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FGFR3 Mutation Analysis in Voided Urine Sample and Its Histopathological Correlation In Transitional Cell Carcinoma of Urinary Bladder.

Urology

KEYWORDS:

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ABSTRACT

Introduction: Bladder cancer is the most expensive and time consuming malignancy because of it high recurrence rate and disease progression. FGFR3 mutation in bladder cancer is associated with non muscle invasive and low grade disease this making it a non invasive marker of for nonaggressive disease.

AIM: The aim of this study was to detect FGFR3 mutation in a single voided urine sample of bladder cancer patients in Indian population and its diagnostic and prognostic performance.

MATERIALS AND METHOD: A prospective observational study was carried out with one-year follow-up of primary bladder tumor patients . Urine samples were collected before TURP. Screening for the most prevalent FGFR3 mutations was done using urinary cells. The prognostic significance of an FGFR3 mutation at the time of the initial diagnosis was determined. The performance of the test in diagnosing and/or predicting recurrence during follow up was assessed .

RESULTS: Of 39 patients studied, 2 (5.62%) had a positive analysis before TUR (FGFR3 MT). There was no statistical significance between MT and WT when compared with grade and stage of tumor. Neither there was any significant risk of tumor recurrence and progression in FGFR3 mutated as compared to wild type group.

Conclusion: Among patients with an FGFR3 mutation in the initial tumor, a noninvasive urine test from a single voided sample before primary resection cannot be valuable in diagnosing or predicting subsequent recurrence or progression.

INRODUCTION

Bladder cancer is one of the most common urological malignancies. As per the Indian cancer registry data in men, it is the ninth most common cancer accounting for 3.9% of all cancer cases in India.[1]Although majority of patients with bladder cancer present with low-grade, noninvasive neoplasia (approximately 70%), recurrence rates for bladder cancer are the highest of any malignancy, and recurrence or progression of the disease is observed in a large percentage of patients. Thus making bladder cancer as most expensive and time consuming malignancy to treat.[2] Currently there are no validated prognostic molecular biomarkers to guide the clinical management of urothelial cancer. Crucial therapeutic decisions are based on risk tables that include tumor size and number, and previous history [3],in addition to histopathological criteria, which are often limited by inter and intra observer variability and have relatively low reproducibility[4]. Cytology remains one of the most commonly used tests for the screening and diagnosis of bladder neoplasms, because it is both cost effective and noninvasive. Urine cytology has a high sensitivity and specificity for the detection of high grade urothelial malignancies but performs far from ideally when it comes to low grade tumors.[5]Cystoscopy, however, is sensitive for low-grade papillary lesions but tend to miss urothelial carcinoma in situ (CIS), with an overall specificity of 88%.[6]Urine cytology and cystoscopy, therefore, seem to complement each other and have been widely used for initial diagnosis and follow up of urothelial tumors patients. However with tumors staged similarly, there is a considerable variation in rates of recurrence, disease progression, and response to treatment. Therefore, biomarkers are needed to improve prognostication of such tumors. Intense work is being done in the field of NMIBC biomarkers with the main goals of reducing the number of invasive cystoscopy evaluations through early diagnosis and identifying those patients with a higher risk of disease progression. The identification of valuable biomarkers would not only improve our understanding of the biological pathways involved in the genesisand progression of the tumor, but would also add information about tumor outcomes. One of such biomarker is FGFR3 mutation commonly seen in axon 7,10 and 15 in bladder cancer. Various studies in past have shown strong association with FGFR3 mutations in bladder cancer with NMIBC and good prognosis.The fact that FGFR3 activation is a key event in the development of noninvasive bladder tumors and since FGFR3 mutations in bladder cancer are related to low stage and grade, they make FGFR3 the first marker for nonaggressive disease.[7] Therefore, a urine based non invasive assay which included FGFR3 mutation detection would significantly increase the detection of early stage bladder cancer. Currently, the most clinically sensitive assays for bladder cancer are based on direct molecular analysis of tumor tissue. Due to the high representativeness of tumor cells present within tissue samples, it is not necessary for these tissue based assays to have high analytical sensitivity, whereas urine DNA is made up predomnantly of normal urothelial DNA, thus requiring a significant increase in analytical sensitivity in order to detect the presence of mutations associated with bladder cancer. However, these urine-based assays have been limited by the technical ability to detect rare events in a dilute medium where thereis a high background of normal DNA. In these assays, FGFR3 mutations are generally found in about 30% of the urine samples, which is 50% concordance with the expected detection in tissue.[8] Others have demonstrated that multiple redundant sampling from a single patient may increase the probability of apositive result.[9] Although this approach can successfully increase sensitivity, it is not applicable to a commercial setting. The aim of this study was to detect FGFR3 mutation in a single voided urine sample of bladder cancer patients in Indian population and its diagnostic and prognostic performance.

