Chapter 2

Analysis of Point Mutations in Clinical Samples of Urothelial Carcinoma

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Abstract

In the last two decades specific point mutations in oncogenes have been identified in urinary bladder cancers. Identification of these mutations in clinical samples (e.g., urine or tumor tissue) can be of use for diagnostic or prognostic purposes. In this chapter we describe how mutations in multiple oncogenes can be identified with a simple assay.

Key words Oncogenic mutations, FGFR3, TERT, PIK3CA, RAS, DNA isolation, Mutation analysis

1 Introduction

1.1 Oncogenic Mutations in Bladder Tumors

In bladder tumors specific point mutations have been found in the *FGFR3, TERT, PIK3CA, HRAS, KRAS,* and *NRAS* oncogenes. Except for *TERT,* the mutations lead to the incorporation of another amino acid in the corresponding protein. This results in a more active protein, and since these proteins are all involved in cell growth and proliferation, the end result is tumor growth. Mutations in the *TERT* gene occur instead in the promoter region and result in more mRNA and more protein and hence active lengthening of telomeres at the end of chromosomes, which is thought to immortalize cells. Table 1 gives an overview of the common mutations in these genes [1–3].

FGFR3 mutations occur in about 60% of bladder tumors, with the highest prevalence in stage Ta (65%,) with 33% in stage T1 (both non-muscle invasive (NMIBC)) and 22% in MIBC. Because of this distribution *FGFR3* together with Ki-67 can be used to predict progression in NMIBC tumors [1]. *TERT* mutations were found in 60–80% of tumors, regardless of stage and grade [1, 2, 4, 5]. Mutations in *PIK3CA* are found in 24% of bladder tumors, with a similarly equal distribution over stage. Finally,

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Table 1
Overview of the relevant genes and their mutation sites

Genes	Mutation sites
HRAS	G12C/S, G12D/V, G13C/R, Q61K, Q61L/R
KRAS	G12C/R/S, G12A/D/V, Q61E
NRAS	Q12R, Q61L/R
PIK3CA	E542K, E545K/Q, E545G, H1047L/R
FGFR3	R248C, S249C, G372C, S373C, Y375C, G382R, A393E, K652E/Q, K652 T/M
TERT[a]	-124 C > T/A; −138 C > T; −146 C > T

[a]Mutations in *TERT* are respective to the ATG start codon

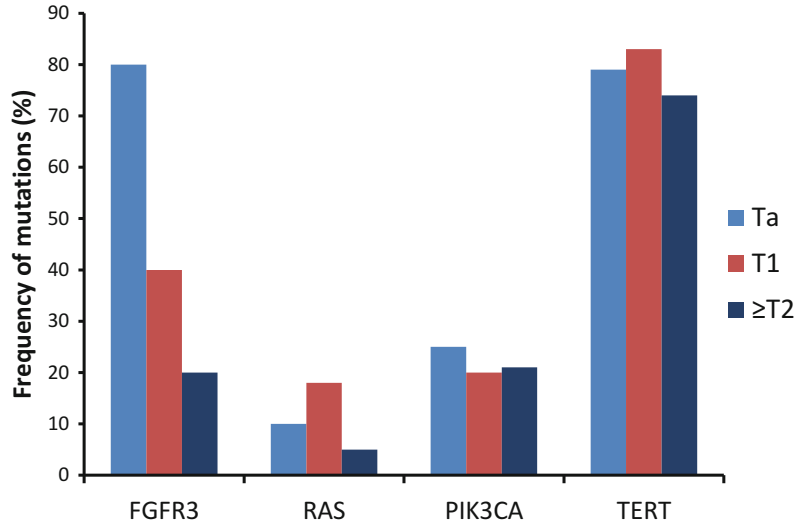

Fig. 1 Frequencies of *FGFR3, RAS, PIK3CA* and *TERT* mutations according to stage

mutations in the three *RAS* genes are relatively uncommon (6–10%) [1]. Figure 1 gives an overview of frequencies according to stage [1–3].

