BRAF and *K-RAS* Mutation in a Greek Papillary and Medullary Thyroid Carcinoma Cohort

NIKOLAOS GOUTAS[1], DIMITRIOS VLACHODIMITROPOULOS[1], MYRTO BOUKA[2], ANDREAS C. LAZARIS[3], GEORGE NASIOULAS[4] and MARIA GAZOULI[2]

[1]Histopathology Department, Evgenidion Hospital, University of Athens, Athens; Departments of [2]Biology and [3]Pathology, School of Medicine, University of Athens, Athens; [4]Molecular Biology Research Center HYGEIA "Antonis Papayiannis", Athens, Greece

Abstract. *Background: The genes RAS and BRAF have been shown to be frequently mutated in human thyroid carcinomas. The aim of this study was to genotype a cohort of 55 sporadic papillary thyroid carcinomas (PTC) and 44 sporadic medullary thyroid carcinomas (MTC) for the K-RAS codon 12 and BRAF codon 600 mutations. Materials and Methods: K-RAS and BRAF mutations were characterized by an enhanced polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR-RFLP). Results: The K-RAS codon 12 mutation was found in 54.5% of the PTC and 40.9% of the MTC cases tested. The BRAF V600E mutation was detected in 27.3% of the PTC and 68.2% of the MTC samples. No significant association between K-RAS and BRAF mutations and clinicopathological parameters was found. Conclusion: These data indicate that K-RAS and BRAF mutations were a frequent genetic event in our samples of sporadic PTC and MTC.*

Thyroid cancer is the most common endocrine malignancy. It is histologically classified as papillary thyroid cancer (PTC), follicular (FTC), anaplastic (ATC) and medullary (MTC), and accounts for approximately 80%, 15%, 2% and 3% of thyroid malignancies, respectively (1). PTC, FTC and ATC derive from follicular thyroid epithelial cells, whereas MTC derives from calcitonin-secreting parafollicular C cells.

Both genetic and epigenetic alterations have been shown to play an important role in the initiation and progression of thyroid carcinomas. Over the past two decades, various genetic alterations have been identified in different thyroid neoplasms. The classical genetic alterations commonly seen in thyroid cancer include *Ras* mutations (2), *RET*/*PTC* rearrangements (3) and *PAX*8-peroxisome proliferator-activated receptor γ (PPARγ) fusion oncogene (4). Several activating *Ras* mutations, also widely found in other cancers, occur mainly in FTC and PTC (5, 6). *RET* mutations are primarily responsible for familial MTC and some sporadic MTC (7, 8). Recently, mutations of *BRAF*, both point mutations (9) and gene rearrangements (10), have been identified. Specifically, an apparently unique mutation V600E (formerly designated V599E) amino acid substitution, has been described in more than 800 PTCs so far, with a prevalence of 20-69% in the different studies (11). Interestingly, *BRAF* mutations are reported to be restricted to PTC and to anaplastic or poorly differentiated carcinomas arising from papillary carcinomas (12). However, even if the role of *RAS* and *BRAF* mutations is well established in PTC cases, it is not known whether *RAS* and *BRAF* mutations also play a role in the tumorigenesis of other types of thyroid neoplasms, such as ATC, benign hyperplasia and MTC.

In this paper, a genotype study of *K-RAS* and *BRAF* gene mutations in a Greek cohort of sporadic PTC and MTC carcinomas is reported.

Materials and Methods

Human thyroid tissues. Paraffin-embedded tumor blocks from thyroidectomy specimens of patients with thyroid neoplasms were retrieved for analysis of *K-RAS* and *BRAF* gene mutations upon approval of the hospital's Ethics Committee. All the patients signed an informed consent form in which they approved the sample collection to be used for genetic analyses. A total of 99 specimens were studied. These included 55 PTC (45 female and 10 male, mean age 43.54±12.98 years), and 44 MTC samples (33 female and 11 male, mean age 50.93±15.18 years).

