RESEARCH ARTICLE

Oncogenic Activation of *Fibroblast Growth Factor Receptor-3* and *RAS* Genes as Non-Overlapping Mutual Exclusive Events in Urinary Bladder Cancer

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Abstract

Background: Urinary bladder cancer is a common malignancy in the West and ranks as the 7th most common cancer in our region of Kashmir, India. *FGFR3* mutations are frequent in superficial urothelial carcinoma (UC) differing from the *RAS* gene mutational pattern. The aim of this study was to analyze the frequency and association of *FGFR3* and *RAS* gene mutations in UC cases. Materials and Methods: Paired tumor and adjacent normal tissue specimens of 65 consecutive UC patients were examined. DNA preparations were evaluated for the occurrence of *FGFR3* and *RAS* gene mutations by PCR-SCCP and DNA sequencing. Results: Somatic point mutations of *FGFR3* were identified in 32.3% (21 of 65). The pattern and distribution were significantly associated with low grade/stage ($p<0.05$). The overall mutations in exon 1 and 2 in all the forms of *RAS* genes aggregated to 21.5% and showed no association with any clinic-pathological parameters. In total, 53.8% (35 of 65) of the tumors studied had mutations in either a *RAS* or *FGFR3* gene, but these were totally mutually exclusive in and none of the samples showed both the mutational events in mutually exclusive *RAS* and *FGFR3*. Conclusions: We conclude that *RAS* and *FGFR3* mutations in UC are mutually exclusive and non-overlapping events which reflect activation of oncogenic pathways through different elements.

Keywords: Bladder cancer - *FGFR3* - *RAS* - gene mutations - Kashmir - mutually exclusive

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Introduction

Urothelial carcinoma (UC) is a common malignancy. Worldwide, it is the seventh most prevalent cancer, accounting for 3.2% of all malignancies (Beaglehole et al., 2004). The highest incidence is seen in industrialized countries and geographic areas where infection with Schistosoma haematobium is endemic (Pelucchi et al., 2006). UBC is the fourth most incident cancer in males and ninth in females. Men have a higher risk of bladder cancer than women, by a rate ratio of at least 3:1. The American Cancer Society estimates that 70,980 adults were diagnosed with bladder cancer in 2009, leading to 14,330 adult deaths in the United States. A detailed 5 year study of the bladder cancer cases revealed that bladder cancer ranks as the 7th leading cancer and accounts for 5.9% of all prevalent cancers in the Kashmiri population (Arshad et al., 2012).

UC arises primarily from the transitional cells of the bladder mucosal epithelium (90% of cases) and may present as a noninvasive, papillary tumor protruding from the mucosal surface that is readily resectable. However, about one-third of incident bladder cancers present as solid, non papillary tumors, which originate from in situ dysplasia and carcinoma in situ. These tumors invade the bladder wall and have a high propensity for metastasis (Knowles et al., 1999; Malkowicz et al., 2007). This stark difference in morphology and survival implicates separate oncogenic pathways for noninvasive vs. muscle-invasive cancer (Dinney et al., 2007).

There have been major efforts to understand the molecular pathogenesis of both groups of bladder cancers to establish the basis for their divergent clinical behavior and to provide potential markers for disease monitoring and targets for therapy. Particularly, the combination of histopathological findings and molecular genetic events has led to the concept of the two-pathway model for bladder carcinogenesis, with TP53 responsible for the pathway leading to dysplasia, carcinoma in situ (CIS) lesions and invasive tumors (Dinney et al 1994; Hartmann et al., 2002; Nagata et al., 2016). The other arm of the model is represented by *FGFR3*: the identification of

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mutations in the *FGFR3* gene in a substantial proportion of primary bladder tumors of low stage and grade (Hartmann et al., 2002) has been the most exciting discovery in the recent years. A substantial part of these *FGFR3* wild type tumors present with mutations in one of the *RAS* genes (Juanpere et al., 2012).

Activating mutations of *FGFR3* are found in the germline in several autosomal dominant human skeletal dysplasia syndromes (Vajo et al., 2000; Billerey et al., 2001). The same activating point mutations that accounted for the skeletal anomalies in these syndromes were found in multiple myeloma (Bellus et al., 2000) and carcinomas of the bladder (Chesi et al 1997; Cappellen et al 1999; Billerey et al., 2001; Sibley et al., 2001), prostate (Kimura et al., 2001), and cervix (Billerey et al., 2001).