1.2 The Use of Oncogenic Mutations in Clinical Practice

Analyses of the mutations in DNA isolated from tumor tissue is important for predicting possible progression, as is the case for *FGFR3* mutations. In addition, mutations in *PIK3CA* and the *RAS* genes may be of use as companion diagnostic for targeted therapies as the pathways in which these genes function will be constitutively active downstream of the mutant protein and hence upstream inhibition will be ineffective. In addition, targeted therapies with small molecule inhibitors or monoclonal antibodies are in

clinical trials for tumors with an activated *FGFR3* gene. Likewise there are multiple small molecule inhibitors for the active PIK3CA protein. Finally, the high frequency with which *FGFR3* and *TERT* mutations occur makes them ideal for the identification of bladder tumor cells in DNA isolated from urinary cell pellets, both for patients under surveillance for recurrences after resection of a primary NMIBC as well as for patients presenting with hematuria to rule out that a bladder tumor is the cause of hematuria [1–3, 6, 7]. This chapter provides background information per mutation type and illustrates how mutation analysis can be carried out from voided urine or tumor tissue [8].

2 Materials

2.1 DNA Isolation from Urine

1. 50 ml centrifuge tubes.
2. Phosphate-buffered saline.
3. 1.5 ml Eppendorf vials.
4. QIAamp mini and Blood kit.
5. Ethanol (96–100%).
6. Qubit 2.0 Fluorometer device.
7. Qubit dsDNA HS Assay Kit.

2.2 DNA Isolation from Formalin-Fixed Paraffin-Embedded (FFPE) Tissue

1. Disposable 1 mm biopsy punch.
2. Xylene.
3. Ethanol 100%.
4. Ethanol 70%.
5. Lysis buffer; 10 mM Tris–HCl, pH 8.0, 100 mM EDTA, pH 8.0, 50 mM NaCl, 0.5% SDS.
6. Proteinase K, 20 mg/ml.
7. Chelex.

2.3 Mutation Analysis

2.3.1 Materials

1. KAPA2G Robust Hotstart ReadyMix.
2. Shrimp alkaline phosphatase.
3. Exonuclease-I.
4. SNaPshot Multiplex kit (Applied Biosystems, Life Technologies, UK).
5. Formamide.
6. Automatic sequencer ABI PRISM 3130 XL Genetic Analyzer or similar.
7. GeneScan Analysis Software version 2.4.2 (SoftGenetics LLC).

3 Methods

The mutations described above are good diagnostic candidates for early detection and disease surveillance. For the analysis one needs 10–100 ml of voided urine from a (potential) patient.

3.1 DNA Isolation from Urine

1. Transfer the urine sample to a 50 ml centrifuge tube.
2. Centrifuge the 50 ml tube at 1500 × *g* for 10 min.
3. Resuspend the pellet in 900 μl phosphate-buffered saline (PBS) and transfer this to a 1.5 ml Eppendorf tube.
4. Centrifuge the 1.5 ml tube at 3000 × *g* for 5 min.
5. Discard the supernatant; pellets can be kept at −80 °C until DNA isolation.
6. DNA from urine pellets is isolated using the QIAamp mini and Blood kit. Before starting, bring samples to room temperature.
7. Add 20 μl proteinase K (20 mg/ml) and 200 μl buffer AL to the sample. Mix for 15 s using the pulse-vortex and incubate for 10 min at 56 °C.
8. Centrifuge the tubes shortly to remove drops from the inside of the lid.
9. Add 200 μl ethanol (96–100%) to the sample and mix again using the pulse-vortex. Again centrifuge the tubes to remove drops from the lid.
10. Add the mixture from the 1.5 ml tube to the QIAamp Mini spin column in a 2 ml collection tube without wetting the rim.
11. Centrifuge the column at 6000 × *g* for 1 min and place the spin column in a clean 2 ml collection tube. Dispose the tube with the eluate.
12. Apply buffer AW1 to the spin column and centrifuge at 6000 × *g* for 1 min, dispose the tube with the eluate.
13. Apply buffer AW2 (500 μl) to the spin column and centrifuge at 20,000 × *g* for 3 min, discard the eluate.
14. For DNA elution transfer the spin column to a 1.5 ml Eppendorf vial and add 100 μl AE buffer to the spin column and incubate at room temperature for 5 min. Centrifuge at 6000 × *g* for 1 min. N.B. the DNA is in the eluate.
15. Measure the DNA concentration from the eluate according to manufacturer's protocol using the Qubit 2.0 Fluorometer device and corresponding Qubit dsDNA HS Assay Kit.

3.2 DNA Isolation from Formalin-Fixed Paraffin-Embedded (FFPE) Tissue

1. Use a hematoxylin-eosin-stained section of the tissue block for selection of an area with tumor cells.
2. Take a 1 mm punch from the selected region using a punch tool.

