteins and downstream effectors in urothelial cells. All mutant forms of FGFR3 were constitutively phosphorylated in the absence of ligand (Figure 3a). The level of activation of the K652E receptor was higher than the S249C and Y375C mutants (Figure 3a, Supplementary Figure 4a). Similar to results in NIH-3T3, K652E FGFR3 differed from the other mutants in that both the 130 kDa and 120 kDa forms were phosphorylated. Consistent with their constitutive activation, ligand-independent dimerization of S249C and Y375C FGFR3 was observed by western blotting under non-denaturing conditions (Figure 3b). Some differences were observed between the size of dimers formed by S249C and Y375C FGFR3, possibly as the result of differential glycosylation. K652E FGFR3 did not spontaneously form dimers, as this mutation does not produce unpaired cysteines.

Signaling was examined in cells at various degrees of confluence, in full and depleted medium (Figure 3c, Supplementary Figure 4b). In all conditions tested, no differences were observed between mutant and control cells regarding the levels of activated p38, JNK, SRC and AKT (data not shown). Constitutive phosphorylation of FRS2 α was detected in mutant FGFR3-expressing cells in all conditions. Increased phosphorylation of ERK1/2 was observed in mutant-expressing cells at subconfluence in depleted medium and at confluence in full medium. However, in exponentially growing cells in full medium, ERK1/2 was equally activated in all cell types, presumably due to stimulation *via* other growth factor

receptors. Similarly, increased PLC γ 1 phosphorylation in FGFR3 mutant cells was only observed after confluence or starvation. Interestingly, in contrast to our findings in NIH-3T3 cells, in TERT-NHUC PLC γ 1 phosphorylation was detected exclusively in cells expressing S249C and Y375C FGFR3, but not K652E.

Overall, these results suggest that although all mutant forms of FGFR3 are constitutively phosphorylated in urothelial cells, they differ in their ability to activate downstream signaling cascades, particularly with regard to PLC γ 1 phosphorylation.

Differential ligand dependence of mutant forms of FGFR3

We next examined whether cells expressing wildtype and mutant FGFR3 were responsive to FGF1 stimulation in terms of receptor activation (Figure 4a, Supplementary Figure 5) and signaling (Figure 4b, Supplementary Figure 5). Phosphorylation of wildtype FGFR3 could be induced by a short incubation with FGF1 (Figure 4a). In contrast, the strong constitutive phosphorylation of S249C FGFR3 was unaffected by treatment with FGF1, suggesting complete ligand-independence. On the other hand, Y375C and K652E FGFR3 showed increased levels of phosphorylation in response to ligand stimulation (Figure 4a).

As expected, FGF1 induced phosphorylation of FRS2 α , ERK1/2 and PLC γ 1 in normal urothelial cells over-expressing wildtype FGFR3 (Figure 4b). Consistent with their complete ligand-independence, FGF1 treatment failed to stimulate signaling further in cells expressing the S249C mutation. However, cells expressing the Y375C mutation, responded to FGF1 treatment with a small increase in ERK1/2 activation, while phosphorylation levels of FRS2 α and PLC γ 1 remained unchanged. Cells expressing the K652E mutated protein displayed the most pronounced response to ligand, with increased phosphorylation of PLC γ 1, ERK1/2 and FRS2 α (Figure 4b). Overall, therefore, the degree of ligand-independence of the different mutant FGFR3 was in the order S249C>Y375C>K652E.

PLC γ 1 phosphorylation contributes to FGFR3-mediated increase in saturation density

To clarify whether the lack of constitutive PLC γ 1 phosphorylation may explain the different phenotypic behavior associated with the K652E mutation, we used a construct encoding a S249C FGFR3 protein with a mutated PLC γ 1 binding site (S249C+Y762F). This mutant protein was able to constitutively activate FRS2 α and ERK1/2, but failed to phosphorylate PLC γ 1 (Figure 5a). When expressed in NIH-3T3, S249C+Y762F FGFR3 was associated with morphological transformation (Figure 5b), increased proliferation ($p=0.004$) (Figure 5c), and increased colony formation in soft agar ($p=0.05$) (Figure 5d), compared to control cells, indicating that PLC γ 1 signaling is not essential for FGFR3-induced transformation in this cell type.

