Mutational Screening of *RET, HRAS, KRAS, NRAS, BRAF, AKT1*, and *CTNNB1* in Medullary Thyroid Carcinoma

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Abstract. *Background: Screening medullary thyroid carcinomas (MTCs) for rearranged during transfection (RET) mutations becomes increasingly important for clinical assessment of the disease. The role of mutations in other genes including RAS (i.e. HRAS, KRAS, and NRAS), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), v-akt murine thymoma viral oncogene homolog 1 (AKT1), and CTNNB1 (β-catenin) is unknown or not fully explored yet for this disease. Materials and Methods: Formalin-fixed and paraffin-embedded (FFPE) material was the primary source for screening 13 sporadic and inherited MTCs and matched non-tumor specimens. Multiplex PCR was included in the PCR protocol. Sequence analysis encompassed mutational hotspot regions in RET exons 5, 8, 10, 11, and 13 to 16; HRAS exons 1 and 2; KRAS exons 1 and 2; NRAS exons 1 and 2; BRAF exon 15; AKT1 exon 2, and CTNNB1 exon 3. Results: We identified RET mutations in seven of 13 MTCs: five RET-positive cases revealed a mutation in exon 16 (M918T) and two a mutation in exon 10 (C618S and C620S). In four of the RET-positive cases, the mutation was inherited, out of which three were reportedly associated with a multiple endocrine neoplasia type 2 (MEN2) syndrome, i.e. MEN2A (C618S), MEN2A/familial MTC (FMTC) (C620S), and MEN2B (M918T). These cases reflect the known MEN2 genotype-phenotype correlation. Three of the five stage IVc MTCs were inherited RET-positive cases. Mutational screening in HRAS, KRAS, NRAS, BRAF, AKT1, and CTNNB1 disclosed one sporadic RET-negative MTC (stage III) with mutation in HRAS codon 13 (G13R). Conclusion: Our study supports the clinical relevance of screening MTC patients for RET mutations. The role of RAS mutations, in particular HRAS mutations, in sporadic RET-negative MTC has not been fully explored yet. Mutations in BRAF, AKT1, and CTNNB1 are likely not to play a role in MTC.*

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Medullary thyroid carcinoma (MTC) comprises approximately 5% of all thyroid cancer types (1). It separates from other thyroid malignancies by its origin from neural crest-derived, calcitonin-secreting parafollicular C cells. Approximately 45 to 65% of MTCs harbor an alteration in the *RET* oncogene which is considered the major genetic event in MTC (2, 3). The *RET* gene is located on 10q11.2. It contains 21 exons encoding the signaling subunit of a cell-surface receptor complex for ligands of the glial derived neurotrophic factor (GDNF) family. The RET protein transmits intracellular signals upon binding of growth factors to the receptor side. Pathways involved regulate growth, survival, differentiation, and migration of neural crest derived cells (4).

In contrast to papillary thyroid carcinomas, which in a minority of cases, in particular in children and upon radiation exposure, show rearrangement of the *RET* gene, MTCs are characterized by point mutations in the *RET* gene. These mutations cluster in exons 5, 8, 10, 11, and 13 to 16 (5, 6). Exons 5 and 8 have been added in recent years to the routine panel of analyzed exons in MTC (5). All *RET* mutations in MTC are gain-of-function mutations. The M918T alteration in exon 16 is the most common mutation in sporadic MTC and in general associated with highest risk for aggressive cancer (2, 7, 8).

An estimated 80% of MTCs are sporadic cases and about 20% are inherited forms (9). Nearly all sporadic cases appear as solitary tumors commonly affecting middle aged adults, whereas familial forms often appear as multiple and bilateral tumors affecting younger age groups. Inherited *RET* mutations are known to be associated with multiple endocrine neoplasia 2A (MEN2A), MEN2B, familial MTC (FMTC), and Hirschsprung disease (4, 10). People with MEN2A have a family history of this disease in over 95% of the cases and have a nearly 100% risk of developing MTC, while MEN2B carriers have a family history of the disease in about 50% of their cases (9). Various kinds of solid tumors, including pheochromocytoma and parathyroid adenoma, are associated with MEN2 syndromes (8). MTC is the most common cause of death in MEN2 carriers (11). Identification of a *RET* germline mutation is the definitive method to distinguish sporadic from inherited cases. Clinically inconspicuous family members of MEN2 carriers can then be tested for inheritance and identified carriers counseled for prophylactic intervention (5).

