Comprehensive Survey of *HRAS*, *KRAS*, and *NRAS* Mutations in Proliferative Thyroid Lesions from An Ethnically Diverse Population

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Abstract. *Background: The distribution and kind of rat sarcoma viral oncogenes homolog (RAS) mutations, as well as their clinical impact on different types of thyroid lesions, vary widely among the different populations studied. We performed a comprehensive mutational survey in the highly related RAS genes HRAS, KRAS, and NRAS in a case series of proliferative thyroid lesions with known BRAF mutational status, originating from an ethnically diverse group. Materials and Methods: Mutational hotspot regions encompassing codons 12, 13, and 61 of the RAS genes were directly sequenced in 381 cases of thyroid lesions. In addition, the putative NRAS hotspot region encompassing codon 97 was sequenced in 36 thyroid lesions. The case series included lesions of Hashimoto's thyroiditis (HT), nodular goiters, hyperplastic nodules, follicular adenomas (FAs), Hurthle cell variants of FA, papillary thyroid carcinomas (PTCs), follicular variants of PTC (FVPTCs), microcarcinomas of PTC (micro PTCs; tumor size ≤1 cm), follicular TCs (FTCs), Hurthle cell variants of FTC, and non-well-differentiated TCs (NWDTCs). Results: We identified RAS mutations in 16 out of 57 (28.1%) FAs, 2 out of 8 (25%) NWDTCs, 8 out of 42 (19.0%) FVPTCs, 2 out of 10 (20.0%) FTCs, 1 out of 12 (8.3%) Hurthle cell variants of FA, 3 out of 46 (6.5%) goiters, 1 out of 18 (5.6%) hyperplastic nodules, 3 out of 56 (5.4%) micro PTCs, 2 out of 115 (1.7%) PTCs, 0 out of 7 (0%) Hurthle cell variants of FTC, and 0 out of 10 (0%) HT lesions. NRAS codon 61 mutation was the predominant form, followed by HRAS codon 61 mutation. Only three mutations affected RAS codons 12 and 13, two of which were identified in goiters. No codon 97 mutation was detected in the examined FVPTCs. An as yet undescribed deletion of KRAS codon 59 was identified in one FA. Discussion: RAS mutations in our case series were commonly associated with follicular-patterned thyroid lesions. Our data suggest that FAs with a RAS mutation may constitute precursor lesions for TC with follicular histology. The newly-discovered KRAS codon 59 deletion is one of the first reported codon deletions in a RAS hotspot region.*

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The rat sarcoma viral oncogenes homolog (RAS) genes *HRAS*, *KRAS* and *NRAS*, are crucial effector molecules in a number of signalling cascades including the mitogen-activated protein kinase (MAPK), the phophatidylinositol 3-kinase (PI3K) and the Ral guanine nucleotide exchange factor (RalGEF) pathways (1). The RAS molecules transmit mitogen signals from tyrosine kinase membrane receptors *via* downstream effectors to transcription factors that ultimately regulate gene expression (2, 3). The RAS genes encode small GTPases that are bound in the inactive stage to GDP and in the active stage to GTP. GEFs and GTPase activating proteins (GAPs) promote stage switching: GEFs support the release of GDP that enables GTP to bind, leading to conformational change of the two switch regions from the inactive to the active stage. Mutations in RAS codons 12, 13, and 61 affect the GTPase activity resulting in a constitutively activated state (4). Of all RAS mutations, 99% target either codons 12, 13 or 61 with variable preference, for instance 66% of all *KRAS* mutations affect codon 12, whereas 95% of all *NRAS* mutations affect codon 61 (5).

