Mutational Analysis of FGFR3 and HRAS Genes in Bladder Cancer and Washing Cell Sediments of Moroccan Patients

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Abstract

Genetic alterations related to carcinogenesis, including punctual mutations, may have a potential as bladder cancer biomarker with high specificity and sensitivity. Specific acquired FGFR3 and HRAS mutations have been found to predominate in bladder cancer. The aim of this study is to determine the incidence of FGFR3 and HRAS mutations in bladder tumors in Morocco and to explore whether these molecular events can be detected in urine sediments of bladder cancer patients. In this order, DNA sequencing of both tissues and urine sediments was used to identify gene mutations. Among 42 DNA tumor specimens, FGFR3 mutations were found in 28.6%. On the sequenced exons 7 and 10 of FGFR3 gene, three different mutations were identified R248C, S249C and Y375C. Whereas, HRAS mutations were detected in 9.5% and two distinct HRAS mutations were found G12C and Q61L. FGFR3 mutations were mutually exclusive with HRAS mutations. FGFR3 mutations were predominant in low stage (p<0.001) and low grade (p=0.045) and this predominance was statistically significant. HRAS mutations were more frequent in low stage than in high stage but there is no significant association with the stage of tumors, whereas the predominance of HRAS mutations in high grade was statistically significant (p<0.001). Therefore, FGFR3 mutation may be considered as an early biomarker for bladder cancer and HRAS mutation may inform on the prognosis of this cancer. Gene mutations were also analyzed in urine and were detected in 56.25% for both FGFR3 and HRAS genes. Thus, mutation detection is feasible in urine sediments and a molecular approach will therefore increase the detection sensitivity.

Keywords: Bladder cancer; Mutations; Urine sediments; FGFR3; HRAS

Introduction

Worldwide, bladder cancer is the most common cancer, approximately accounting 386,300 new cases each year. The highest incidence rates are observed in the countries of Europe, North America, and Northern Africa. In this last area, the age standardized urinary bladder cancer incidence rates was evaluated at 14.5 in males and 2.4 in females [1]. In Morocco, according to the regional cancer registries, bladder cancer is the most common cancer with an incidence of 5.8 and 11.3 per 100,000 persons in Casablanca and Rabat, respectively [2,3].

The majority of malignant bladder tumors are of epithelial origin, and more than 90% correspond to histologically defined “urothelial carcinomas” (UC), formerly called “transitional cell carcinomas” (TCC) [4].

Approximately 75% to 85% of bladder neoplasms are diagnosed to be superficial bladder tumors [5]. Most of these patients with papillary superficial UC (70%) will recur frequently, without developing an invasive neoplasm. Only 2% to 5% of Ta [6] and 20% to 30% of T1 [7] bladder tumors will invade the bladder muscle. The remaining 15% to 25% carcinomas are invasive (T2, T3, T4) or metastatic lesions at the time of initial clinical presentation.

There is a need for improved clinical stratification methods that can distinguish patients with early-stage disease from those with high risk of recurrence. Conventional methods, including urine cytology, histopathology, or tumor-node-metastasis classification, have numerous limitations in early detection and risk assessment.

Contrarily a large scale of novel molecular methods, such as detection of activating mutation and methylation profiling have shown promising results and may have a potential as bladder cancer biomarker with high specificity and sensitivity [8]. Subsequent larger studies point out to the relevance of mutation of bladder cancer [9-11].

Mutations of FGFR3 were identified in a great portion of bladder carcinomas and at low frequency in cervical urothelial cell, and colorectal carcinomas. Many of these mutations occurred at highly conserved sequences in the Ig-like domain III. In non-muscle invasive bladder tumors, mutated FGFR3 leads to constitutive activation of the RAS-MAPK-pathway and consequently to an augmented transduction of growth signals [12,13]. These mutations are found in approximately 70% of low-grade Ta and to a much lower extent of 10-20% in muscle-invasive bladder cancer [14,15].
Somatic FGFR3 mutations in bladder tumors were localized in exon 7, 10 and 15 [16,17], and were shown to occur frequently in low stage and grade tumors [18] (Knowles MA, 2008). It has been shown that FGFR3 mutation analysis in urine samples from bladder cancer patients was possible to detect recurrent tumors [19,20] and the screening for the presence of recurrences using urine-based assays can potentially improve quality-of-life and reduce disease management costs [21,22]. Therefore, detection of FGFR3 mutations in urine could also be employed for general population screening aimed at early detection of primary tumors.