In TERT-NHUC, abolishing PLC γ 1 phosphorylation significantly reduced the increase in saturation density associated with S249C FGFR3 (13% vs. 24%, $p=0.05$) (Figure 6a), suggesting that PLC γ 1 signaling contributes to this phenotype. Confluent TERT-NHUC expressing the S249C+Y762F mutant showed an increase in the number of cells in G0/G1 phase (63% vs. 59%) ($p=0.006$), a decrease in the number of cells in G2/M (27% vs 33%) ($p=0.004$) (Figure 6b), and a lower viability (39% vs. 45%, $p=0.047$) (Figure 6c) compared with S249C cells. These results confirm our hypothesis that lack of PLC γ 1 phosphorylation may contribute to the lower viability and saturation density exhibited by cells expressing the K652E *FGFR3* mutation. However, cells expressing S249C+Y762F FGFR3 achieved higher cell numbers at confluence compared with K652E and control cells ($p=0.05$) (Figures 2a and 6a), suggesting that other signaling pathways also modulate this phenotype and may be differentially activated between K652E and the other mutants.

Discussion

FGFR3 is the predominant FGF receptor in the urothelium, where its expression levels are up to 20-fold higher than other FGFRs (Tomlinson *et al.*, 2005). A number of studies have underlined a link between bladder carcinogenesis and dysregulated, ligand-independent signaling due to activating mutations of *FGFR3*. Despite its important physiological and pathological roles in the urothelium, however, most information regarding the effects of mutant FGFR3 derives from functional investigations performed in other tissues or cell types. This is the first study to specifically investigate the signaling and phenotypic consequences of mutant FGFR3 in normal urothelial cells.

Our results show that in urothelial cells mutant FGFR3 initiates a sequence of phosphorylation events mediated by FRS2 α and PLC γ 1, leading to the specific activation of the ERK1/2 signaling pathway, without simultaneous phosphorylation of JNK or p38. In contrast to findings in NIH-3T3, no differences in the activation of Src and Akt were detected in TERT-NHUC expressing mutant FGFR3. Previous studies have also described differences in FGFR3 signaling between different cell types. For example, whilst the STAT1 signaling pathway mediates the effects of FGFR3 in chondrocytes, STAT1 is not activated by FGFR3 signaling in NIH-3T3 (Sahni *et al.*, 1999), or multiple myeloma cell lines (Ronchetti *et al.*, 2001). Similarly we have not detected STAT1 phosphorylation in normal urothelial cells expressing mutant FGFR3 (unpublished). The diversity of FGFR3 signaling in different cellular contexts may be due to tissue-specificity in the expression of modulators or mediators of signaling, and underlines the crucial importance of investigating the effect of *FGFR3* mutation specifically in the target cells of interest.

We have also shown that, as a result of *FGFR3* mutation, normal urothelial cells reach higher cell numbers at confluence, due to a combination of increased proliferation and viability, and exhibit consistent changes in a number of proteins involved in cell cycle control and survival. Expression of RB, for example, was switched off in confluent control cells, an event consistent with G1 arrest and confluence-induced differentiation in some epithelial cell types (Guy *et al.*, 2001). In contrast, mutant cells retained RB expression and the protein was hyper-phosphorylated. Furthermore, the cell cycle regulator CDK1, which modulates RB phosphorylation (Santamaria *et al.*, 2007; Yamamoto *et al.*, 1995), was up-regulated in cells expressing mutant FGFR3. Complete loss of RB function is associated with both p53-dependent and -independent apoptosis, creating a selective pressure for the acquisition of an anti-apoptotic phenotype (Harbour and Dean, 2000). Indeed, a number of pro-survival factors were found to be up-regulated in cells expressing mutant FGFR3, including BCL2, Bcl-X_L, and MCL1. Thus our observations suggest that at confluence TERT-NHUC cease dividing and, as result of RB downregulation, either differentiate or die. In contrast, cells expressing mutant FGFR3 survive and continue proliferating by phosphorylating RB and inducing mechanisms to escape apoptosis. Some of these events have been observed in bladder tumors *in vivo*. High expression of Cdk1 and hyperphosphorylated Rb has been described in mouse SV40 T-induced bladder tumors (Garcia-Espana *et al.*, 2005) and expression of BCL2 and Bcl-X_L is observed in a large proportion of human bladder carcinomas (Korkolopoulou *et al.*, 2002). Since our observations indicate that these proteins may represent downstream effectors of FGFR3 signaling in urothelial cells, it will be interesting to assess their expression in bladder tumors in relation to *FGFR3* mutation status.