3. Transfer the sample to a 1.5 ml tube and add 800 µl xylene to remove paraffin.
4. Incubate for 10 min at room temperature.
5. Centrifuge the mixture at 16,000 × *g* for 1 min and pipet the xylene supernatant from the mixture.
6. For ethanol rehydration add 800 µl of 100% ethanol to the specimen, vortex and centrifuge at 16000 × *g* for 3 min.
7. Remove the ethanol supernatant without touching the pellet.
8. Pipet 800 µl of 70% ethanol, vortex and centrifuge at 16,000 × *g* for 3 min.
9. Remove the ethanol supernatant as much as possible.
10. Open the tube and air-dry the pellet for 10 min.
11. For the tissue dissolution add 100 µl lysis buffer, 25 µl proteinase K (20 mg/ml) and 25 µl Chelex (Bio-RAD, California, USA) to the pellet.
12. Incubate overnight in heat block at 56 °C.
13. Incubate the mixture at 95 °C for 10 min to deactivate proteinase K, and centrifuge for 1 min at 16,000 × *g*.
14. The supernatant contains the extracted DNA. Transfer into a clean tube leaving the Chelex mixture with the cell debris behind (*see* **Note 1**).
15. Measure DNA concentration as explained for urine DNA (*see* **Note 2**).

3.3 Mutation Analysis

1. For *FGFR3* mutation analysis, set up a multiplex PCR in a final volume of 10 µl containing 5 ng of DNA, 5 µl KAPA2G Robust Hotstart ReadyMix, 18 pmol of exon 7 primers and 10 pmol each of exon 10 and 15 primers (*see* Table 2 for primer sequences)(*see* **Note 3**).
2. After 3 min at 95 °C, 40 PCR cycles are carried out (15 s at 95 °C, 15 s at 55 °C and 20 s at 72 °C) followed by 10 min at 72 °C.
3. Treat the PCR product with 1.5 units of shrimp alkaline phosphatase and two units of exonuclease-I for 60 min at 37 °C to get rid of excess primers and deoxynucleotide triphosphates (ddNTPs), followed by 15 min at 85 °C to inactivate the enzymes.
4. Next, use a SNaPshot Multiplex kit for a single-nucleotide probe extension based on probes that anneal adjacent to the investigated nucleotides (probe sequences in Table 2, *see* **Note 4**).
5. The SNaPshot reaction is carried out in a total volume of 10 µl containing 1 µl of PCR product, 2.5 µl of the Snapshot ready mix, 2 µl of the 5× sequencing buffer and 1 µl of the probe mix.

Table 2
Primer and probe sequence for *FGFR3* mutation analysis

Primer	Sequence (5′- > 3′)	Product size (bp)	pmol			
FGFR3 RI Fw	AGTGGCGGTGGTGGTGAGGGAG	115	18			
FGFR3 RI Rev	GCACCGCCGTCTGGTTGG		18			
FGFR3 RII Fw	CAACGCCCATGTCTTTGCAG	138	10			
FGFR3 RII Rev	AGGCGGCAGAGCGTCACAG		10			
FGFR3 RIII Fw	GACCGAGGACAACGTGATG	160	10			
FGFR3 RIII Rev	GTGTGGGAAGGCGGTGTTG		10			
Probe	**Sequence (5′- > 3′)**	**Size (bp)**	**Strand**	**WT**	**MT**	
S373C	T19 GAGGATGCCTGCATACACAC[a]	39	sense	T[b]	A	2
K652 M/T	T20 CACAACCTCGACTACTACAAGA	42	sense	A	T/C	7
G372C	T29 GGTGGAGGCTGACGAGGCG	48	sense	G	T	2
A393E	T34 CCTGTTCATCCTGGTGGTGG	54	sense	C	A	10
R248C	T46 CGTCATCTGCCCCCACAGAG	66	sense	C	G	8
Y375C	T43 ACGAGGCGGGCAGTGTGT	61	sense	A	G	10
S249C	T36TCTGCCCCCACAGAGCGCT	55	sense	C	T	4
K652Q/E	T50 GCACAACCTCGACTACTACAAG	72	antisense	A	C/G	3
G382R	T56 GAACAGGAAGAAGCCCACACC	76	antisense	C	T	6

[a]T19 etc. indicate the length of the T-tails
[b]The color of the incorporated WT and MT nucleotides corresponds with the color of the peaks in the sequence run

6. Probe extension is for 35 cycles of 10 s at 96 °C, followed by 40 s at 58.5 °C.
7. Treat the Snapshot product with 1 U SAP for 30 min at 37 °C to remove excess ddNTPs and incubate for 15 min at 85 °C to inactivate the enzyme.
8. Add 1 μl of the reaction to 10 μl formamide and denature by incubation at 95 °C for 5 min.
9. Use this mixture for the separation of the product in a 20–25 min run on 36 cm long capillaries using the automatic sequencer. The absence or presence of a mutation is indicated by the fluorescent marker of the incorporated ddNTP.
10. Use GeneScan Analysis Software version 2.4.2 for analysis of the generated data or alternatively software provided by Applied Biosystems.

