High Resolution Melting Analysis for Rapid Detection of PIK3CA Gene Mutations in Bladder Cancer: A Mutated Target for Cancer Therapy

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Purpose: PIK3CA gene mutations have clinical importance and their presence is associated with therapy response. They are also considered as a molecule for targeted therapy. As regards to their importance, genetic variation within a population as well as among different populations, this study was conducted to detect common mutations of exons 9 and 20 and other probable mutations in PIK3CA gene as well as their frequencies in Iranian bladder cancer patients.

Materials and methods: Paired tumor and adjacent normal tissues samples were obtained from 50 bladder cancer patients. Mutations of PIK3CA gene were detected using High Resolution Melting (HRM) analysis which is a highly sensitive, repeatable, rapid, and cost-effective technique. To determine the precision of the HRM analysis, Sanger sequencing analysis was used.

Results: The result showed that mutations were present in 10% (5/50) of the subjects. The majority of these cases (4/5) had the mutation(s) in exon 9, spanning over five different mutations, among which three of them were actually novel mutations. Further analysis showed that 2 cases had simultaneous mutations for exon 9. In addition to novel mutations, the PIK3CA mutation rate observed in Iranian bladder patients was not as frequent as previous reports and COSMIC.

Conclusion: HRM can be used as a rapid and sensitive method for mutation screening. Dysregulation of PIK3CA gene in bladder cancer reveals its potentials as a mechanistic link for cancer development, which in turn suggests its special use in interventional studies for targeted therapy.

Keywords: bladder cancer; high resolution melting; mutation; PIK3CA gene; targeted therapy.

INTRODUCTION

Bladder cancer is one of the most frequent non-cutaneous solid malignancies next to prostate cancer among the most common genitourinary cancers worldwide[1]. It is caused by both genetic and environmental factors. According to Iranian cancer registry report, it represents the fourth most common cancer and even the second most common in some regions in men with less frequency in women. Although, the occurrence of bladder cancer is increasing, but this increase rate is up to six times higher in developed countries[2,3]. Since close continuous surveillance by cystoscopy and other monitoring interventions are highly popular, i.e. for earlier detection of its recurrence, bladder cancer is considered to be one of the most costly cancers in health care systems.

Urothelial carcinoma is the most common type of bladder tumors that originate from epithelial cells. It consists of two major types: non-muscle-invasive (NMIBC) which accounts for approximately 75% of the cases and muscle invasive (MIBC)[4,5]. Remarkable inter-individual variations make it difficult to provide efficient therapeutic cares for a given patient, because of the significant heterogeneity of urothelial carcinomas in terms of its clinical and genetic backgrounds. Such heterogeneity partly originates from different changes in different genes that affect signal transduction cascades, most notably PI3K/mTOR/AKT pathways. These dysregulations are potentially accountable for the initiation and progression of both NMIBC and MIBC[6,7].

Advances in our understanding of molecular pathogenesis of cancer have led to new approach of targeted therapies for many cancers. One of the most deregulated pathways in cancer is phosphatidylinositol 3-kinase signaling pathway (PI3K). This signaling pathway is involved in regulation of a number of normal processes such as cell growth, apoptosis, proliferation, and survival and will be activated by interacting with tyrosine kinase receptors[8-10]. Based on subunit structure, function and substrate selectivity, the phosphoinositol-3-kinase family is divided into three different classes. Class IA PI3K, which is heterodimers, consists of a regulatory (p85) and catalytic (p110) subunits and is the one most clearly implicated in tumorigenesis and cancer progression. Catalytic subunit p110 is encoded by PIK-3CA gene located on 3q26.3. P110α is important in the induction of the signaling cascade called PTEN/AKT pathway. The genetic aberration of this gene has been associated with different cancer development. Muta-

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Table 1. List of primer sets for HRM, Sanger sequencing