Interestingly, we found that the ability of mutant FGFR3-expressing cells to reach a higher saturation density was partially dependent on PLC γ 1 phosphorylation, as this was reduced in cells expressing S249C FGFR3 with a mutation disrupting the PLC γ 1-binding site. This finding is consistent with a previous report showing that abolishing PLC γ 1 signaling

attenuates, but does not completely eliminate, the ability of mutant FGFR3 to transform Ba/F3 hematopoietic cells (Chen *et al.*, 2005), indicating that transformation in this cell type requires both PLC γ -dependent and -independent mechanisms, possibly relating to MAPK activation. Although mutant FGFR3 has not as yet been implicated in dysregulation of contact inhibition of cell proliferation and movement, PLC γ 1 activation could potentially modulate cytoskeletal reorganization and cell adhesion through PKC signaling and release of calcium from the endoplasmic reticulum (Wells and Grandis, 2003). Furthermore, in some cell types FGF can signal through non-canonical pathways involving a number of cell-cell and cell-matrix adhesion molecules (Murakami *et al.*, 2008).

As expected for a normal cell type, TERT-NHUC require further genetic alterations for the acquisition of a completely transformed phenotype, and FGFR3 mutation alone was not sufficient to induce anchorage independent growth in soft agar or tumor formation in nude mice (unpublished). However, the observed proliferative and survival advantage under confluent conditions in culture suggests that *FGFR3* mutation as an initiating or early genetic event could contribute to clonal expansion or the development of hyperplasia within the urothelium *in vivo*.

Interestingly, the majority of UC with *FGFR3* mutations also show an up to 20-fold increase in the levels of FGFR3 protein and over-expression of the wildtype receptor is found in 42% of bladder tumors without detectable *FGFR3* mutation, indicating that this may be an additional mechanism for FGFR3 dysregulation (Tomlinson *et al.*, 2007a). In this study, FGFR3 expression in transduced cells was comparable to levels in the bladder tumor cell line TCC97-7 and representative of the range of over-expression observed in bladder tumors (Tomlinson *et al.*, 2007b). It is currently unclear whether during malignant transformation FGFR3 up-regulation precedes or follows its mutation. In this study, no proliferative or survival advantage was observed in cells over-expressing wildtype receptor, although phosphorylation and signaling could be induced by FGF1 stimulation, suggesting that over-expression of wildtype FGFR3 may be advantageous in those tumors with high levels of endogenous or exogenous ligand.