Genotyping. Genomic DNA from paraffin-embedded tumor blocks was extracted using a commercial kit (Nucleospin Tissue, Macherey-Nagel, Germany). The presence of a mutation at codon 12 of *K-RAS* was determined by an enriched polymerase chain reaction followed by restriction fragment length polymorphism

Correspondence to: Maria Gazouli, Ph.D., Department of Biology, School of Medicine, University of Athens, Michalakopoulou 176, 11527 Goudi, Athens, Greece. Tel: +30 210 7462231, e-mail: mgazouli@med.uoa.gr

Key Words: BRAF, K-RAS, mutation, papillary, medullary, thyroid carcinoma.

analysis (PCR-RFLP) according to the method originally described by Kahn *et al.* (13) and modified by Nagasaka *et al.* (14). Mutation at codon 600 of *BRAF* was screened by an enriched PCR-RFLP analysis, as described by Nagasaka *et al.* (14).

Statistical analysis. All statistical calculations were made using the GraphPad InStat software (version 3.00; GraphPad Software, Inc., San Diego, CA, USA). Differences in frequencies were evaluated by the Chi-square test or Fisher's exact test, where appropriate. All reported *p*-values are two-sided and a *p*-value of <0.05 was considered to be statistically significant.

Results

Mutation of codon 12 of *K-RAS* was found in 30 of the 55 PTC (54.5%) and in 18 of the 44 MTC (40.9%) samples tested. All the mutated samples were heterozygous. The V600E mutation of *BRAF* was found in 15 of the 55 PTC (27.3%) and in 30 of the 44 (68.2%) MTC samples tested. Only one of the mutated samples was homozygous. Concordant mutations of *BRAF* and *K-RAS* were found in 8 PTC and in 5 MTC samples. Analysis of the unaffected thyroid tissue demonstrated the somatic origin of the mutations.

As shown in Table I, no significant correlation between *K-RAS* and *BRAF* mutations and clinicopathological parameters was found.

Discussion

It is well-known that *K-, H-* and *N-RAS* genes are activated at a relatively high frequency in human thyroid cancers. *K-, H-* and *N-RAS* point mutations in PTCs have been described with a prevalence of 20-30% in different studies (15, 16), whereas the limited studies carried out so far failed to find *RAS* mutations in MTCs (17, 18). Our findings concerning PTCs are in agreement with several studies conducted in North American and other European populations (6, 19, 20), but diverged from the results of an Italian series (21, 22). Regarding MTCs, in contrast with previous studies (17, 18), we found a high frequency of the *K-RAS* codon 12 point mutation in our samples (40.9%). Less is known about MTCs, but our results suggest that *K-RAS* might play an important role in the carcinogenesis of sporadic MTC.

Concerning the *BRAF* gene, our study demonstrated that mutation at the hot-spot codon 600 occurred in 27.3% and in 68.2% of PTCs and MTCs, respectively. Regarding the PTC cases, our data confirm previous findings (9, 19, 22) and provide evidence for a leading role of the *BRAF* gene in the pathogenesis of sporadic PTC. However, our results are in sharp contrast to the limited previous studies which did not find any *BRAF* mutation in MTC (23). Since there are few studies examining the frequency of *K-RAS* and *BRAF* mutations in sporadic MTCs, it is possible to speculate that other genetic factors and/or environmental setting may account for these significant differences in mutation prevalence in this type of thyroid carcinoma. More ad hoc-designed molecular epidemiological studies are needed to clarify these aspects.