FGFR3 belongs to a family of structurally related tyrosine kinase receptors. Fibroblast growth factor receptors regulate cell growth, differentiation, and angiogenesis (Hernandez et al., 2009, Powers et al., 2007). Somatic mutations of the gene were reported in approximately 40% of the bladder tumors analyzed. These mutations are significantly associated with low tumor grade and low tumor stage (Sibley et al., 2001; Ornitz et al., 2002).

The four cellular *RAS* genes encode four highly homologous 21 kDa proteins: *HRAS*, *NRAS*, *KRAS4A* and *KRAS4B*. Activating *RAS* mutations occur in ~30% of human cancers. *HRAS* mutations predominate in bladder cancer (Dinney et al., 2007). Many studies have examined only *HRAS* and have reported a wide range of mutation frequencies (0-70%) that may reflect true differences in the tumors examined or technical differences between assays. Currently, there is agreement from several studies that the frequency for H-*RAS* is in the range of 10-20% (van Rhijn et al., 2001). Few studies have screened *NRAS* and *KRAS*2, (Uchid et al 1995; Olderoy et al 1998; Przybojewska et al., 2000; Ayan et al., 2001).

FGFR3 and *RAS* are in the same signal transduction pathway, which might be a possible explanation for the hypothesis of mutual exclusiveness of mutations in these genes in this study. We analyzed the mutations of *FGFR3* gene and *RAS* gene family (*HRAS*, *NRAS*, and *KRAS*) to explore the association of *FGFR3* & *RAS* gene alterations as two genetic events in the development of UC.

Materials and Methods

Subjects in molecular analysis

This prospective study was conducted in Department of Immunology and Molecular Medicine, at the Sher-I-Kashmir Institute of Medical Sciences (SKIMS) in Kashmir, India. The Ethical Committee of SKIMS Deemed University approved the study. All patients signed the written informed consent. A total of sixty-five (n=65) consecutive urinary bladder tumors surgically resected either by TURBT and radical cystectomy and their adjacent normal tissues were used for the mutational analysis of the *FGFR3* and *RAS* gene. All the samples resected by urological surgeon were confirmed to be histologically bladder cancers. A recurrence was defined as the presence of histologically proven bladder cancer at a positive cystoscopy after a complete previous resection. Almost all the patients had attended the hospital with a clinical presentation of haematuria, a hallmark of bladder cancer. The clinico-pathologic characteristics of these patients are listed in the Table 2. In this study 84.4% (n=55) of the cases were males and 15.6% (n=10) were females with a male: female ratio of 6:1. On the basis of age, the patients were grouped into two categories, less than 50 years (<50) and greater than or equal to 50 years of age (≥50). The number of cases in the age group of ≥50 (n=45; 70%) exceeded than <50 years (n=20; 30%).

Based on the smoking status, 50 patients were smokers who were inclusively males (76.9 %) and 15 were non-smokers (23.1%). Based on the differentiation, there were 06 (7.6%), 27 (41.5%), 24 (38.4%) and 08 (12.3%) cases with grade I, II, III, and IV respectively. Histological breakup of the bladder cancer cases were pTa 21(32.3%) and pT1 and pT2 sharing equal number of cases as 22 (33.8%) each. All the cases of bladder cancer cases were histologically confirmed to be transitional cell carcinoma (TCC) except one rare case of adenocarcinoma. Clinical and operative findings revealed that superficial bladder cancer cases were more (n=40; 61.5%) than the muscle invasive type (n=25; 38.5%). Among all the cases, 49 (75.3%) were primary tumors while as 16 (24.7%) were confirmed to be recurrent bladder tumors.