Gain-of-function mutations that play a critical role in some other thyroid and non-thyroid lesions such as the common V600E mutation in exon 15 of *BRAF*, or mutations in exon 1 (codons 12 and 13) and exon 2 (codon 61) of the related RAS genes *HRAS, KRAS*, and *NRAS* have not been fully explored yet in MTC (12, 13). Similarly, abundance of mutations in *AKT1* and *CTNNB1* which are key regulator genes of the phosphatidylinositol 3-kinase (PI3K)/AKT and canonical Wnt/β-catenin pathways, respectively, are virtually unknown in MTC (14, 15). *AKT1* is the most ubiquitously expressed AKT isoform and harbors a mutational hotspot in exon 2 (codon 17) that is mutated at lower frequencies in different tumor types (14, 16). This mutation activates *AKT1* by localizing it to the plasma membrane. Recently, it has been shown that molecules of the PI3K/AKT pathway are preferentially activated in inherited *RET*-positive cases (17). *CTNNB1* mutations clustering in exon 3 are commonly found in poorly differentiated and undifferentiated thyroid carcinomas (18). These mutations frequently involve serine and threonine residues that are potential phosphorylation sites for β-catenin degradation.

The frequency of MTC among thyroid malignancies in Saudi Arabia is within the range described for other regions. A survey of the frequency of thyroid cancer cases admitted to the King Faisal Specialist Hospital and Research Center, Riyadh revealed an incidence of 5.3% for MTC (19). A study on the pattern of thyroid cancer at the King Abdulaziz University, Jeddah reported a frequency of 6.7% for MTC (20). We performed a genetic screening for *RET, HRAS, KRAS, NRAS, BRAF, AKT1*, and *CTNNB1* in 13 MTCs to establish a comprehensive genetic profile of genetic mutations for this disease with the prospect to improve the clinical management of this type of cancer.

Materials and Methods

MTC cases. We examined 13 MTC cases which were treated by partial or total thyroidectomy in the period of 1999 to 2010 at the King Abdulaziz University Hospital, Jeddah (9 MTCs) and at the King Faisal Specialist Hospital and Research Center, Jeddah (4 MTCs). The vast majority of patients originated from Saudi Arabia and other Arabic countries. All cases were primary tumors except one late recurrence (PT-072-10). Histopathological diagnosis and staging of MTCs was performed by an oncologic pathologist (JM) according to established criteria (21, 22). Clinicopathological and follow-up data were compiled from patients' charts. The study was approved by the Ethical Review Boards of both institutions.

Mutational screening. Genomic DNA was extracted from 10 μm sections of formalin-fixed and paraffin-embedded (FFPE) tumor and non-tumor specimens using conventional xylene/ethanol treatment, overnight incubation with proteinase K, and subsequent DNA purification utilizing the QIAmp DNA FFPE tissue kit (Qiagen, Hilden, Germany). This method yielded DNA of sufficient quality in all cases including older FFPE samples from 1999 (PT-73-10D, PT-74-10D, and PT-110-10D). Genomic DNA from fresh-frozen samples was obtainable for genetic screening in two cases (TM-2001-10, TM-2384-10). Non-tumor specimens were available in all cases except one (PT-110-10) and used to investigate possible germline mutations. DNA concentration was measured with the Nanodrop device (Thermo Scientific, Wilmington, DE, USA).

Gene specific PCR primers were designed for use primarily with DNA from FFPE material and to flank mutational hotspots regions in *RET* exons 5, 8, 10, 11, and 13 to 16 (8); *HRAS* exons 1 and 2, *KRAS* exons 1 and 2, *NRAS* exons 1 and 2; *BRAF* exon 15, *AKT1* exon 2, and *CTNNB1* exon 3 (Table I). The *RET* primers flanked virtually all known hotspot regions described so far for the gene (8). PCR were performed in 20 μl volumes each containing 2 μl 10X buffer, 0.1% 2-mercaptoethanol, 0.0125% bovine serum albumin (BSA), 3 mM $MgCl_2$, 10 nmol of each dNTP, 10 pmol forward primer, 10 pmol reverse primer, 1.25 units hot start DNA polymerase, and 1 μl of DNA template (100 to 300 ng) or 1 μl of multiplex PCR product. DNA templates were excluded in negative controls. The standard PCR protocol included an initial denaturation step at 95˚C followed by 5 touch-down cycles with an annealing temperature decreasing 2˚C per cycle from 68˚C to 60˚C, then 40 cycles followed with 30 s at 95˚C, 30 s at 58˚C, and 30 s at 72˚C. The final step was performed for 10 min at 72˚C. Multiplex PCR containing pooled primers for *HRAS, KRAS,* and *NRAS* or *AKT1*, and *CTNNB1* were performed with the same standard PCR protocol except that only 15 instead of 40 cycles were conducted at 58˚C annealing temperature. Positive and negative controls for multiplex PCR were subjected to 40 cycles. PCR products were checked by electrophoresis on 2% agarose gels.