MATERIALS AND METHODS

This was an observational study carried out in Dept. of Urology and Molecular Biology, Amrita Institute of Medical Sciences in year 2014-2015 after obtaining the approval of the Institute Ethical Review Board committee.

Patient Selection

Patients undergoing TURBT for primary bladder lesions diagnosed and confirmed on imaging (US/CECT/MRI abdomen and pelvis) or cystoscopywere included in study population.

Sample Collection

Voided urine was collected one day prior to the planned procedure in a 50ml plastic container and was kept at -20°Celsius. FGFR3 mutation analysis was performed on the samples. All resected specimens were subject to histopathology study. Histopathology was reported by a single dedicated uropathologist in the Dept. of Pathology,AIMS, Kochi. Pathologic features included histologic subtype, 2004 WHO tumor classification, and TNM staging.

Protocol for FGFR3 mutation analysis

DNA extraction from the urine samples Urine samples were centrifuged at 5,000 rpm for 10 minutes. The cell pellet obtained was washed once with 10 ml of 1X phosphate buffered saline, supernatant was discarded and DNA was extracted from the cell pellets using the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Germany). The DNA obtained was quantified using the Multiskan GO microplate spectrophotometer (Thermo Scientific, USA).

FGFR3 Mutation analysis

For detecting mutations in the exon 7 (R248C or S249C), exon 10 (G372C or Y375C) and exon 15 (K652E) the regions flanking the corresponding mutations of the FGFR3 gene were PCR-amplified using the primers listed in the Table. 1.

Primer	Sequence (5'->3')	Product size
Exon 7 primers		
FGFR3 Exon 7	FAGTGGCGGTGGTGGTGAGGGAG	161 bp
FGFR3 Exon 7 R	TGTGCGTCACTGTACACCTTGCAG	
Exon 10 primers		
FGFR3 Exon 10 F	CCTCAACGCCCATGTCTTTTCAGC	208 bp
FGFR3 Exon 10 R	CTTGAGCGGGAAGCGGGAGATCTTG	
Exon 15 primers		
FGFR3 Exon 15 F	TGGTGACCGAGGACAACGTGATG	167 bp
FGFR3 Exon 15 R	AGGGTGTGGGAAGGCGGTGTTG	

Table.1: Sequence of the PCR primers and product sizes

All PCR reactions were performed in 50 µl PCR reaction volume containing 10 pm of respective forward and reverse primers,1x concentration of the Emerald Amp GT PCR Master Mix (Takara Bio Inc, Japan) and approximately 20ng of the Genomic DNA isolated from the urine samples as template DNA. The thermocycling conditions for the amplification were an initial denaturing cycle at 94°C for 5 minutes followed by 30 cycles of denaturing at 94°C for 15 seconds, annealing at 59°C for 15 seconds, and extension at 72°C for 30 seconds. Final extension was at 72°C for 10 minutes. The PCR products were electrophoresed in a 3% agarose gel to assess PCR efficacy and product size.Before sequencing, the PCR products were purified using the ExoSAP-IT PCR clean-up kit (Assymetrix, USA). Sequencing was performed with one of the PCR primers using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) where 5µL of the purified PCR products served as template. The thermocycling conditions for the cycle sequencing consisted of 25 cycles of denaturing at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. The resulting cycle sequencing products were purified using the EDTA-Ethanol method and were analyzed on an ABI 3130 XL Genetic Analyzer from Applied Biosystems. The data obtained was analyzed with Sequencing Analysis Software, version 5.2 (Applied Biosystems) or FinchTV software (Geospiza).

STATISTICAL ANALYSIS

All data collected were entered in Microsoft excel program and were analysed using SPSS software version 20. To test the significance of the association of FGFR3 with different factors Fisher Exact test was applied.