Notably, our data indicate a clear hierarchy for specific mutations in relation to ligand-independence, signalling and phenotypic effect, that reflects the frequency at which they are detected in bladder tumors (S249C>Y375C>K652E), thus suggesting that the spectrum of *FGFR3* mutations observed in UC may relate to selection for their potency. In contrast, all forms of mutant FGFR3 were equally transforming in NIH-3T3, underlining the danger of extrapolating to urothelial cells conclusions drawn from functional studies in other cell lines. Cell type- and mutation-specific differences in the effects of mutant FGFR3 are also emphasized by previous studies. In NIH-3T3, in line with our results, all types of mutant FGFR3 were highly phosphorylated and able to induce transformation (Agazie *et al.*, 2003; Bernard-Pierrot *et al.*, 2006; Chesi *et al.*, 2001; Hart *et al.*, 2001; Ronchetti *et al.*, 2001; Webster and Donoghue, 1997). In other cell lines, however, the Y375C mutant was only moderately phosphorylated and partially ligand-dependent (Adar *et al.*, 2002; Bonaventure *et al.*, 2007; Harada *et al.*, 2007), which is consistent with our observations in TERT-NHUC. In HEK293T, the K650E mutation was highly phosphorylated, completely ligand-independent and able to activate PLC γ 1 and STAT1 (Gibbs and Legeai-Mallet, 2007; Harada *et al.*, 2007; Su *et al.*, 1997), while in multiple myeloma cell lines it showed only weak phosphorylation, was partially ligand-dependent and unable to activate STAT1 signaling even after FGF stimulation (Ronchetti *et al.*, 2001). An earlier functional study in BaF3 cells also indicated partial ligand-dependence of the K650E mutant (Naski *et al.*, 1996). To our knowledge, the degree of ligand-independence of the S249C mutant has not been examined previously, although the adjacent mutation R248C was shown to be fully

activated in BaF3 cells (Naski *et al.*, 1996) but only moderately phosphorylated in HEK293 cells (Bonaventure *et al.*, 2007; Gibbs and Legeai-Mallet, 2007).

As K652E FGFR3 displayed a high level of constitutive phosphorylation when expressed in normal urothelial cells, the reasons for the less intense signaling and phenotypic effect is not completely clear. Differential localization of K652E FGFR3 and signaling from distinct cellular compartments could explain some of these differences. Interestingly, in both NIH-3T3 and TERT-NHUC we detected phosphorylation of the 120 KDa form of K652E, but not of S249C or Y375C FGFR3. This observation is consistent with previous reports showing that the immature form of K650E FGFR3 protein may be activated and may signal from the endoplasmic reticulum rather than the cell membrane (Lievens and Liboi, 2003). However, in TERT-NHUC K652E was able to activate FRS2, an observation consistent with membrane rather than cytoplasmic localization (Lievens *et al.*, 2006). Another possible explanation of the differences between K652E and the other mutants is that dimerization of the receptor, which does not occur with the K652E mutation, may be essential for the physical interaction with some downstream signaling effector or for *trans*-phosphorylation of some tyrosine residues, such as Y762. This may explain why in urothelial cells K652E was capable of phosphorylating PLC γ 1 only after stimulation with FGF1.

In conclusion, we have elucidated the signaling pathways activated by mutant FGFR3 in normal urothelial cells and identified putative downstream targets. We have demonstrated that mutant FGFR3 confers both proliferative and survival advantages to urothelial cells, and that this effect is partially PLC γ 1-dependent. We have also demonstrated a clear correlation between the degree of ligand-independence and constitutive signaling of specific mutant FGFR3 proteins that is directly related to the frequency of these mutations in bladder cancer, implying a proportional selective advantage for cells carrying these mutations.

Materials and Methods

Cell culture

Telomerase-immortalized normal human urothelial cells (TERT-NHUC) (Chapman *et al.*, 2006) were cultured in Keratinocyte Growth Medium 2 (KGM2) (Promocell GmbH., Heidelberg, Germany) supplemented with provided growth factors, 0.09 mM CaCl₂, and 30 ng/ml cholera toxin (Sigma-Aldrich Company Ltd., Poole, UK). For FGF1 stimulation experiments, cells were treated with 20 ng/ml recombinant human FGF1 and 10 μ g/ml heparin (R&D Systems Europe Ltd., Abingdon, UK) after 1 h incubation in depleted medium (KGM2 without added growth factors). NIH-3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 10% FCS (Invitrogen Ltd, Paisley, UK) and 2 mM L-glutamine. Both cell lines were cultured in a humidified atmosphere at 37°C in 5% CO₂.