HRAS mutations predominate in bladder cancer and vary widely from 0% to 45% between studies [23,24]. In most cases including bladder cancer, the somatic missense Ras mutations found in cancer cells introduces amino acid substitutions at positions 12, 13, and 61. These changes impair the intrinsic GTPase activity and confer resistance to GAPS, thereby causing cancer-associated mutant Ras proteins to accumulate in the active, GTP-bound conformation [25].

These mutations have been shown to occur in all stages and grades [26]. In addition, it has been reported that RAS and FGFR3 mutations were mutually exclusive [26]. Preliminary results show that a combined test for mutation of FGFR3 and HRAS could potentially detect 75% of primary tumours; including 88% of the pTa-T1G1-2 tumors but only 36% of the high-grade and –stage malignancies [15].

The relationship that exists between FGFR3 and HRAS guide us to conduct the present study which aims to determine the incidence of FGFR3 and HRAS mutations in bladder tumors in Morocco. To investigate the potential of these alterations as markers for noninvasive detection of primary tumors; including 88% of the pTa-T1G1-2 tumors but only 36% of the high-grade and –stage malignancies [15].

The corresponding hematoxylin-eosin-stained sections were examined at the Anatomopathology department at the same hospital; all the samples were confirmed to be histologically urothelial carcinoma (UC) of bladder. Tumors were graded according to the WHO (World Health Organization) criteria [28] as follows: 13 tumors were low grade and 29 tumors were high grade; and staged according to the TNM (Tumor Node Metastasis) guidelines [29] as follows: 9 pTa, 25 pT1, 7 pT2 and 1 pT4. Four DNA from urinary inflammatory lesions and Forty DNA blood samples from healthy volunteers were included as controls.

**Materials and Methods**

**Characteristics of the patients and tissue samples**

DNA from Forty two patients with bladder disease was available from our laboratory DNA bank. The study design and population has been previously described [27]. Briefly bladder cancer patients underwent transurethral resection of bladder tumor (TURBT) from January 2010 to June 2011, at the Urology department of Military Hospital of Instruction Mohamed V (MHIMV) in Rabat, Morocco. The mean age of these patients was 64.8 ± 10.1, the median age was 67 years (range 42-84) and 38 of them were male (male:female 7:6:1). Paired tumor and urine samples were collected.

| Exon | Sequence (5’ > 3’) | T (°C) | Fragment size (bp) | Reference |
|------|--------------------|--------|--------------------|-----------|
| FGFR3 | | | | |
| 7 | F AGTGGCCGCTGGTGCTGAGGAG | 63 | 116 | (Bakkar et al., [14]) |
| | R CAGCAGCCGCTCTGGTGG | | | |
| 10 | F CAAGGCCTAGCTCCTTCGAG | 59 | 165 | (Bakkar et al., [14]) |
| | R GAGGCCAGGCTTCTTG | | | |
| 15 | F AGGACAACGTGATGAAAGATCG | 59 | 154 | (Bakkar et al., [14]) |

Exons 7, 10 and 15 of the FGFR3, and exon 1 and 2 of the HRAS genes were amplified using the previously described specific primers [14,30] (Table 1). PCR mix was prepared in a final volume of 25 µl containing 1 µl of genomic DNA, 1X PCR buffer, 3 mM, 0.2 mM of each dNTP, 0.2 µM of each primer and 0.625 units AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA).