Purified PCR products were subjected to cycle sequence reactions using the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Nested primers overlapping with the PCR primers were utilized for sequence reactions. The purified sequencing products were finally resolved by capillary electrophoresis on an ABI PRISM 3130 Sequencer. Sequences were screened for gene alterations using a combination of manual readout of sequences and the online NCBI's BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table I. *Primer sequences used for mutational screening in MTC.*

Forward Primer[a]	Sequence[b]	Reverse primer[a]	Sequence[b]	Product size (bp)
RET-ex5-F	TCGCCTGCACTGACCAAC	*RET*-ex5-R	GAGCACCTCATTTCCTGG	285
RET-ex8-F	TGCTGTTCCCTGTCCTTG	*RET*-ex8-R	AGAAGCAGACCTGGAGCA	317
RET-ex10-F	ACACTGCCCTGGAAATATG	*RET*-ex10-R	TCAGATGTGCTGTTGAGAC	261
RET-ex11-F	AGCATACGCAGCCTGTAC	*RET*-ex11-R	GTCATCTCAGCTGAGGAG	216
RET-ex13-F	CGTTTGCAACCTGCTCTG	*RET*-ex13-R	TGCAGCTGGCCTTACCAT	153
RET-ex14-F	TCCTGGAAGACCCAAGCT	*RET*-ex14-R	ATATGCACGCACCTTCATC	285
RET-ex15-F	CTCGTGCTATTTTTCCTCAC	*RET*-ex15-R	ATCTTTCCTAGGCTTCCC	232
RET-ex16-F	CTCCTTCCTAGAGAGTTAG	*RET*-ex16-R	GTGTTTCTGTAACCTCC	171
HRAS-c12/13-F	CAGGAGACCCTGTAGGAG	*HRAS*-c12/13-R	TATCCTGGCTGTGTCCTG	224
HRAS-c61-F	TGTCCTCCTGCAGGATTC	*HRAS*-c61-R	GTACTGGTGGATGTCCTC	189
KRAS-c12/13-F	AACCTTATGTGTGACATGTTC	*KRAS*-c12/13-R	TCCTGCACCAGTAATATGC	215
KRAS-c61-F	AATCCAGACTGTGTTTCTCC	*KRAS*-c61-R	TTAAACCCACCTATAATGGTG	216
NRAS-c12/13-F	AAAGTACTGTAGATGTGGCTC	*NRAS*-c12/13-R	GTGAGAGACAGGATCAGG	233
NRAS-c61-F	GCATTGCATTCCCTGTGG	*NRAS*-c61-R	TAATATCCGCAAATGACTTGC	244
BRAF-F	CATAATGCTTGCTCTGATAGGAA	*BRAF*-R	AGTAACTCAGCAGCATCTCAG	243
AKT1-F	ACATCTGTCCTGGCACAC	*AKT1*-R	CCATCTGAATCCCGAGAG	255
CTNNB1-F	GCTGATTTGATGGAGTTGG	*CTNNB1*-R	CTCTTACCAGCTACTTGTTC	235

[a]ex, exon; c12/13, codons 12 and 13; c61, codon 61; [b]primer sequences from 3' to 5'.

Table II. *Clinicopathological and genetic features of 13 MTCs.*

Case	Gender	Age at diagnosis (years)	Tumor size (cm)	Vascular invasion	Distant metastasis	pTNM	Stage	Syndrome	Follow-up (years)	Gene mutation	
										Tumor	Non-tumor
PT-073-10	F	58	1.5	No	No	T1N0M0	I	NR	0	Neg	NT
PT-284-10	F	68	0.1, MF	No	No	T1N0M0	I	NR	2.3	Neg	NT
PT-110-10	M	60	2.2	No	No	T2N0M0	II	Sporadic	0.2	Neg	NT
PT-285-10	F	42	2.5	NR	No	T2N0M0	II	NR	5.5	Neg	NT
PT-113-10	F	25	3.2	NR	No	T2N0M0	II	FMTC/MEN2[b]	2.2	RET C620S	RET C620S
PT-069-10	F	52	3.5	No	No	T2NxM0	II	Sporadic	2.1	RET M918T	Neg
TM-2001-10	M	30	7.0	NR	No	T3NxM0	II	Sporadic	0.2	RET M918T	Neg
PT-112-10	M	57	2.2	NR	No	T2N1M0	III	Sporadic	8.4	HRAS G13R	Neg
PT-072-10[a]	M	34	0.5	No	Retrosternum	TXN1M1	IVc	MEN2A	26	RET C618S	RET C618S
PT-074-10	M	45	2.2	Yes	Neck	T2N0M1	IVc	NR	0.2, DOD	RET M918T	RET M918T
TM-2384-10	M	67	7.0	No	Lung, liver	T3N0M1	IVc	Sporadic	0.4	Neg	NT
PT-070-10	M	25	5.8	No	Mediastinum	T4aN1M1	IVc	NR	2.5	RET M918T	Neg
PT-071-10	F	23	2.5	Yes	Lung, liver	T4aN1M1	IVc	MEN2B	1.0	RET M918T	RET M918T