Expression vectors and transduction of cell lines

Site-directed mutagenesis on FGFR3 IIIb cDNA was used to create the S249C, S249C +K510A ('kinase-dead', S249C KD) (Monsonigo-Ornan *et al.*, 2002), S249C+Y762F, Y375C, and K652E mutations. The presence of the correct mutation was verified by sequencing and wildtype and mutant FGFR3 were cloned into a retroviral expression vector (pFB; Stratagene, La Jolla, CA) containing a hygromycin resistance cassette (Tomlinson *et al.*, 2005). The expression vectors were transfected into Phoenix A cells using siPORT XP-1 (Ambion, Huntingdon, UK). TERT-NHUC and NIH-3T3 cells were incubated with retroviral supernatant containing 8 μ g/ml polybrene and selected with hygromycin 48 h after transduction.

Phenotypic assays

For all phenotypic assays, $1-3 \times 10^3$ cells/cm² were seeded in triplicate and each experiment was repeated at least three times. All experiments were carried out shortly after transduction (<30 population doublings). To investigate anchorage-independent growth, cells were cultured in medium containing 0.3% agarose with weekly feeding. Colonies bigger than 200 μ m were counted on day 28, after staining with 8 mM p-iodonitrotetrazolium violet (Sigma-Aldrich). For growth curves, cells were counted with a Z2 Coulter™ analyzer (Beckman Coulter Ltd., High Wycombe, UK). The Guava EasyCyte™ Plus System 3 (Guava Technologies, Inc., Stamford, UK) was used to examine cell viability with the ViaCount® dye-exclusion test (Guava Technologies), and cell cycle profile with propidium iodide staining. Cell cycle profiles were analysed using ModFit LT 2.0 software (Verity Software House, Topsham, US).

Western blotting

Protein extraction and quantification was carried out as described (Tomlinson *et al.*, 2005). Heat-denatured proteins were separated by 7.5-12.5% SDS-PAGE, transferred to Hybond-C membrane (GE Healthcare Life Sciences, Buckinghamshire, UK), and incubated with 1:1000 primary antibodies. For detection of FGFR3 dimers, the denaturation step was omitted. Primary antibodies used were: anti-FGFR3 clone B9, anti-ERK1/2, anti-phospho-ERK1/2 (Tyr 204), anti-RB, and anti-CDK1 from Insight Biotechnology Ltd. (Wembley, UK), anti-phospho-PLC γ 1 (Tyr 783), anti-PLC γ 1, anti-phospho-FRS2 α (Tyr 436), anti-phospho-SRC family (Tyr 416), anti-phospho-p38 MAPK (Thr 180/Tyr 182), anti-phospho-RB (Ser 807/811), anti-phospho-AKT (Ser 473), anti-MCL1, anti-BCL2 and anti-BCL-X_L from New England Biolabs Ltd. (Hitchin, UK). Bound primary antibodies were detected using HRP-conjugated secondary antibody and the ECL Plus kit (GE Healthcare Life Sciences). Blots were reprobed with anti-actin clone AC-40 (Sigma-Aldrich Company Ltd) or anti-tubulin alpha (MorphoSys UK Ltd., Kidlington, UK) as loading control.

Immunoprecipitation

For detection of phosphorylated FGFR3, protein lysates were incubated overnight at 4°C with 2 μ l of rabbit anti-FGFR3 cytoplasmic antibody (Sigma-Aldrich Company Ltd), followed by 2 h incubation at 4°C with protein A-sepharose® beads (Sigma-Aldrich Company Ltd). Eluted proteins were separated by 7.5% SDS-PAGE. Phosphorylated FGFR3 was detected using 1:1000 anti-phosphotyrosine antibody clone 4G10 (Millipore, Hampshire, UK) in 5% BSA (Sigma-Aldrich).