Primers	Sequence	Amplicon size
PIK3CA - EX9F- HRM	AATTAAGGGAAAATGACAAAGAACAGC	114
PIK3CA-EX9R- HRM	ATTTTAGCACTTACCTGTGACTCC	
PIK3CA-EX20F-HRM	AAGAGGCTTTGGAGTATTTCATG	99
PIK3CA-EX20R-HRM	CATGCTGTTTAATTGTGTGGAAG	
PIK3CAEX9F-sequencing	CCAGAGGGGAAAAATATGACA	196
PIK3CA-EX9-sequencing	CATTTTAGCACTTACCTGTGAC	
PIK3CA -EX20-sequencing	CATTTGCTCCAAACTGACCA	387
PIK3CA-EX20R-sequencing	GGTCTTTGCCTGCTGAGAGT	

tions in the PIK3CAgene have been reported in head and neck squamous cell carcinomas (HNSCC), breast, colorectal, gastric, endometrial and many other cancers with various frequencies[11-16]. Most of the PIK3CA gene mutations are located at 3 hot spot codons in exon 9 (E542K, E545K) and exon 20 (H1047L). These exons are responsible for coding helical and kinase domains. PIK3CA has been proposed as an oncogene, due to its high mutation frequency, being heterozygous missense changes and clustering mutations in 2 hot spot exons. These characteristics may be related to kinase activity. Oncogenic mutation of PIK3CA in human cancers with its clinical importance makes it a target for cancer therapy

Studies on urothelial carcinoma confirmed that the PIK3CA gene changes are present in 13 to 35% of the cases and this rate decreases when the tumor's stage and grade are increasing. Also, it has been reported that other genes such as FGFR3 are involved in superficial low-grade bladder cancer that is more frequently associated with PIK3CA gene mutations[5,17-21]. In addition to mutations, gene amplification or increased copy gains have been reported. It has been reported in recent studies that PIK3CA mutations can independently hamper the therapeutic response to anti-EGFR biological therapies[22].

Among the mutation detection methods, High Resolution Melting (HRM) is a powerful technique in molecular biology. It is a simple polymerase chain reaction (PCR)-based method used for the discovery of DNA sequence variations by measuring changes in the melting curve of a DNA without the need for any post-PCR handling. In other words, HRM analysis is the quantitative analysis of the melt curve of a DNA fragment. Unlike other methods, HRM has many advantages, such as high sensitivity, repeatabiliy, rapid turn-around time that greatly diminishes contamination risk, and low cost [23].

Considering the importance of molecular targeted therapy, conflicting responses of bladder cancer cells owing to mutations in some genes in signaling pathways and some inhibitors were assessed in clinical trials in bladder cancer patients. More importantly, since there are considerable genetic variation within a population and also among different populations, this study was conducted to detect common mutations of exons 9 and 20 and other probable mutations in PIK3CA gene as well as their frequencies in Iranian bladder cancer patients by using HRM analysis which is a useful method for mutation screening with high analytical sensitivity.

Material and Methods

Patients and tissue samples

Bladder tumor and adjacent normal tissue samples were obtained from 50 Iranian individuals who underwent transurethral bladder tumor resection or radical cystectomy at the Sina and Imam Khomeini Hospitals. Cancerous and their adjacent non-cancerous samples as normal control both from the bladder were rapidly frozen in liquid nitrogen following collection and were stored at -80 until subsequent RNA and DNA extraction.

Two experienced pathologists appraised the grade, stage, and nodal status. Of the 50 patients, 45 were males and 5 were females with the mean age of 67.3 ± 10 years. None of the patients received previous treatment such as BCG therapy, radiotherapy or chemother-

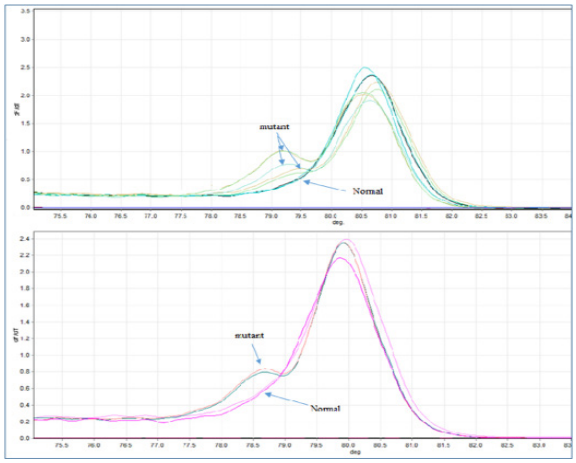

Figure 1. Examples of melting curves of wild and mutant samples. Shoulders on curves demonstrate nucleotide changes in PIK3CA gene.