RESULTS

39 patients underwent TURBT for primary bladder tumor during the study period. Their median age was 66.18 years. 95% of the study group were male. Hematuria was main presenting symptom in 34(87%). 12(31%) patients had multiple tumors and 27 (69%) had single bladder tumor. 18(46%) patients had tumor size < 3cm and 21(54%) had tumor size ≥ 3cm. At primary diagnosis of bladder TCC, 28% of tumors were stage Ta, 53% were T1 and 18% T2. 54% tumors were high grade , 44% low grade and only one was PUNLMP. FGFR3 mutations were detected in 2 patients (FGFR3 MT) before TUR in the initialtumor.Bothpatientsshowed mutationson Exon 10 . Sequence analysis of the Exon 10 of FGFR3 gene in one patient in MT group showed GGC→TGC heterozygous mutation in the codon 372(G372C)(Fig 1). Sequence analysis of the Exon 10 of FGFR3 gene of the second patient in MT group showed TAT→TGT homozygous mutation in the codon 375(Y375C) (Fig 2). No significant correlation was found between the FGFR3 MT with tumor multiplicity (p=0.526), tumor stage (p=1.00) and tumor grade (p=0.490) Table(2). Out of 39 patients in study group 7(17.94) patients were T2 high grade tumors and subsequently underwent radical cystectomy with urinary diversion or chemo radiation. 32 (82.05%) patients with non muscle invasive tumors were followed up for a period of 1 year. After the end of follow-up, 11 (34.37%) patients had relapsed, 100% in the FGFR3mutation group and 30% in the FGFR3wild-type group. However there was no statistical significance between MT and WT in tumor recurrence (p=0.111).(Table3). We observed both patients in the FGFR3 MT 2(100%)and 5(55.6%) patients in the FGFR3 WT group progressed during the one year of the study which was not statistically significant(p=0.491).(Table 4)

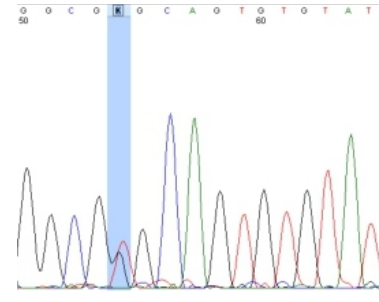


Fig 1: GGC→TGC heterozygous mutation in the codon 372(G372C). According to IUPAC codes 'K' stands for Keto group which is present in G or T

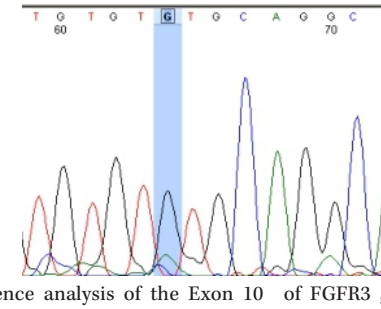


Fig 2:Sequence analysis of the Exon 10 of FGFR3 gene of the second patient in MT group showedTAT→TGT homozygous mutation in the codon 375(Y375C) (Fig 18)

FGFR3 FACTOR	CATEGORY	MUTANT(MT) n(%)	WILD(WT) n(%)	p Value
	MULTIPLE	1(8.3)	11(91.7)	0.526
MULTIPL	SINGLE	1(3.7)	26(96.3)	
ICITY	Ta	0(0)	11(100)	
	T1	2(9.5)	19(90.5)	

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	T2	0(0)	7(100)	
	PUNLMP	0(0)	1(100)	
GRADE	LOW	0(0)	17(100)	0.490
	HIGH	2(9.5)	19(90.5)	

TABLE 2:Correlation of FGFR3 mutation with Tumor Multiplicity, Stage and Grade

For Fischer's Exact test Ta and T1 were combined together.

For Fischer's Exact test PUNLMP and Low grade were combined together.

FGFR3 FACTOR	CATEGORY	MUTANT(MT) n(%)	WILD(WT) n(%)	p Value
	PRESENT	2(100)	9(30)	0.111
RECURRENCE @1 YR	ABSENT	0(0)	21(70)	

TABLE 3: Recurrence in Patients with respect to the presence or absence of FGFR3 mutations in the initial tumor.

FGFR3 FACTOR	CATEGORY	MUTANT(MT) n(%)	WILD(WT) n(%)	p Value
	PRESENT	2(100)	5(55.6)	0.111
PROGRESSION @1 YR	ABSENT	0(0)	0444.4)	

Table 4: Tumor progression in patients with respect to the presence or absence of FGFR3 mutations in the initial tumor

DISCUSSION

It has been shown that urine based FGFR3 mutation analysis can detect recurrence, although its sensitivity may be limited if samples have few tumor cells [9]. Recently, several studies have evaluated the sensitivity and specificity of FGFR3mutation analysis in voided urine for detection of recurrence of superficial bladder cancer. In these studies, screening for mutated FGFR3 was performed using various assays.