Statistical analysis

Significant differences between cell types were assessed using the Mann-Whitney test, with the SPSS 12.0 statistical analysis software (SPSS Inc., Chicago, US). A $p < 0.05$ was accepted as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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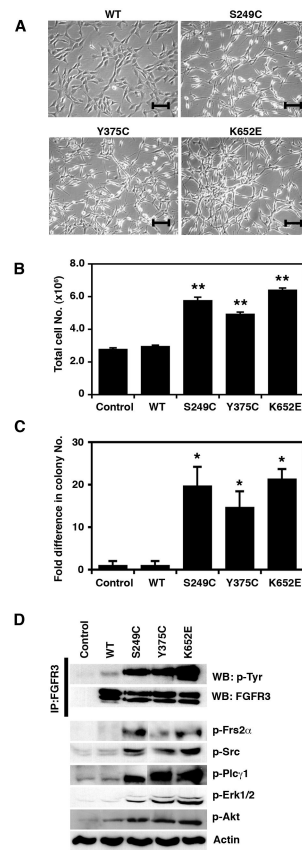


Figure 1.

Effects of mutant FGFR3 in NIH-3T3 cells. **(a)** Morphology of cells expressing wildtype (WT), S249C, Y375C, and K652E FGFR3. Line bars indicate 100 μ m. **(b)** Representative experiment showing the total cell numbers reached on day 7 by cells expressing WT or mutant FGFR3 and by control cells. **(c)** Fold difference in the number of colonies formed in soft agar by cells expressing WT or mutant FGFR3 and control cells. **(d)** Constitutive phosphorylation of mutant FGFR3 and ligand-independent activation of signaling cascades. Significant differences to control cells (transduced with an empty pFB vector) are indicated as ‘*’ ($p < 0.05$) or ‘**’ ($p < 0.01$)

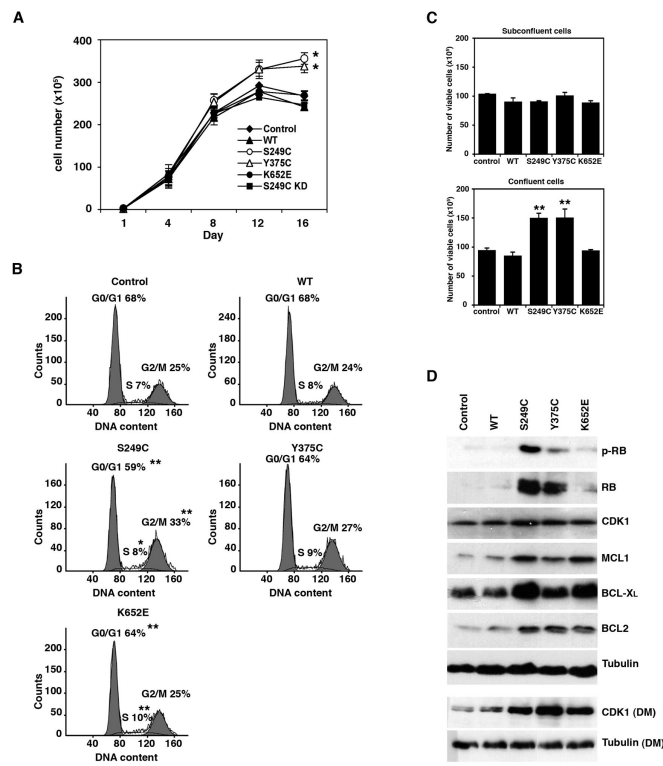


Figure 2. Effects of mutant FGFR3 in TERT-NHUC. **(a)** Saturation density of confluent cells. **(b)** Flow cytometric analysis of cell cycle profiles of confluent cells (day 12). **(c)** Representative experiment showing the number of viable cells expressing WT or mutant FGFR3, compared to control cells, at subconfluence (day 4) and confluence (day 12). **(d)** Expression levels of proteins involved in cell cycle and cell survival in confluent cultures (day 12); proteins were extracted from cells grown in full medium unless otherwise specified (DM, depleted medium). Significant differences to control cells (transduced with an empty pFB vector) are indicated as ‘*’ ($p < 0.05$) or ‘**’ ($p < 0.01$)