KRAS is one of the most commonly mutated molecules in cancer, reflecting its critical key regulatory functions, not only for neoplastic but also for normal development, as findings in mice indicate that *KRAS*, in its splice variant encoding K-RAS4B, is indispensable for normal development (6, 7). Yet in some tumor types, including follicular TC (FTC) and anaplastic TC, *NRAS* is preferentially mutated whereas in Hurthle cell variant of FTC, and medullary TC (MTC), *HRAS* is the predominately mutated variant (8, 9). A variety of factors, including embryological origin of tissue, differential interactions with regulators/effectors, mode of intracellular processing and subcellular location, may contribute to tumour type specificity of the different RAS isoform mutations (4). Mutations outside the hotspot regions of codons 12, 13 and 61 are far less frequent and preferentially target the C-terminal, membrane-oriented domain; however, *NRAS* point-mutations at the arginine of codon 97 (R97) affecting the allosteric site of lobe 2 have been reported (5, 10). The aim of the current study was to delineate the frequency, distribution, and clinicopathological impact of RAS mutations in proliferative thyroid lesions originating in an ethnically diverse population. The cases were previously characterized by their v-raf murine sarcoma viral oncogenes homolog B (*BRAF*) mutational status, revealing the highest prevalence for *BRAF* mutations in papillary TC (PTC) (~63%) (11). BRAF is the major downstream effector of the RAS molecules within the MAPK signalling pathway.

Materials and Methods

Proliferative thyroid lesions. We examined 381 cases of proliferative thyroid lesions from 376 patients which were treated surgically in the period between January 1995 to June 2011 at the King Abdulaziz University Hospital, Jeddah, and the King Faisal Specialist Hospital and Research Center, Jeddah, Saudi Arabia, or were referral/consultant cases from other regional hospitals (11). For five patients, non-cancerous as well as cancerous thyroid lesions were evaluated in this study. Sixty one percent were Saudi Arabian patients, 27% originated from other Middle East and North African countries, 8% originated from other world regions, and nationality was not reported for 4%. Histopathological diagnosis and staging of thyroid lesions was performed by an oncologic pathologist (JM) according to established criteria (12, 13). A detailed overview of demographic and clinicopathological data is provided elsewhere (11). The young mean age of the patients in our case series can be attributed to the young population structure of the region. This study was approved by the Research Ethics Committee of the King Abdulaziz University, Faculty of Medicine (#358-10), and the Institutional Review Board of the King Faisal Specialist Hospital and Research Center (#IRB2010-07). Written informed consent for participation in the study was obtained from prospective study participants.

Mutational screening. Only specimens with no-less than 70% of proliferative thyroid cells were included in the mutational screening. Genomic DNA was extracted in the majority of cases from 10-μm sections of formalin-fixed and paraffin-embedded (FFPE) material and in a minority of cases (n=116) from freshly-preserved specimens according to established protocols. The standard PCR protocol including primer sequences for *HRAS*, *KRAS* and *NRAS* codons 12, 13 and 61 was described earlier (14). For mutational screening of the putative hotspot region of codon 97, primers were used to amplify the 3' region of *NRAS* coding exon 2 and the 5' region of *NRAS* coding exon 3. The primer sequences were as follows: *NRAS*-ex2-97-F: GGTGAAACCTGTTTGTTGG, *NRAS*-ex2-97-R: TTCCCTA GTGTGGTAACC, *NRAS*-ex3-97-F: AAACTCCTGGGTTCAAGC, *NRAS*-ex3-97-R: TAACTCTTGGCCAGTTCG. For this mutational screening, 36 follicular variants of PTC (FVPTCs) were selected based on sufficient DNA availability. PCR products were confirmed by electrophoresis on 2% agarose gels. Purified PCR products were subjected to cycle sequence reactions using nested primers overlapping with the PCR primers and BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Purified sequencing products were finally resolved by capillary electrophoresis on an ABI PRISM 3130 Sequencer. Sequences were screened for mutations using a combination of manual readout of electropherograms and the NCBI's online BLAST database (15). Sequence results for *NRAS* codon 61 were of insufficient quality in one NWDTC and were excluded from analysis.

Protein structure prediction. The iterative threading assembly refinement (I-TASSER) server for automated protein structure and function prediction was used to model for entire protein structures of wild-type and mutant *KRAS* (16). I-TASSER employs state-of-the-art algorithms and displays three dimensional structures in pdb format. Figures of aligned protein structures were made by using PyMOL (17), which performs sequence alignment followed by structural alignment and refinement.