We assessed the FGFR3 mutation analysis in a single voided urine sample in Indian population and its diagnostic and prognostic performance observed during follow up of one year.

In our study FGFR3 mutations were seen in 6.25% of all NMIBC. Both mutations were detected in Exon 10, one heterozygous G372C mutation (G→T transversion at codon 372) and one homozygous Y375C mutation (A→G transition at codon 375). Non of the muscle invasive tumors had FGFR3 mutation. Previous studies have shown FGFR3 mutations in 70% of all patients presenting with an NMIBC and at higher frequencies in tumors of low grade and low stage.The proportion of patients with a mutation (6.25%) is lower than in most previously published studies. FGFR3 mutations occur predominantly in superficial/papillary low-grade bladder tumors and therefore the proportion of FGFR3 mutation patients can only be compared after stratification for stage and/or grade. Even so, there are relatively large variations between reported series, ranging from 43% to 77% in patients with superficial tumors[10].

Currently, the most clinically sensitive assays for bladder cancer are based on directmolecular analysis of tumor tissue. Due to the high representativeness of tumor cells present within tissue samples, it is not necessary for these tissue based assays to have high analytical sensitivity, whereas urine DNA is made up predominantly of normal urothelial DNA, thus requiring a significant increase in analytical sensitivity in order to detect the presence of mutations associated with bladder cancer[10]. All previous studies on FGFR3 mutation analysis were either performed on resected tissue samples or multiple urine samples from single patient. Although this approach can successfully increase sensitivity, it is not applicable to a commercial setting. Studies have shown quantitative PCR assay has sufficient analytical sensitivity to detect FGFR3 mutations in tissue where the mutant to normal ratio is high, it fails to produce similar results in urine, where the mutant to normal DNA ratio is much lower. Also, there are more than 11 different point mutation sites within this gene, and therefore identification of all possible mutations is difficult in a single urine sample.[11]

In the study presented here, clinical sensitivity in urine for FGFR3 by quantitative PCR was 6.66%. In a similar study John M Millholland et al showed clinical sensitivity in urine for FGFR3 by quantitative PCR was 11.6%.[10] However in same study author has showed that using the small-molecule FGFR3 assay ,mutations can be detected in 55.8% of the same urine samples , demonstrating that superior analytical sensitivity will ultimately improve clinical performance. We in this study followed quantitative PCR protocol for detection of FGFR3 mutations due to limited resources.

Approximately 80% of all patients with bladder cancer present with NMI tumors, of which 10% to 20% progress to muscle invasive disease. 70% of patients with NMI BC have at least 1 recurrence within 5 years12 .In our study 82% patients were NMIBC out of which recurrence was seen in 34.34% at the end of one year follow up. 21.8% progressed to higher T stage on follow up. Low rate of recurrence in this study is attributed to short follow up period.

Recurrence was seen in 100% in FGFR3 mutant group and 30% in WT group. However, we found that patients with a positive FGFR3 mutation analysis were not significantly more likely to relapse during the 1 year follow up than patients with wild-type tumors (p=0.111).In a similar study by Kompier LC et al [13] stated that recurrence risk was not significantly different for patients with a mutant or wild-type primary tumor. Such apparent discrepancies might be partly explained by different lengths of follow up and sample size as well as differences in patient characteristics.

100% patients in MT group progressed to higher stage disease as compared to 55.6 % in WT at the end of follow up after 1 year. However there was no statistical significance between two groups (p=0.491). A prospective study including 221 superficial tumors indicated that FGFR3 status is predictive of disease progression in pT1 and high-grade malignancies[14]. Progression in this study group may be due T1 and high grade disease which are independent predictors of tumor progression.

CONCLUSION

FGFR3 mutation is associated with non muscle invasive bladder cancer however there is not statically significant risk of tumor recurrence and progression in FGFR3 mutated as compared to wild type group.

Detection of FGFR3 mutations in single voided urine sample could not be employed for general population screening aimed at early detection of primary tumors. Single urine sample fails to produce similar results in urine where the mutant to normal DNA ratio is much lower as compared to tumor tissue. Thus requiring a significant increase in analytical sensitivity in order to detect the presence of mutations associated with bladder cancer. Therefore multiple urine sample from single person pooled together may improve FGFR3 assay sensitivity. It would be interesting to perform a larger trial with urine samples pooled during a 24-hour period to validate these findings.