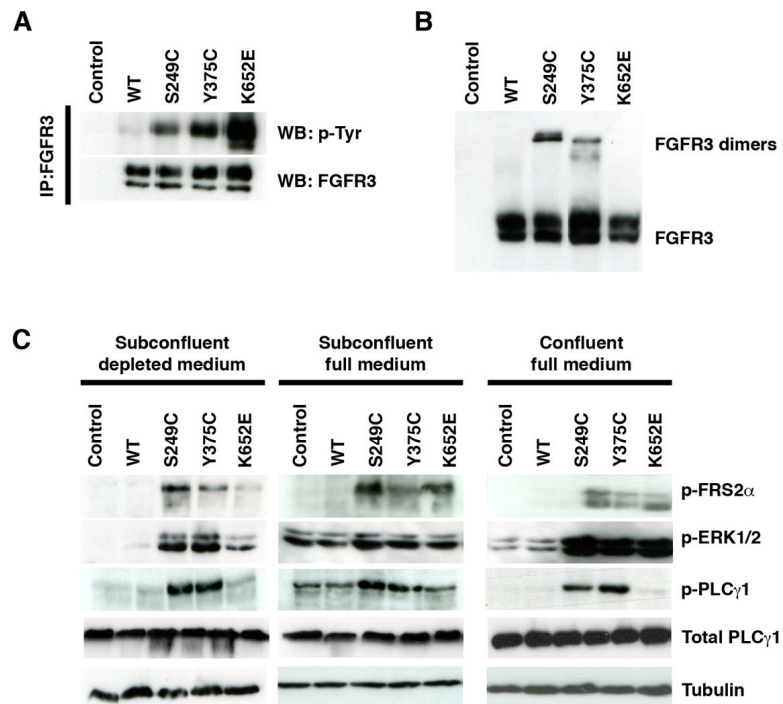


Figure 3. Constitutive activation of mutant FGFR3 and downstream signaling cascades in TERT-NHUC. **(a)** Constitutive phosphorylation of mutant FGFR3. **(b)** Western blot of cell lysates run under non-denaturing conditions showing ligand-independent dimerization of S249C and Y375C FGFR3. **(c)** Downstream signaling activated by mutant FGFR3 in confluent (day 12) and subconfluent (day 4) cells in full medium, and in subconfluent cells after 1 h in depleted medium.

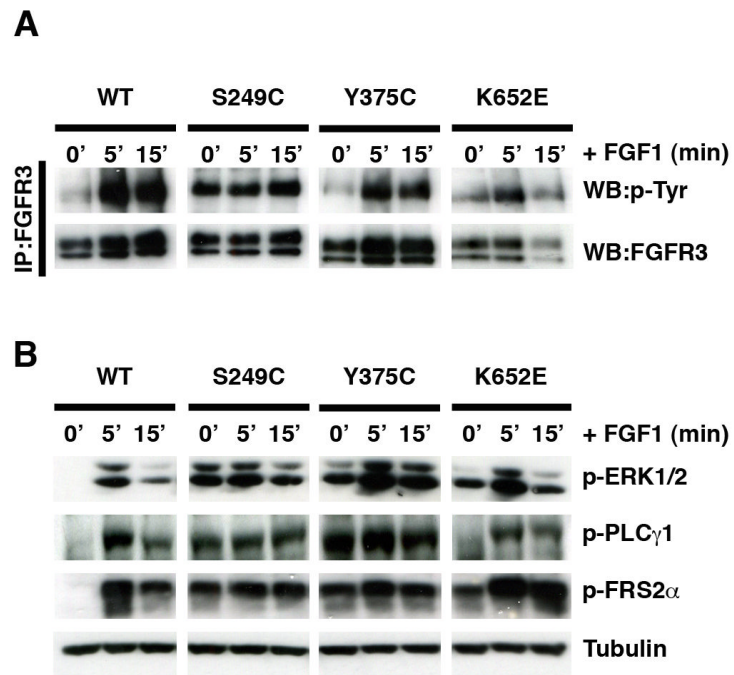


Figure 4. Ligand independence of different forms of mutant FGFR3 expressed in TERT-NHUC. Receptor phosphorylation (**a**) and signaling activation (**b**) in starved subconfluent cells, before and after stimulation with FGF1.