M: male; F: female; DOD: died from disease; LN: lymph node; MF: multifocal; Neg: negative for gene alteration; NR: not reported; NT: not tested; [a]Mutational screen on a late recurrence; [b]Penetrance of MEN2A not assessed.

Results

Mutational screening was performed in 13 cases of MTC that were treated at two main hospital centers in Jeddah during the period 1999 to 2011 (Table II). Seven of the patients were males and six females. Tumor size in the 12 primary cases was on average 3.1 cm (±2.2 cm). Lymph node and distant metastases were diagnosed in 6 patients. The majority of cases were stage II or greater. The follow-up period ranged between 0 and 26 years and there was one reported death.

A *RET* mutation in exon 16 affecting codon 918 was identified in five patients. This mutation resulted in substitution of methionine by threonine (M918T). In two cases, the alteration was inherited and in one case reportedly associated with MEN2B. Two *RET* mutations were identified in exon 10 at codons 618 and 620, respectively, resulting in substitution of cysteine by serine in both cases. These two exon 10 mutations were inherited and associated with MEN2A and FMTC/MEN2A, respectively. Three out of the four inherited *RET*-positive cases had stage IVc. In

comparison to the nine sporadic cases, the four inherited cases were on average younger (32 years *vs*. 51 years).

Screening of exon 1 and 2 of *HRAS, KRAS*, and *NRAS* disclosed one mutation affecting *HRAS* codon 13 leading to substitution of glycine by arginine (G13R). This mutation was confined to a stage III, sporadic *RET*-negative tumor. No mutations were observed in *BRAF* exon 15, *AKT1* exon 2, and *CTNNB1* exon 3.

Discussion

We identified mutations in *RET* exons 10 and 16 in 7 of 13 MTC cases. Exon 10 is localized in the cysteine-rich extracellular domain in which mutations in cysteine residues cause *RET* activation by ligand-independent dimerization (23). Inherited mutations in exon 10 of *RET* which are most commonly C618S and C620s are typically associated with MEN2A (24). In contrast, exon 16 is localized in the intracellular tyrosine kinase domain in which inherited mutations, most commonly M918T, are typically associated with MEN2B. The inherited *RET* cases associated with a MEN2 syndrome in our series displayed the expected genotype-phenotype correlation (8), *i.e*. association of exon 10 mutation with MEN2A and FMTC/MEN2A, respectively, and exon 16 mutation with MEN2B. In general, *RET* testing is a mandatory tool in the clinical assessment of sporadic or apparently sporadic MTCs, and of MEN2 carriers. In addition, depending on the type of *RET* mutation, a certain risk level for developing aggressive cancer can be assessed (8). *RET* testing can gain further clinical importance, as recent studies reveal that efficiency of different *RET* inhibitors against MTC is associated with the type of *RET* mutation (25, 26).

Our mutational screening in hotspot regions of *HRAS, KRAS, NRAS, BRAF, AKT1,* and *CTNNB1* revealed a mutation in *HRAS* in one of the five sporadic *RET*-negative MTCs. Only two larger studies have reported on mutational screening of *RET, HRAS*, and *KRAS* in sporadic MTC so far (27, 28). In both studies *HRAS* mutations were more common than *KRAS* mutations. Remarkably, whereas one of these studies revealed *HRAS* mutations in 56% of sporadic *RET*-negative MTCs (14 out of 25 cases), the other study detected the mutation only in 5% (2 out of 37 cases). This later study was conducted on a selected patient group having either symptomatic disease or disease progression. Regarding the frequency and distribution of RAS mutations among thyroid lesions, MTC shares the prevalence for *HRAS* mutations with Hurthle cell carcinomas, whereas follicular and anaplastic thyroid carcinomas reveal a trend for *NRAS* mutations (13). Taken together, besides the increasing importance of identifying the type of *RET* mutation in an MTC, mutational and clinical assessment of other genes in *RET*-negative cases warrants further exploration.

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