REFERENCES

1. Gupta P, Jain M, Kapoor R, Muruganandham K, Srivastava A, Mandhani A. Impact of age and gender on the clinicopathological characteristics of bladder cancer. Indian J Urol. 2009 Apr;25(2):207-10.
2. Sangar VK, Ragavan N, Matanhelia SS, Watson MW, Blades RA. The economic consequences of prostate and bladder cancer in the UK. BJU Int. 2005 Jan;95(1):59-63.
3. Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L,

Newling DW, Kurth K. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol. 2006 Mar;49(3):466-5; discussion 475-7. Epub 2006 Jan 17.

4. Tosoni I, Wagner U, Sauter G, Egloff M, Knönagel H, Alund G, Bannwart F, Mihatsch MJ, Gasser TC, Maurer R. Clinical significance of interobserver differences in the staging and grading of superficial bladder cancer. BJU Int. 2000 Jan;85(1):48-53.
5. Bastacky S, Ibrahim S, Wilczynski SP, Murphy WM. The accuracy of urinary cytology in daily practice. Cancer. 1999 Jun 25;87(3):118-28.
6. Chang S, Kim Y, Kim M. Urinary Survivin Test Compared to the Nuclear Matrix Protein (NMP)-22 Test and Urine Cytology for the Diagnosis of Bladder Cancer. Korean Journal of Urology. 2006;47(10):1041-45.
7. Miyake M, Sugano K, Sugino H, Imai K, Matsumoto E, Maeda K, Fukuzono S, Ichikawa H, Kawashima K, Hirabayashi K, Kodama T, Fujimoto H, Kakizoe T, Kanai Y, Fujimoto K, Hirao Y. Fibroblast growth factor receptor 3 mutation in voided urine is a useful diagnostic marker and significant indicator of tumor recurrence in non-muscle invasive bladder cancer. Cancer Sci. 2010 Jan;101(1):250-8. doi: 10.1111/j.1349-7006.2009.01334.x. Epub 2009 Sep 1.
8. Fernandez CA, Millholland JM, Zwarthoff EC, Feldman AS, Karnes RJ, Shuber AP. A noninvasive multi-analyte diagnostic assay: combining protein and DNA markers to stratify bladder cancer patients. Res Rep Urol. 2012 Mar 5;4:17-26. doi: 10.2147/RRU.S28959.
9. Zuiverloon TC, Tjin SS, Busstra M, Bangma CH, Boevé ER, Zwarthoff EC. Optimization of nonmuscle invasive bladder cancer recurrence detection using a urine based FGFR3 mutation assay. J Urol. 2011 Aug;186(2):707-12. doi: 10.1016/j.juro.2011.03.141. Epub 2011 Jun 17.
10. Millholland JM, Li S, Fernandez CA, Shuber AP. Detection of low frequency FGFR3 mutations in the urine of bladder cancer patients using next-generation deep sequencing. Res Rep Urol. 2012 Jun 27;4:33-40. doi: 10.2147/RRU.S32736. eCollection 2012. Erratum in: Res Rep Urol. 2012;4:47.
11. van Oers JM, Lurkin I, van Exsel AJ, Nijsen Y, van Rhijn BW, van der Aa MN, Zwarthoff EC. A simple and fast method for the simultaneous detection of nine fibroblast growth factor receptor 3 mutations in bladder cancer and voided urine. Clin Cancer Res. 2005 Nov 1;11(21):7743-8.
12. Holmäng S, Hedelin H, Anderström C, Johansson SL. The relationship among multiple recurrences, progression and prognosis of patients with stages Ta and T1 transitional cell cancer of the bladder followed for at least 20 years. J Urol. 1995 Jun;153(6):1823-6; discussion 1826-7.
13. Kompier LC, van der Aa MNM, Lurkin I, Vermeij M, Kirkels WJ, Bangma CH, et al. The development of multiple bladder tumour recurrences in relation to the FGFR3 mutation status of the primary tumour. J Pathol. 2009 May;218(1):104–12 .
14. Burger M, van der Aa MN, van Oers JM, Brinkmann A, van der Kwast TH, Steyerberg EC, Stoehr R, Kirkels WJ, Denzinger S, Wild PJ, Wieland WF, Hofstaedter F, Hartmann A, Zwarthoff EC. Prediction of progression of non-muscle-invasive bladder cancer by WHO 1973 and 2004 grading and by FGFR3 mutation status: a prospective study. Eur Urol. 2008 Oct;54(4):835-43. doi: 10.1016/j.eururo.2007.12.026. Epub 2007 Dec 26.