DNA amplification was performed on Gen Amp PCR system 9700 apparatus (Applied Biosystems) under the following conditions: 9 min at 95°C fo initial denaturation, 35 cycles of 30s at 94°C for, 30s at specific annealing temperature of each primer set (Table 1) and 30s 72°C, followed by a final elongation step of 7 min at 72 °C. Water was used as negative control. Ten µl of PCR products were loaded into 2% agarose gel and spered by electrophoresis in TBE buffer. The gels were then stained with ethidium bromide and visualized under UV illumination.

**DNA sequencing**

PCR products were purified by Exosap-IT clean up system (USB, USA) and the sequencing was performed with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA), according to manufacturer's protocol, on an ABI 3130XL DNA analyzer (Applied Biosystems, Foster city, CA, USA).

The sequences obtained were then matched with gene reference sequence collected from the GeneBank database. The sequence alignment was performed using Clustal W program in BioEdit Software. According to the position of targeted mutation, we used either forward or reverse primer in sequencing. If a mutation was identified, matched DNA urine sediments were also sequenced. Results were confirmed by sequencing both strands.

**References**

1. Bakkar et al., [14]
2. [16,17]
3. [21,22]
4. [23,24]
5. [19,20]
6. [27]
Statistical analysis

The association between mutations and stage/grade was evaluated by calculating p values according to χ² test using MedCalc version 9. P<0.05 was considered significant.

Results

Oncogenic mutations of FGFR3 and HRAS in bladder cancer cases

Exon 7, 10 and 15 of the FGFR3, and exon 1 and 2 of the HRAS genes were screened for mutations in DNA from 42 bladder cancer cases and paired urine sediments. The various FGFR3 and HRAS mutations identified are listed in Table 2.

Overall, 28.6% (12 of 42) of the tumors contained an FGFR3 mutation. The FGFR3 mutation in exon 15 was not detected in any of the 42 urinary bladder cancer cases. On the sequenced exons 7 and 10 of FGFR3 gene, three different mutations were identified R248C, S249C and Y375C (Table 2), which are known mutation hotspots in bladder cancer. In exon 7, The S249C mutation consisting in the substitution of the nucleotide C by the nucleotide G at position 746 occurs more frequently and was detected in 21.4% (9/42) of analyzed cases. Whereas, the R248C (substitution of C by T at position 742 in exon 7) and Y375C (substitution of A by G at position 1124 in exon 10) were detected only in 2.3% (1/42) and 4.7% (2/42) respectively.

The HRAS mutations were screened for mutations in 42 bladder cancer cases and were detected in 9.5% (4 of 42) of the tumors. Two distinct HRAS mutations were found G12C (substitution of G by T at position 34 in exon 1) and Q61L (substitution of A by T at position 182 in exon 2) and were distributed equally among the tumors (Table 2).

Table 2: Frequencies of individual mutations of GFR3 and HRAS genes in primary tumors of 42 patients.

| Exon | Nucleotide position and base change | Amino acid change | Number of tumors |
|------|-----------------------------------|------------------|-----------------|
| FGFR3 gene | | | |
| 7 | 742 C>T | Arg/Cys (R248C) | 1 |
| 7 | 746 C>G | Ser/Cys (S249C) | 9 |
| 10 | 1124 A>G | Tyr/Cys (Y375C) | 2 |
| HRAS gene | | | |
| 1 | 34G>T | Gly/Cys (G12C) | 2 |
| 2 | 182 A>T | Gin/Leu (Q61L) | 2 |

The HRAS mutations were screened for mutations in 42 bladder cancer cases and were detected in 9.5% (4 of 42) of the tumors. Two distinct HRAS mutations were found G12C (substitution of G by T at position 34 in exon 1) and Q61L (substitution of A by T at position 182 in exon 2) and were distributed equally among the tumors (Table 2).

In total, 38.1% (16/42) of the analyzed tumors had mutation of either HRAS gene or FGFR3 gene. All the mutated cases harbored no more than one mutation and the FGFR3 mutations were mutually exclusive with HRAS mutations.