Table 3
Primer and probe sequence for *TERT* mutation analysis

Primer	Sequence (5′- > 3′)	Product size (bp)	pmol			
TERT Fw	AGCGCTGCCTGAAACTCG	155	10			
TERT Rev	CCCTTCACCTTCCAGCTC		10			
Probe	**Sequence (5′- > 3′)**	**Size (bp)**	**Strand**	**WT**	**MT**	**pmol/ reaction**
TERT; −124 C > T	T20 GGCTGGGAGGGCCCGGA[a]	37	sense	G	A/T[b]	10
TERT; −138C > T	T27 GGAGGGGGCTGGGCCGG	44	sense	G	A	5
TERT; −146 C > T	T39 CTGGGCCGGGGACCCGG	56	sense	G	A	15

[a]T20 etc. indicate the length of the T-tails
[b]The color of the incorporated WT and MT nucleotides corresponds with the color of the peaks in the sequence run

The *TERT* and *PIK3CA* mutation analyses are similar to the *FGFR3* mutation analysis. The *TERT* PCR covers the two most frequent sites for *TERT* mutations in the promoter. Primer and probe sequences for *TERT* mutation analysis are given in Table 3. Figure 2 illustrates the results of a *TERT* mutation analysis.

The *PIK3CA* PCR covers the hotspot mutation sites in the gene (E542K, E545G, E545K, and H1047R) [1]. Primer and probe sequences for PIK3CA mutation analysis are given in Table 4. Mutations in the *HRAS*, *KRAS,* and *NRAS* genes are not very common in bladder tumors. We therefore combined the most prevalent mutations in one assay as explained in Kompier et al. [1]. This assay covers 96% of the possible *RAS* mutations in bladder tumors. Details on primers and probes for the different mutations are depicted in Tables 2–5.

4 Notes

1. Take care to get rid of the Chelex beads as they may interfere with subsequent steps.
2. Do not use a spectrophotometer for determining DNA concentration. Especially with FFPE samples many proteins are still present in the DNA solution. Proteins absorb ultraviolet light in the 230 and 280 nM range and high concentrations may result in absorption at 260 nM and hence the DNA concentration may be overestimated.
3. Make up with water to 10 μl, if necessary. Optional: add a small drop of paraffin oil to prevent evaporation.
4. T-tails are added to the probe to allow separation and visualization of the different probes on the sequencer.

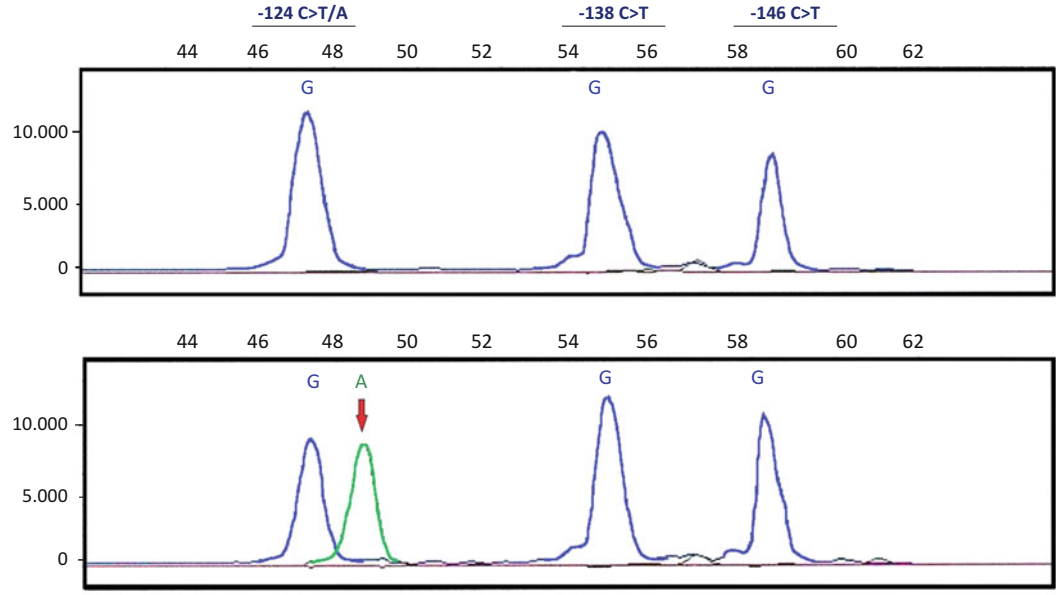

Fig. 2 Example of a wild type and mutant type (lower panel) *TERT* mutation analysis result