PCR-SSCP analysis

The single-strand conformation polymorphism (SSCP) analysis of the amplicons of exon 7, 10, and 15 of *FGFR3* and exon 1 and 2 of *HRAS*, *NRAS* and *KRAS* gene was performed on 6% non-denaturing polyacrylamide gel (PAGE) utilizing non-radioactive silver staining. polymerase chain reaction (PCR) of the same exons of both the genes was performed using previously described specific primers shown in Table 1. PCR amplification was carried out in a 50 µL volume container with 50 ng of genomic DNA, 1XPCR buffer containing 15 mM MgCl2, 100 µM each of dATP, dGTP, dTTP, dCTP, and 1.5 U of Taq DNA polymerase (Biotools; Madrid, Spain), and 1 µM of forward and reverse primers (Genescript; Piscataway, NJ, USA). The PCR products were run on 2% agarose gel and analyzed under an ultraviolet illuminator. PCR products mixed in denaturing buffer (95% formamide, 10 mM NaOH, 0.05% xylene-cyanol FF and 0.05% bromophenol blue) in 1:1 ratio were heat denatured at 95°C for 5 min, immediately cooled on ice for 20 min, 6 µl of which were loaded on 6% PAGE and eletrophoresed in 0.5× Tris-borate EDTA buffer at ± 17°C at 4W constant power for 18-22 h. Gels were then silver stained and subsequently photographed. The purified PCR amplicons of the tumor samples showing mobility shift on SSCP analysis and randomly chosen normal samples were used for direct DNA sequencing, using the automated DNA sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems, Life Technologies; Carlsbad, CA, USA).

Results

Overall mutations of *FGFR3* identified in this study aggregated to 32.3% (21/65) the data (of *FGFR3*

Table 1. Primers Used for Screening Different Exons of FGFR3 and RAS Family of Genes

Amplicon	Primer sequence*	Annealing Temp. (°C)	Product size (bp)
FGFR3	F 5'–AGTGGCGGTGGTGGTGAGGGAG-3'	65	161
exon 7	R 5'-TGTGCGTCACTGTACACCTTGCAG-3'		
FGFR3	F 5'-CAACGCCCATGTCTTTGCAG-3'	62	199
exon 10	R5-CGGGAAGCGGGAGATCTTG-3'		
FGFR3	F 5'-GACCGAGGACAACGTGATG-3'	60	160
exon 15	R 5'-GTGTGGGAAGGCGGTGTTG-3'		
HRAS	F 5'–CAGGAGACCCTGTAGGAGGA-3'	60	139
exon1	R 5'–TCGTCCACAAAATGGTTCTG-3'		
HRAS	F5'–TCCTGCAGGATTCCTACCGG-3'	55	194
exon 2	R5'GGTTCACCTGTACTGGTGGA-3'		
KRAS	F5'–GGCCTGCTGAAAATGACTG-3'	55	162
exon 1	R5'–GTCCTGCACCAGTAA-3'		
KRAS	F5'–TTCCTACAGGAAGCAAGT-3'	55	128
exon2	R5'–CACAAAGAAAGCCCTCCCCA-3'		
NRAS	F5'GACTGAGTACAAACTGGTGGTGG-3'	60	118
exon 1	R5'–GGGCCTCACCTCTATGGTG-3'		
NRAS	F5'–GGTGAAACCTGTTTGTTGGA-3'	55	103
exon 2	R5'–ATACACAGAGGAAGCCTTCG-3'		

Table 2. Clinico-epidemiological Variables of UBC Patients Used for Mutational Analysis

Variable	Parameter	Cases N=65, n%	
Sex[a]	Males:	55(84.4)	
	Females:	10(15.6)	
Age	≤50	45	-70
	>50	20	-30
Dwelling[b]	Rural:	45	-70
	Urban:	20 (30)	
Smoking status[c]	Smokers:	50	-76.9
	Nonsmokers:	15(23.1)	
Differentiation	I:	6	-7.6
grade	II:	27	-41.5
	III:	24	-38.4
	IV:	8	-12.3
Histological	S:	40(61.5)	
types[d]*	MI:	25(38.5)	
Site[e]	RPL:	30(46.1)	
	LRL:	24(36.9)	
	BN	4(6.1)	
	O:	7(10.7)	
Size	≤ 3cm:	45(53.8)	
	> 3cm:	20(46.2)	
Lymph node	NO:	60(92.3)	
status	YES:	5(7.6)	
Status[f]	NR:	49(75.3)	
	R:	16(24.7)	
Stage	PTa:	21(32.3)	
	PT1:	22(33.8)	
	PT2:	22(33.8)	

[a]Age/Sex: M = Male, F = Female; [b]Rural/Urban: R = Rural, U = Urban; [c]Smoking Status: S = Smokers; NS = Non Smokers; [d]Histopathological Type: MI = Muscle Invasive, S = Superficial ; [e]Site: LRL=Left posterior lateral, RPL=Right posterior lateral; O=Orifice, BN=bladder neck; [f]Status: NR=Non recurrent; R=Recurrent; *All cases were histologically confirmed as transitional cell carcinoma except one case of adenocarcinoma