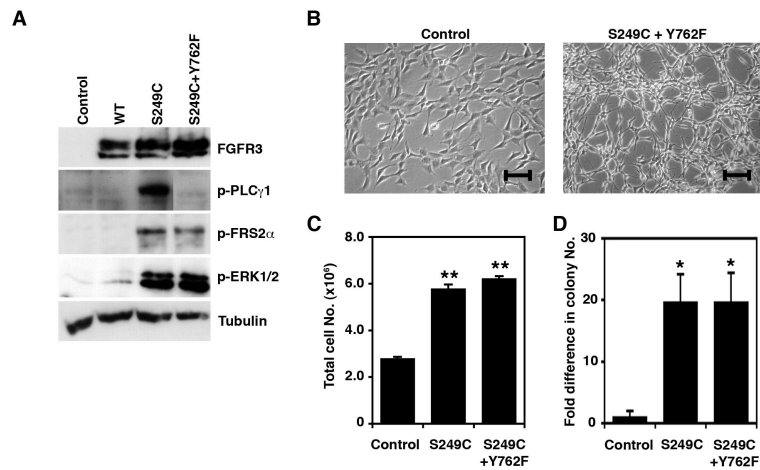


Figure 5.

Contribution of Plc γ 1 phosphorylation to FGFR3-mediated transformation in NIH-3T3. **(a)** Activation of signaling cascades in cells expressing S249C FGFR3 or S249C with a mutation in the PLC γ 1 binding site (S249C+Y762F). **(b)** Morphology of NIH-3T3 cells expressing S249C+Y762F FGFR3 and control cells. Line bars indicate 100 μ m. **(c)** Representative experiment showing total cell numbers reached on day 7 by NIH-3T3 cells expressing S249C and S249C+Y762F FGFR3, compared with controls **(d)** Fold difference in the number of colonies >200 μ m formed in soft agar by NIH-3T3 cells expressing S249C and S249C+Y762F FGFR3, compared with control cells. Significant differences to control cells (transduced with an empty pFB vector) are indicated as ‘*’ (p<0.05) or ‘**’ (p<0.01)

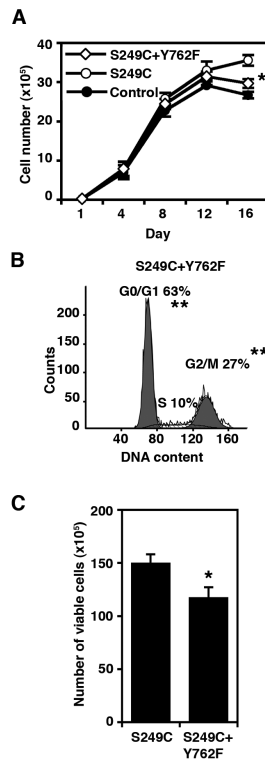


Figure 6.

Contribution of PLC γ 1 phosphorylation to saturation density in TERT-NHUC. **(a)** Growth curves showing saturation density achieved by cells expressing S249C+Y762F FGFR3, compared with cells expressing S249C FGFR3 and control cells. **(b)** Flow cytometric analysis of cell cycle profiles of confluent cells (day 12) expressing S249C+Y762F FGFR3. **(c)** Representative experiment showing the number of viable cells at confluence (day 12) in cultures expressing S249C+Y762F FGFR3 compared with cells expressing S249C FGFR3. Significant differences to S249C cells are indicated as ‘*’ ($p < 0.05$) or ‘***’ ($p < 0.01$)