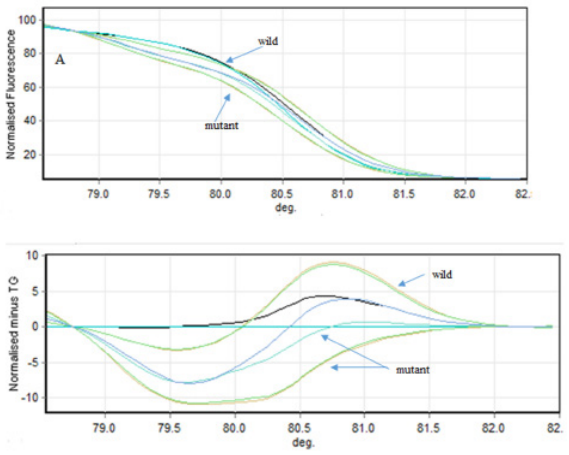

Figure 2. Normalized (A) and differenc (B) graphs of wild and mutant samples.

Table 2. Demographics and Clinical Characteristics of participants

Clinicopathologic parameters	Total		Wild type		Muted		*P*-value
	Number	percent	Number	percent	Number	percent	
Age (year)							0.353
≤67	4	8	3	6.7	1	20	
>67	46	92	42	93.3	4	80	
Stage							0.616
I	21	42.9	18	40.9	3	60	
II	20	40.8	19	43.2	1	20	
III	6	12.2	5	11.4	1	20	
IV	2	4.1	2	4.5	0	0	
Grade							0.584
Low	12	24.5	10	22.7	2	40	
High	37	75.5	34	77.3	3	60	
Tumor type							0.638
Non-invasive muscle	21	42	18	40.0	3	60	
Invasive muscle	29	58	27	60.0	2	40	
Family history of Bladder cancer							0.566
No	44	78	40	88.9	4	80	
Yes	6	12	5	11.1	1	20	
Family history of other cancer							0.736
No	49	98	44	97.8	5	100	
Yes	1	2	1	2.2	0	0	
Smoker							0.690
No smoker	23	46	20	44.4	3	60	
≤10 years	1	2	1	2.2	0	0	
>10 years	26	52	24	53.3	2	40	
Occupational exposure							0.004
Yes	18	36	13	28.9	5	100	
No	32	64	32	71.1	0	0	

apy prior to surgery and sample collection. All participants in this research signed written informed consent. This study was approved by Research Review Board and also the Ethics Committee of Tehran University of Medical Sciences (TUMS) (Ethic code: IR.TUMS.REC.1395.2895).

DNA Extraction

Genomic DNA from cancerous and normal frozen tissue samples were extracted by High Pure PCR Template preparation kit (Roche) according to manufacturer instructions. The quality and quantity of DNA were assessed by spectrophotometry with NanoDrop -2000 (Thermo, USA). DNA template samples were diluted to 20-30 ng /µL for using in HRM analysis.

Primer Design

Specific set of primers for each of the PIK3CA exons 9 and 20 that embrace hotspot regions were designed for HRM analysis with pre-amplification. For Sanger sequencing, other sets of primers for exons 9 and 20 were used to have a suitable length of amplicons. Primers sets were checked by primer-BLAST and Oligoanalyzer software. **Table 1** shows the primers sequences and amplicon lengths.

High Resolution Melting analysis

HRM analysis with pre-Amplification was performed on Rotor-Gene Q 5-plex HRM (Qiagen, Germany) using 0.1 ml strip tubes (QIAGEN, GmbH) in the presence of EvaGreen, the fluorescent DNA intercalating dye. The reaction was conducted in a 20 µL final volume containing 4 µL 5X Hot FIREPOL EvaGreen qPCR mix (Solis BioDyne), 0.5 µL of 10 pmol/µL primers (forward and reverse) and 20-30 ng/µL DNA sample. For each exon, one mixture was prepared. Two verified samples with and without mutation were used as a positive and negative control, respectively. HRM was performed in duplicate on all samples.