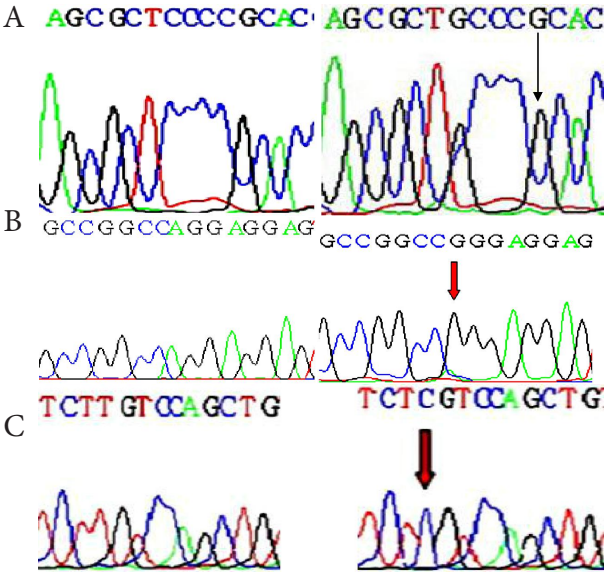

Figure 1. Partial Electropherogram Sequences. A) Forward of the normal (Left) and mutant in exon 7 of the FGFR3 gene codon 249(TCC→TGC, right). B) Forward of the adjacent normal (Left) and mutants in (Right) exon 2 of the HRAS codon 61 CAG→CGG. C) Reverse of the adjacent normal and mutant in exon 2 of NRAS oncogene codon 61 (CAA>CGA

mutations) has been previously published by our lab (Arshad et al., 2010). We detected six different single-nucleotide substitutions in 21 of the 65 bladder carcinomas (Table 3). These mutations affected codons 248, 249, 372, 375,417 and 652 (*FGFR3*b isoform numbering) [Figure 1a, b, c]. All six types of mutations except one in codon 417 identified in bladder carcinomas were identical to the germinal activating mutations responsible for thanatophoric dysplasia, a lethal form of dwarfism. Mutations were found more frequently in lower grade and stage. A strong correlation between *FGFR3* mutation pattern and low grade and stage bladder tumors was observed ($p<0.05$).

The overall mutations in exon 1 and 2 of all the forms of *RAS* genes including *HRAS*, *NRAS* and *KRAS* identified in this study aggregated to 21.5%(14/65) . The mutations found were only seen in the two hot spot codons (12 and 61) of *RAS* and all the mutations that were identified in this study were of missense nature. These mutations were found in *HRAS* and *NRAS* only whereas no mutation was detected in *KRAS*. In total there were nine mutations in *HRAS* (three in codon 12, six in codon 61), five in *NRAS* (two in codon 12 and three in codon 61) [Table 4]. Of the 14 mutations detected, six were A:T >G:C transitions,

Table 3. Clinico-epidemiological Variables of Bladder Cancer Patients Versus the Mutant Phenotypes of the *FGFR3* and *RAS* G enes

Variable	Parameter	Cases N=65	(%)	Mutants (n = 21*) FGFR3 gene	(%)	P value	Mutants (n=14*) RAS gene	(%)	P value
Sex	Males:	55	-84.4	17	-30.9	0.572	11	-20	0.479
	Females:	10	-15.6	4	-40		3	-30	
Age	≤50	20	-30	5	-25	0.401	5	-25	0.651
	>50	45	-70	16	-35.5		9	-20	
Dwelling	Rural:	45	-70	15	-33.3	0.604	10	-22.2	0.841
	Urban:	20	-30	8	-40		4	-20	
Smoking status	Smokers:	50	-76.9	15	-33	0.468	13	-26	0.11
	Nonsmokers:	15	-23.1	6	-40		1	-13.3	
Differentiation	I:	6	-7.6	3	-50	0.044	1	-16.6	
grade	II:	27	-41.5	13	-48.1		6	-22.2	0.984
	III:	24	-38.4	4	-20		5	-20.8	
	IV:	8	-12.3	1	-12.5		2	-25	
Histological type[a]	S:	40	-61.5	17	-42.5	0.026	9	-22.5	0.811
	MI:	25	-38.5	4	-16		5	-20	
Site[b]	RPL:	30	-46.1	11	-36.6	0.576	7	-23.3	
	LRL:	24	-36.9	7	-29.1		4	-16.6	0.5
	BN	4	-6.1	2	-50		1	-25	
	O:	7	-10.7	1	-14.2		2	-28.7	
Size	≤ 3cm:	45	-53.8	17	-37.7	0.157	6	-13.3	0.014
	> 3cm:	20	-46.2	4	-20		8	-40	
Lymph node	NO:	60	-92.3	20	-33.3	0.54	13	-21.6	0.639
status	YES:	5	-7.6	1	-20		2	-40	
Status[c]	NR:	49	-75.3	15	-30.6	0.609	10	-20.4	0.517
	R:	16	-24.7	6	-37.5		4	-25	
Stage	PTa:	21	-32.3	12	-57.1	0.008	4	-19	
	PT1:	22	-33.8	6	-27.2		4	-18.1	0.722
	PT2:	22	-33.8	3	-13.6		6	-27.2	