No mutation was detected in the four urinary inflammatory lesions and control cases for both FGFR3 and HRAS genes.

Association between mutations and clinicopathological parameters

In our studied cohort, thirty-four of 42 studied bladder tumors (80.95%) were non muscle invasive tumors (9 pTa and 25 pT1), whereas 8 tumors (19.05%) were muscle invasive tumors (7 pT2 and 1 pT4). This distribution of tumors in our study was consistent with previous epidemiological studies on the stage, supporting the validity of our cohort of patients.

The distribution of FGFR3 and HRAS mutations according to stage and grade was represented in Figure 1A and 1B; respectively. FGFR3 mutations were predominant in low stage (2/12 pTa and 10/12 pT1) (p<0.001) and low grade (8/12) (p=0.045) and this predominance was statistically significant. HRAS mutations were more frequent in low stage (3/4 pT1) than in high stage but there is no significant association with the stage of tumors, whereas the predominance of...
HRAS mutations in high grade (4/4) was statistically significant (p<0.001).

Mutations in urine samples from patients with bladder cancer

To assess the value of the individual markers for noninvasive detection of bladder cancer, once a mutation was detected in the tumor DNA, we analyzed DNA isolated from paired urine sediments. Overall, mutations were detected in 56.25% (9 of 16) of mutated cases for both FGFR3 and HRAS genes. In FGFR3 gene, mutations in urine sediments were identified in 58.33% (7 of 12) of mutated cases in paired tumors, whereas HRAS mutations were found in 50% (2 of 4) of mutated cases.

Regarding the frequency of HRAS mutations, our data were comparable with other studies [33,34] and two distinct HRAS mutations in exon 1 and 2 were found in 9.5% (4 of 42) of the analyzed tumors. HRAS mutations were more frequent in low stage than in high stage but there is no significant association with the stage of tumors, whereas the predominance of HRAS mutations in high grade was statistically significant (p<0.001). In previous study, Activating HRAS mutations were detected with an estimated overall frequency of 10-15% without a clear association with tumor grade or stage [15,26].

In bladder cancer, the common HRAS gene mutations occur in the hotspot codons 12, 13 (exon1), or 61 (exon2). These mutations cause specific substitutions to amino acid and result in the loss of GTPase activity [35]. This enzyme enables hydrolysis of RAS protein from active conformation (RAS-GTP) to inactive form (RAS-GDP). In the lack of GTPase activity, the active form is permanently maintained, thereby inducing activation of downstream kinase cascades that results in continuous mitogenic signaling leading to uncontrolled cell division and the formation of a tumor [36].

As for RAS, upstream FGFR3 is also able to activate the MAPK signaling pathway. Superficial papillary tumors are characterized by a high frequency of FGFR3 mutations [24,34] leading to constitutive activation of the RAS-MAPK pathway [12,13]. In our study, as well as in other, FGFR3 and HRAS mutations were mutually exclusive in bladder cancer, probably due to their redundancy in the same signaling pathway [26].

Cytology is the non-invasive test widely used for patient follow-up, whereas this method had several limits in detection of non-muscle invasive bladder cancer [37]. Numerous studies have shown that it is possible to detect bladder oncogene mutations in DNA isolated from urine sediments [33,38]. In our study, the sensitivity of mutation identification is the fraction of mutations detected in urine divided by mutations found in tumor biopsies. The FGFR3 mutation is detected in 58.3% of urine samples. This frequency is comparable to other studies [33]. For the HRAS gene mutation, the sensitivity in the present study (50%) was higher than previous reports [38,39]. In some cases, mutations were detected in tumors but not in the corresponding urine. DNA isolated from urine sediments may not be sufficient for a
molecular analysis due to the limited numbers of bladder tumor cells exfoliated in urine [33]. Thus, tumor cell population should be enriched for a better outcome in detection of molecular events. This may be achieved either by improving the procedure for urine sampling or by using filter devices or antibody-based assays to selectively capture the tumor cells [40,41].

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