Results

Hotspot regions in coding exons 1 and 2 of *HRAS, KRAS*, and *NRAS* were screened for mutations in 381 cases of proliferative thyroid lesions. In addition, the putative *NRAS* hotspot region encompassing codon 97 that is split between its second and third base into coding exons 2 and 3 was sequenced in 36 FVPTCs. The entire case series comprised of 10 Hashimoto's thyroiditis (HT) lesions, 46 nodular goiters, 18 hyperplastic nodules, 57 follicular adenomas (FAs), 12 Hurthle cell variants of FA, 115 PTCs, 42 FVPTCs, 56 microcarcinomas of PTC (micro PTCs; tumor size ≤1 cm), 10 FTCs, seven Hurthle cell variants of FTC, and eight NWDTCs (Table I).

RAS mutations were most commonly identified in tumors with follicular-patterned histology including FAs (16 mutations out of 57 cases) and FVPTCs (8 mutations out of 42 cases) (Table I). Fifty-three percent (20 out of 38 mutations) of all detected RAS mutations affected *NRAS* codon 61 and 32% (12 out of 38 mutations) affected *KRAS* codon 61 (Table I). We identified only three mutations RAS codons 12 and 13 in our study, affecting *HRAS* codon 13 and *KRAS* codon 12 in two goiters and *KRAS* codon 13 in an FVPTC. The most common type of mutation was a glutamine-to-arginine substitution at *NRAS* codon 61 (15

Table I. *HRAS, KRAS, NRAS and BRAF mutational status in 381 cases of proliferative thyroid lesions.*

Thyroid lesion	Number of cases	*BRAF*[a] Mutations		RAS Mutations		*HRAS*		*KRAS*		*NRAS*	
		N	(%)	N	(%)	c12/13	c61	c12/13	c61	c12/13	c61
HT	10	0	(0)	0	(0)	0	0	0	0	0	0
Goiter	46	0	(0)	3	(6.5)	1 GGT→CGT (Arg)	0	1 GGT→GAT (Asp)	0	0	1 CAA→AAA (Lys)
Hyperplastic	18	0	(0)	1	(5.6)	0	1 CAG→CGG (Arg)	0	0	0	0
FA	57	1	(1.8)	16	(28.1)	0	3 CAG→CGG (Arg) 1 CAG→AAG (Lys)	0	1 A59del 1 CAA→CGA (Arg)	0	8 CAA→CGA (Arg) 2 CAA→AAA (Lys)
Hurthle cell FA	12	0	(0)	1	(8.3)	0	1 CAG→CGG (Arg)	0	0	0	0
FTC	10	1	(10.0)	2	(20.0)	0	1 CAG→CGG (Arg)	0	0	0	1 CAA→AAA (Lys)
Hurthle cell FTC	7	0	(0)	0	(0)	0	0	0	0	0	0
PTC	115	72	(62.6)	2	(1.7)	0	0	0	1 CAA→CGA (Arg)	0	1 CAA→CGA (Arg)
FVPTC	42	7	(17.0)	8	(19.0)	0	1 CAG→CGG (Arg)	1 GGC→CGC (Arg)	0	0	5 CAA→CGA (Arg) 1 CAA→AAA (Lys)
Micro PTC	56	10	(17.9)	3	(5.4)	0	2 CAG→CGG (Arg) 1 CAG→AAG (Lys)	0	0	0	0
NWDTC	8	1	(12.5)	2	(25)	0	1 CAG→CGG (Arg)	0	0	0	1 CAA→CGA (Arg)

FA: Follicular adenoma; FTC: follicular thyroid carcinoma; FVPTC: follicular variant of papillary TC; HT: Hashimoto`s thyroiditis; micro PTC: microcarcinoma of PTC, tumor size ≤1 cm; NWDTC: non-well differentiated TC; [a]*BRAF* mutations as reported in (11), wherein Hurthle cell variants of FA were grouped with FAs and Hurthle cell variants of FTC with FTCs; c12/13, codons 12/13; c61, codon 61.