[a]Histopathological Type: MI = Muscle Invasive, S = Superficial ; [b]Site: LRL=Left posterior lateral, RPL=Right posterior lateral; O=Orifice, BN=bladder neck; [c]Status: NR=Non recurrent; R=Recurrent

Table 4. Point Mutations in the FGFR3 Gene in Bladder Carcinomas

Gene	Exon	CN	aa change	Base Change	No.of mutations	Frequency %
FGFR3	7	248	Ser → Cys	CGC>TGC	3	14
FGFR3	7	249	Ser → Tyr	TCC>TGC	12	57.1
FGFR3	10	372	Gly → Cys	GGC>TGC	1	4.7
FGFR3	10	375	Tyr → Cys	TAT >TGT	1	14.2
FGFR3	7	417	Arg → Cys	TCC>ACC	1	4.7
FGFR3	15	652	Lys → Glu	AAG>GAG	3	4.7
HRAS	1	12	Gly → Cys	GGC>TGC	3	14
HRAS	2	61	Gly → Leu	CAG>CTG	2	9.4
HRAS	2	61	Gly → Arg	CAG>CGG	4	18.8
NRAS	1	12	Gly → Cys	GGT>TGT	2	9.4
NRAS	2	61	Gln → Leu	CAA>CTA	1	4.7
NRAS	2	61	Gly → Arg	CAA>CGA	2	9.4

five were G:C > T:A transversions, three were A:T > T:A transversions. No significant association between tumor grade or stage and mutation was apparent (P>0.72 for each) (Table 2). There was no significant association between distribution of *RAS* gene mutation with any other clinicopathological parameters (P>0.05) (Table 3) but when the pattern and distribution was stratified for *HRAS* only, mutations was found to be significantly associated with smoking (P<0.05).

In total, 53.8% (35 of 65) of the tumors studied had mutation of either a *RAS* gene or *FGFR3*. This accounted for 04 of 06 (66%) of G-I tumors, 19 of 27 (70%) for G-II, 09 of 24 (35%) for G-III and 3 of 08(37%) for G-IV. Stage wise overall pTa tumors accounted for 16 of 21 (77%), 10 of 22 (45%) for pT1 and 09 of 22 (41%) pT2 (Table 3, Figure 2). Examination of the distribution of *RAS* and *FGFR3* mutations revealed that these events were totally mutually exclusive in both tumors and none of the samples showed both the mutation events of either *RAS* or *FGFR3*. The conventional odds ratio for *RAS* mutation in the presence/absence of *FGFR3* mutation in a tumor was estimated as 0 and the χ^2 test for independence was highly significant (P<0.0001). The relationship between stage or grade and tumor genotype was further analyzed (*FGFR3*wt/*RAS*mut and *FGFR3*mut/*RAS* wt), rather than *RAS* and *FGFR3* mutations, to see whether this affected

Table 5. The Relationship Between Tumor Genotype and Stage/Grade and Smoking Status

Parameters	FGFR3 mut/ RASwt	FGFR3 wt/ RAS mut	FGFR3 wt/OR (95% CI)	p value
Stage				
pTa +p T1≥ pT2	18	8	1	0.06
	3	6	4.5	
Grade				
G-I + G-II	16	7	1	0.11
G-III+G-IV	5	7	3.2	
Smoking				
Smokers	16	3	1	0.22
Non-smokers	5	1	4.06	

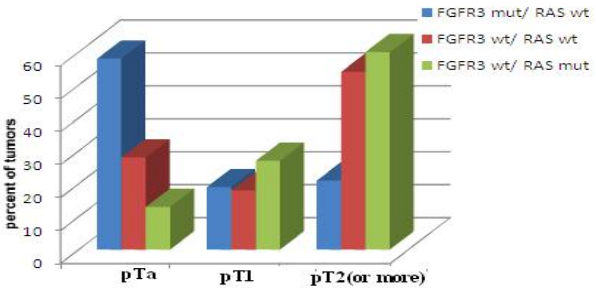

Figure 2. Cumulative Distribution of RAS and FGFR3 Mutations in Stages/Grades of UC

associations.