Cycling for pre-Amplification and melting conditions were as follows: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 60°C, and 20 seconds at 72°C, followed by melting from 75°C to 85°C. The temperature was increased by 0.1°C/sec. All data and melting curves from different samples were analyzed using Rotor-Gene Q series software version 2.0.2. To confirm the findings resulted by HRM analysis, the samples were subjected to sequencing. For PCR reaction, primers for exons 9 and 20 were added to PCR master mix (Takara) separately, and DNA was added. PCR was performed on SensoQuest lab cycler. Finally, PCR products were applied for sequencing.

Statistical analysis

Using Fisher´s exact test or Pearson chi-square, the results were statistically analyzed by SPSS version 21 and

Table 3. Mutations in exon 9 and 20 of PIK3CA gene

PIK3CA	nucleotide change	protein (codon,amino acid)	mutation type
Exon 9	c.1571G>A	R524K	Missense
	c.1578T>A	N526K	Missense
	c.1624G>A	E542K	Missense
	c.1633G>A	E545K	Missense
	c.1638G>A	E547R	Missense
Exon20	c.3140 A>T	H1047L	Missense

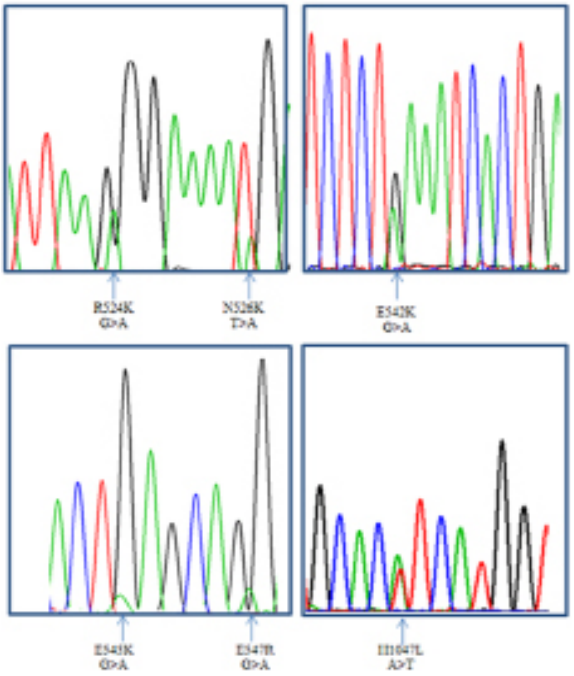

Figure 3. Mutations in exon 9 and 20 of PIK3CA gene. First and second rows show normal and mutant sequences, respectively.

P value was set at 0.05 or less to indicate statistically significant difference. Kolmogorov- Smirnov test was performed to assess the normality of quantitative data.

RESULTS

A total of 50 bladder cancer patients including 45 (90%) men and 5 (10%) women participated in this study. PIK3CA hot spot mutations and PIK3CA gene expression at mRNA level were examined. **Table 2** shows the demographic and clinicopathological characteristics of all the patients. Their median age was 67 years (33 to 87 years). There were 26 patients (54.5%) with smoking habit and 98% of them were cigarette smoker for more than 10 years and 13.6% were opium-addict. Among these patients, 14% were suffering from diabetes and 54% had cardiovascular and respiratory diseases. The rate of occupational exposure was about 38%.

PIK3CA mutations

Hot spot mutations in exons 9 and 20 of PIK3CA gene were screened in tumors and adjacent non-tumor tissues of all the subjects using HRM analysis. Mutation 10% (5/50) was detected in PIK3CA gene in tumor tissues. Melting curve and normalized graph for HRM are as shown in **Figures 1and 2**. Direct Sanger sequencing confirmed and documented the exact mutational parameters (Figure 3). In addition to hot spot mutations, E542K and E545K in exon 9, three novel mutations in the helical domain of PIK3CA gene that lead to amino acid substitution in R524K and N526K, and E547R were also evident. It was notable that two samples harbored two and three mutations simultaneously. No mutation was found in non-tumor tissues. Most of the mutations were clustered in helical domain of PIK3CA, whereas only one mutation (H1047L) was located in the kinase domain. Nucleotide changes in exons 9 and 20 PIK3CA gene are shown in **Table 3**.