Table 4
Primer and probe sequence for *PIK3CA* mutation analysis

Primer	Sequence (5′- > 3′)	Product size (bp)	pmol			
PIK3CA ex9-Fw	AGTAACAGACTAGCTAGAGA	139	0.5			
PIK3CA ex9-Rev.	ATTTTAGCACTTACCTGTGAC		0.5			
PIK3CA ex20-Fw	GACCCTAGCCTTAGATAAAAC	109	1			
PIK3CA ex20-Rev	GTGGAAGATCCAATCCATTT		1			
Probe	**Sequence (5′- > 3′)**	**Size (bp)**	**Strand**	**WT**	**MT**	**pmol/ reaction**
E542K	T17 ACACGAGATCCTCCTCTCT*	35	sense	G[a]	A[b]	1.5
E545G	T21 CCTCTCTCTGAAATCACTG	40	sense	A	G	5
E545G	T25 ATCCTCTCTCTGAAATCACT	45	sense	G	A	3
H1047R	T30 GAAACAAATGAATGATGCAC	50	sense	A	G	3

[a]T17 etc. indicate the length of the T-tails
[b]The color of the incorporated WT and MT nucleotides corresponds with the color of the peaks in the sequence run

Table 5
Primer and probe sequence for *HRAS, KRAS, NRAS* mutation analysis

Primer	Sequence (5′- > 3′)	Product size (bp)	pmol
HRAS exon1 Fw	CAGGAGACCCTGTAGGAGG	139	6
HRAS exon1 Rev	TCGTCCACAAAATGGTTCTG		6
HRAS exon2 Fw	GGAGACGTGCCTGTTGGA	140	3
HRAS exon2 Rev	GGTGGATGTCCTCAAAAGAC		3
KRAS exon1 Fw	GGTCCTGCTGAAAATGACTG	163	3
KRAS exon1 Rev	GGTCCTGCACCAGTAATATG		3
KRAS exon1 Fw	CCAGACTGTGTTTCTCCCTT	155	3
KRAS exon1 Rev	CACAAAGAAAGCCCTCCCCA		3
NRAS exon1 Fw	GGTGTGAAATGACTGAGTAC	128	3
NRAS exon1 Rev	GGGCCTCACCTCTATGGTG		3
NRAS exon2 Fw	GGTGAAACCTGTTTGTTGGA	103	3
NRAS exon2 Rev	ATACACAGAGGAAGCCTTCG		3

(continued)

Table 5
(continued)

Probe	Sequence (5′- > 3′)	Size (bp)	Strand	WT	MT	pmol/ reaction
HRAS pos.34	T17 CTGGTGGTGGTGGGCGCC[a]	35	Sense	G[b]	C/T/A	5
HRAS pos.182	T18 GCATGGCGCTGTACTCCTCC	38	antisense	T	G/C/A	1.5
KRAS pos.34	T25 GGACTCTTGCCTACGCCCAC	45	antisense	C	G/A/T	5
HRAS pos.35	T31 CGCACTCTTGCCCACACCG	50	antisense	C	G/A/T	7
NRAS pos.182	T33 GACATACTGGATACAGCTGGAC	55	sense	A	G/C/T	5
KRAS pos.181	T41 CTCATTGCACTGTACTCCTCTT	63	antisense	G	T/C	2
HRAS pos.181	T46 CATCCTGGATACCGCCGGC	65	sense	C	A/G	7
KRAS pos.35	T49 AACTTGTGGTAGTTGGAGCTG	70	sense	G	C/T/A	2
HRAS pos.37	T55 CAGCGCACTCTTGCCCACAC	75	antisense	C	G/A/T	7
NRAS pos.34	T62 CTGGTGGTGGTTGGAGCA	80	sense	G	C/T/A	2

[a]T17 etc. indicate the length of the T-tails
[b]The color of the incorporated WT and MT nucleotides corresponds with the color of the peaks in the sequence

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