Associations between tumor genotype and stage/grade were lower than those between mutations and stage and grade as compared to the strong association observed in *FGFR3* (Table 5). Tumor genotypes also detected mutual exclusiveness of *FGFR3* and *RAS*. When the relationship between tumor genotype and smoking status was observed (*FGFR3*wt/*RAS* mut and *FGFR3*mut/*RAS* wt), odds ratios were higher for smokers (OR, 4.06; 95% CI, 0.75-21.91) than for non-smokers, but no significant association was found. Interestingly, pTa tumors harbored 12 of 21 (57.1%) mutations in *FGFR3* gene and 4 of 21 (19%) in *RAS* gene. Thus an overall mutation frequency of both genes in pTa tumors aggregate to 16 of 21 (76.2%). The combined mutational frequency of both the *FGFR3* and *RAS* genes in low grade/stage lesions summed up to 65% (26 of 40), with 17 of the 40 in *FGFR3* and 09 of the 40 in the *RAS* gene respectively.

Discussion

Urothelial carcinoma (UC), the common histological subtype of bladder cancer, presents as a papillary tumor or as an invasive. The majority of known genetic events have been described in muscle invasive UC, such as TP53 and RB1 mutation, are associated with poor prognosis. Genetic studies till date within the very large group of low-grade superficial tumors has not been fully elucidated. In this group, the only frequent genetic alterations described are LOH affecting chromosome 9 and mutations of *FGFR3*. Few candidate genes and genome-wide approaches have been followed whereby it is argued that a 'cancer pathway' perspective is useful to integrate findings from both approaches. According to this view, papillary cancers typically exhibit activation of the MAPK pathway, as a consequence of oncogenic mutations in *FGFR3* or *RAS* genes (Theodorescu et al 1991; Hart et al., 2000).

The involvement of *RAS* gene mutations in low grade/ stage tumors (Knowles et al., 1999), lead the present idea of this study to assess whether such mutations are found in tumors with *FGFR3* mutation, a genetic event that might be predicted to result in activation of similar downstream signaling pathways. Mutual exclusion of genetic events was observed in nine multiple myeloma cell lines with a t(4;14) translocation, where four *FGFR3* mutations and four *RAS* gene mutations were found, leading to the suggestion that these events may play an analogous role in the pathogenesis of multiple myeloma (Jebar et al., 2005). In this study we have examined a series of 65 bladder tumor samples for mutations in *FGFR3*, *HRAS*, *KRAS* and *NRAS* to provide a comprehensive assessment of all three *RAS* genes and to examine the possibility that *RAS* and *FGFR3* mutation are mutually exclusive. The overall mutations observed in bladder tumors in this study had a mutation of either *FGFR3* or *RAS* which aggregated to 53.8% (35 of 65). This accounted for 69.6% in low grade tumors (G-I & II) and 35.5% in high grade tumors (G-III & IV). Stage wise maximum frequency of both gene mutations were detected in pTa tumors which accounted for 77% and pT2 or higher stage accounted for 33%.

The distribution of *RAS* and *FGFR3* mutations revealed that these events were totally mutually exclusive in both tumors and none of the samples showed both the mutation events of either *RAS* or *FGFR3* and mutations were non-overlapping in both genetic events. Thus the finding in present study is finely in tune with the only study conducted, where mutations in *RAS* and *FGFR3* were also found to be mutually exclusive genetic events (Jebar et al., 2005). An interesting finding observed in this study is the marked differences in the pattern of mutations in *FGFR3* and *RAS* in relation to phenotype of the tumors. *FGFR3* is strongly associated with low tumor grade and low tumor stage; several studies describe very similar profiles (Olderoy et al 1998; Chesi et al., 2001; Smal et al., 2014) while as *RAS* mutations observed in this study were found throughout the low grade/stage to high grade/stage and thus do not seem to be associated with stage or grade as observed in other studies (Theodorescu et al 1991; Lindgren et al., 2006; Al Hussain et al., 2013). Therefore, our results demonstrate that there is some degree of biological equivalence of these events particularly in non-invasive tumors. The relationship between TP53 and *FGFR3* mutations shows an inverse relationship and are almost mutually exclusive in UC (Zhang et al 1991; Bakkar et al., 2003; Smal et al., 2014). These events appear to characterize the two major groups of UCs, which may define two alternative pathways in the pathogenesis of these cancers. TP53 is associated with tumors of high grade and stage and with carcinoma in situ which is a high-risk superficial lesion believed to represent a precursor for invasive UC.