The relationships between tumors characteristics and the mutations were analyzed. There was no statistically significant correlation between age, grade, stage, and other indices concerning the mutations. While occupational exposures showed a significant relationship (P = .005), no relationship was found when smoking habit was compared with the mutations' status.

DISCUSSION

In the present study, HRM being a sensitive, rapid and cost-effective and simple approach was used to screen PIK3CA gene mutation, which is a key component of the PI3K pathway. HRM with Sanger sequencing showed new mutations in addition to hot spot mutations in PIK3CA gene. The PI3K signaling pathway is associated with a number of oncogenes and multiple receptor classes for several basic cellular functions. It has been proposed to be the most commonly activated signaling pathway in human cancers[24-25]. There are several lines of evidence that show different mutations at PIK3CA gene to be one of the main mechanisms responsible for PI3K pathway activation in cancer cells [5,9,26]. Therefore, it has been considered that cancer accompanied by PIK3CA mutations could be the potential candidate for targeted therapeutic intervention with catalytic subunit (p110α) specific-inhibitor. Moreover, PIK3CA mutations can independently hinder the therapeutic response to anti-receptor tyrosine kinase therapy[27].

Common hot spot mutations were examined in PIK3CA gene in our patients since the pattern of genetic makeup from health to disease states is highly dependent on the population racial background and as such more information are also needed for targeted therapy. To the best of our knowledge, this is the first study that examined the PIK3CA mutations in Iranian bladder cancer patients.

Out of the patients, almost 10% (5/50) cases harbored PIK3CA mutations in relation to the prevalence of 13 to 35% reported in previous studies[5,9,10,17,18]. In the present study, nearly 75% of subjects had high-grade tumor that explains the lower frequency of mutation in the study. This frequency has been observed to decrease in the case of advanced stage and grade tumors. However, a much higher frequency of 18% for these mutations has also been reported for high-grade tumor of the bladder cancer[18]. However, considering the fact that the high frequency (35%) of PIK3CA gene mutations is present in superficial bladder tumors (low grade, low stage) and the low frequency is present in bladder cancer cases with muscle invasive tumors and advanced stage and grade[5,10], PIK3CA gene mutations were postulated to be early event in bladder carcinogenesis process. On the other hand, the disagreement in PIK3CA mutations rate among different reports can be partly explained by ethnic variation, population base diversity, and methodological parameters including preferences in mutation detection techniques and histopathological heterogeneity within individual tumor type.

The majority of the PIK3CA mutations in the present research were clustered in helical domain (exon 9) of PIK3CA gene which is in agreement with previous findings of bladder cancer[5,9,28]. Besides, hot spot codons E542K and E545K mutations are available at Catalogue of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic), hence, three novel mutations: R524K, 526K, and E547R were reported.

Remarkably, in one sample, three mutations were present at the same time. Although, occupational exposures have shown statistically significant correlation with mutation presence (P = .005), there was no significant relationship between mutations and other clinicopathological parameters. The habit of tobacco smoking has been reported to be the most well-known risk factor for bladder cancer development, but no significant relationship was observed between this habit and mutations, whereas more than half of the cases in the present study were smoker. The lack of significant correlation may be as a result of the relatively small number of samples in this study. According to some reports, it seems that PIK3CA gene mutations' specific location in association with, or development of, different cancers have a particular implication that may partly translate their heterogeneity at the molecular level. For example, in contrast to bladder cancer, breast cancer mutations in the kinase domain (exon 20) are more common than in the helical domain as the mutation rate sharply rises to 25-40% [12,16,24,25,28,29]. Also, in colon cancer, heterogeneity may depend on different histological aspects of the lesion[30].

CONCLUSIONS

To the best of our knowledge, this is the first study on PIK3CA mutations in Iranian bladder cancer patients. PIK3CA hot spot mutations and three new variants were detected in 10% of the cases. Also, HRM analysis was confirmed to be a rapid and low-cost method for mutation screening. Since PIK3CA gene is considered as a target for cancer therapy and the mutations of this gene can resist some anti-tumor agents for individualized medicine in our patients, a better understanding of both general and specific events that took place at the cellular and molecular level in bladder cancer is needed. This comprehensive study would make it possible to design new targeted and tailored interventions by considering both the subject who has the disease and the disease itself.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this study.

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