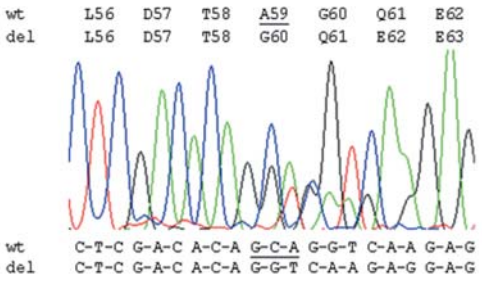

Figure 1. *A new 3-base-pair deletion at KRAS codon 59 identified in a follicular adenoma results in deletion of alanine 59 (p.A59del). Nucleotides and codon affected by the deletion are underlined.*

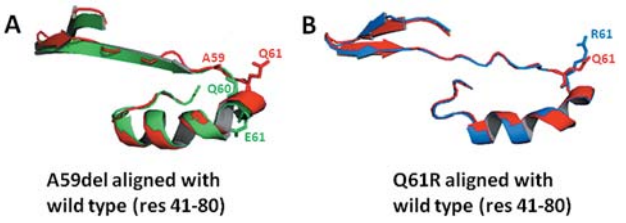

Figure 2. *Homology modeling of KRAS residues 41-80 including, highlighted side chains at the critical mutational region. Structural models of the entire KRAS proteins (wild-type, A59del, and Q61R) were predicted by I-TASSER and taken as templates. Figures made by PyMOL. A: A59del (green) aligned with wild-type (red). B: Q61R (blue) aligned with wild-type (red).*

CAA→CGA; Q61R) followed by a glutamine-to-arginine substitution at *HRAS* codon 61 (10 CAG→CGG; Q61R). One FA exhibited an almost hemi/homozygous *NRAS* codon 61 mutation. An as yet undescribed *KRAS* mutation resulting in deletion of codon 59 (A59del) was identified in an FA (Figure 1). This mutation was confined to the tumour. Sequence analysis of *NRAS* codon 97 disclosed no mutation in the analysed FVPTCs.

Patients with a RAS mutation in an FA did not differ significantly from other patients with an FA in terms of basic demographic and clinicopathological data including mean age (~38 years), gender distribution (~3 females:1 male), and tumor size (~3.5 cm). Distribution of tumor stages in TCs harbouring a RAS mutation was as follows: stage I: 3 micro PTCs, 2 FTCs, 1 PTC and 6 FVPTCs; stage II: 1 FVPTC; stage III: 1 FVPTC; stage IV: 1 PTC and 2 NWDTCs. All RAS and *BRAF* mutations proved to be mutational exclusive.

Discussion

Gain-of-function mutations in the hotspot regions of the RAS genes in the vast majority of cases affect either codon 12, 13, or 61, or, with far lesser prevalence, codon 59. These mutations result in inhibition of GTP hydrolysis, either by diminishing GTPase activity, or, in the case of codon 59 point-mutations, by modulating the rate of guanine nucleotide exchange (4). Although the frequency of RAS mutations varies among different cancer types, a number of studies has shown that mutations in *NRAS* codon 61 predominate in follicular thyroid lesions over other RAS mutations (5, 18, 19). In particular, a glutamine-to-arginine substitution (CAA→CGA; Q61R) is the most common form, followed by a glutamine to lysine substitution (CAA→AAA; Q61K) (5, 20). *In vivo* experiments demonstrated that GTP hydrolysis is strongly inhibited by direct RAS/RAF interaction in Q61L and Q61K mutants, resulting in dysregulation of the pathway (21). The dominant mutational variant in thyroid tumors, Q61R, exhibits a transforming capacity similar to Q61L and Q61K mutants (22).