The finding of *FGFR3* mutation in a population of tumors that is virtually distinct from these is compatible with the previous finding that *FGFR3* mutations are strongly associated with low grade and stage UC and with

lower frequency of recurrence (Billerey et al., 2001; van Rhijn et al., 2004). This distinction of *FGFR3* and TP53 mutant UCs fits well with the proposed two pathway model for UC development and provides a specific marker for the pathway leading to the development of low grade superficial cancer. Mutations in *FGFR3* gene in this study defined a specific distribution for the pathway of papillary tumorigenesis, while as it is documented that TP53 mutations cause carcinoma in situ (CIS) and subsequent development of invasive tumors. However, a good percentage of papillary tumors are *FGFR3* wild type in our study but present with the same phenotype as mutant tumors. A substantial part of these *FGFR3* wild type tumors present with mutations in one of the *RAS* genes, and from all low grade (pTa/G1) papillary tumors, this study found ~77% were defined by a mutation in either *FGFR3* (~57%) or *RAS* (19%). The absolute mutual exclusivity of *FGFR3* and *RAS* gene mutations is thought to reflect activation of the same pathway by either event. This observation clearly suggests that MAPK pathway activation may be an obligate event in most of these tumors. Several RTKs that function upstream of *RAS* are constitutively active in urothelial carcinomas like *HRAS*, *FGFR3* and EGFR and ERB2/3/4. The frequency of *HRAS* gene mutations (14.5%; 09 of 65) was highest among the all forms of *RAS*.

Several recent studies indicate that the oncogenic role of activated *FGFR3* gene bu mutations is mediated by the *RAS* signaling pathway. Forced expression of *FGFR3* mutants in NIH-3T3 cells resulted in cellular transformation and mitogen-activated protein kinase (MAPK) activation, resembling the transfection effects observed with activated *HRAS* (van Rhijn et al., 2001; Agazie et al., 2003). Inhibition of MAPK activity by specific inhibitors reversed the transformation phenotype. Hart and co-workers showed that only the myristylated mutant form of *FGFR3*, a membrane-bound form, was capable of activating MAPK and transforming the NIH-3T3 cells (Kanai et al 1997). Activated *FGFR3* seemed to be linked to *RAS* through adaptor proteins (that is, growth factor receptor-bound protein 2 (GRB2)- son of seven less (SOS) complexes) that are common to the RTK activation pathway (Hart et al., 2001). The fact that *HRAS* and *FGFR3* gene mutations occur in about 30% and 70% of the low-grade non-invasive papillary tumors, respectively, strongly indicates that the constitutive activation of the RTK-*RAS* pathway is responsible for the genesis of an overwhelming majority of this tumor variant. It is still unclear whether both *HRAS* and *FGFR3* mutations can co-exist in the same tumors. This seems unlikely based on data from other epithelial tumor types where components of the same signaling pathways are rarely mutated simultaneously, perhaps because this does not add any selective advantage to the affected cells.

Interestingly, unlike *FGFR3* mutation, no obvious relationship of mutational pattern of a *RAS* gene with tumor grade and stage has been found. This implies that although both events may fulfill at least one function that precludes selection for both, there is a difference, possibly in the strength and/or duration of signals generated that allows *RAS* mutation to contribute equally well to the development of both major tumor groups. In summary unlike *FGFR3* mutation, no obvious significance of mutation of a *RAS* gene with tumor grade/ stage has been found. This depicts that although events may fulfill at least one function that precludes selection for both, there is a possible difference in the strength of signals generated that allows *RAS* mutation to contribute equally well to the development of both major tumor groups.

It is concluded that *FGFR3* and *RAS* are mutually exclusive genetic events in UC, suggesting that both provide the same selective advantage most likely activation of MAPK pathway. Studies of much larger series of tumors are now needed, together with comparisons of the downstream effects of *RAS* and *FGFR3* signaling in urothelial cells.

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