In comparison to *BRAF* mutations, which are virtually absent in non-malignant thyroid lesions (Table I), RAS mutations occur with varying frequency in malignant as well as non-malignant thyroid lesions and are preferentially associated with follicular histology. Consistent with this, RAS mutations, and in particular *KRAS* and *NRAS* codon 61 mutations, in PTC are typically associated with the follicular variant (20, 23-25). A molecular genetics study on FVPTC has shown that this variant shares genetic alterations with both FTC and conventional PTC, *i.e.* mutations involving *BRAF* exon 15 and *NRAS* codon 61, as well as gene fusions of rearranged during transfection (*RET*) with different partner genes and gene fusions of paired box-8 with peroxisome proliferator-activated receptor gamma (*PAX8-PPARγ*) (26). A recent population-based survey on the clinical behaviour of FVPTC found that this variant accounts for about one third of all PTCs and represents a clinically unique entity, with features shared with PTC and FTC (27).

The association of RAS mutations with progressive tumour features is controversially discussed. Whereas a number of studies found correlation of RAS mutations with tumour progression in FTC and PTC (18, 28), other studies correlated RAS mutations with encapsulated rather than with infiltrative FVPTC (20, 25, 29). In contrast, *BRAF* mutations are commonly associated with infiltrative tumours (25). The association of RAS mutations with clinicopathological features in NWDTC seems to be different as these tumors are frequently aneuploidous (30). In the case of radioiodine-refractory thyroid tumours, the RAS mutational status may gain clinical importance as those with a RAS mutation are likely to respond well to the newly-developed MAPK kinase (MEK)1 and MEK2 inhibitor Selumetinib (31).

No HT was found to have a RAS mutation in our case series. These findings coincide with those of other studies

indicating that HT can be clearly distinguished from PTC by the absence of any RAS mutation (32, 33). The frequency of RAS mutations in histologically-examined nodular goiters varies between the studies, *i.e.* 0% (out of 49 cases) (34-36), 4% (1/25 *NRAS* c61) (37), 7% (1/46 *HRAS* c13, 1/46 *KRAS* c12, 1/46 *NRAS* c61) in this study, 10% (1/10 *KRAS* c13) (38), 21% (4/19 *HRAS* c12) (39), 25% (1/5 *KRAS* c13) (40). Of note, in contrast to thyroid tumors with follicular histology, the majority of goiters in these studies exhibited mutations in RAS codons 12 and 13 , *i.e.* five *HRAS* and three *KRAS* mutations in codons 12 and 13 in contrast to two *NRAS* codon 61 mutations.

The molecular pathogenesis of FA and its role in histopathogenesis of TC has not been fully-elucidated yet. It has been hypothesized that a subset of FAs bearing atypical nuclear features of TC with follicular histology and are characterized by NRAS codon 61 mutations may be regarded as precursor lesions of their malignant counterparts (41). Yet studies directly comparing the frequency of RAS mutations amongst thyroid lesions with follicular histology are rare (19, 42). We assume that FAs with a RAS mutation may represent precursor lesions for their malignant counterparts; however, a fraction of FVPTCs harbouring a *BRAF* mutation (17% in our study) may not develop through the FA sequence and undergo different histopathogenesis.

The newly-identified *KRAS* A59del in an FA in our study series affects the GTPase domain. Mutations in *KRAS* codon 59 are found sporadically in a number of solid and haematological types of cancer and are commonly point-mutations resulting in A59E, A59T, or A59G substitutions (8). The *KRAS* A59del places the glycine residue of codon 60 into the position of codon 59 and the glutamate residue of codon 62 into the position of codon 61. Further studies are needed to reveal if these substitutions bear similar functional properties as the known *KRAS* A59G and Q61E point-mutations. An initial protein structure and homology alignment prediction assumes that the orientation of side chains of critical amino acids in the mutational region is impaired in the A59del variant (Figure 2).

Conclusion

In conclusion, RAS mutations, and in particular *NRAS* codon 61 mutations, are preferentially associated with follicular-patterned thyroid lesions, supporting the hypothesis that benign variants with a RAS mutation may represent precursor lesions for their malignant counterparts. In view of the fact that RAS mutations are common in follicular-patterned TCs, screening for RAS mutations in patients with advanced tumors may advance clinical relevance. The novel *KRAS* A59del detected in an FA is one of the first single codon deletions reported so far for a mutational hotspot region of a RAS gene.

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