As far as the genotype-phenotype correlation is concerned, discordant and scant data were generated in previous studies (12, 24). Indeed, Nikiforova *et al.* (12) reported that an older age and a higher stage, and not the presence of lymph-nodal and distant metastases, are associated with *BRAF* mutation. In a comparable series by Namba *et al.* (24), *BRAF* mutation was found to be slightly correlated with the stage, and, more significantly, with distant metastases, but not with age. Additionally, Garcia-Rostan *et al.* (25) reported that *RAS* mutations are a marker for an aggressive thyroid cancer phenotype and indicate a possible benefit of *RAS* genotyping to identify thyroid carcinoma subsets associated with poor prognosis. On the other hand, Capella *et al.* (26) suggested that *RAS* mutations are not related to thyroid cancer prognosis. In view of these controversial data, it is difficult to indicate any possible association between *RAS* and *BRAF* mutation and age, gender or aggressiveness of the tumor. The results obtained in the present study do not allow us to confirm any of the reported correlation. Indeed, in our cases, no statistically significant differences in age, gender, stage or metastasis between *RAS* and/or *BRAF* mutated and nonmutated PTCs and MTCs were found.

In conclusion, our results, together with previous genetic studies on thyroid neoplasms, are consistent with the concept that thyroid carcinomas inherently harbor a *BRAF* mutation which, together with mutations in *RAS, RET* as well as *RET/PTC* rearragements, are all implicated in the Ras→Raf→MEK→MAP/ERK signaling pathway leading to cell growth, proliferation and transformation, and this demonstrate a common link between all the thyroid neoplasms.

References

1 Hundahl SA, Fleming ID, Fremgen AM and Menck HR: National cancer data base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985-1995. Cancer *83*: 2638-2648, 1998.

2 Bongarzone I and Pierotti MA: The molecular basis of thyroid epithelial tumorigenesis. Tumori *89*: 514-516, 2003.

3 Tallini G: Molecular pathobiology of thyroid neoplasms. Endocr Pathol *13*: 271-288, 2002.

4 McIver B, Grebe SK and Eberhardt NL: The *PAX8/PPAR gamma* fusion oncogene as a potential therapeutic target in follicular thyroid carcinoma. Curr Drug Targets Immune Endocr Metabol Disord *4*: 221-234, 2004.

5 Vasko V, Ferrand M, Di Cristofaro J, Carayon P, Henry JF and de Micco C: Specific pattern of *RAS* oncogene mutations in follicular thyroid tumors. J Clin Endocrinol Metab *88*: 2745-2752, 2003.

Table I. *Correlation between KRAS and BRAF mutations and various clinicopathological parameters in papillary and medullary thyroid cancers.*

Papillary thyroid cancer	*K-RAS* codon 12 mutation				*p*	*BRAF* mutation				*p*
	Positive		Negative			Positive		Negative		
	n	(%)	n	(%)		n	(%)	n	(%)	
Age										
< 45 years	15	50	12	48	1.000	6	40	20	50	0.557
≥ 45 years	15	50	13	52		9	60	20	50	
Gender										
Female	25	83.3	20	80	1.000	13	86.7	32	80	0.710
Male	5	16.7	5	20		2	13.3	8	20	
Tumor size										
<10 mm	10	33.3	9	36	0.649	7	46.6	12	30	0.106
10-40 mm	19	63.3	16	64		7	46.6	28	70	
>40 mm	1	3.3	0	0		1	6.6	0	0	
Stage										
I	9	30	7	28	0.734	6	40	9	22.5	0.401
II	14	46.7	14	56		7	46.7	22	55	
III	7	23.3	4	16		2	13.3	9	22.5	
IV	0	0	0	0		0	0	0	0	
Nodal metastasis										
No	24	80	21	84	0.741	13	86.7	32	80	0.710
Yes	6	20	4	16		2	13.3	8	20	
Medullary thyroid cancer	*K-RAS* codon 12 mutation				*p*	*BRAF* mutation				*p*
	Positive		Negative			Positive		Negative		
	n	(%)	n	(%)		n	(%)	n	(%)	
Age										
<45 years	7	38.9	14	53.9	0.372	14	46.7	5	35.7	0.534
≥45 years	11	61.1	12	46.1		16	53.3	9	64.3	
Gender										
Female	13	72.2	20	76.9	0.737	23	76.7	10	71.4	0.722
Male	5	27.8	6	23.1		7	23.3	4	28.6	
Tumor size										
<10 mm	5	27.8	9	34.6	0.342	10	33.3	4	28.6	0.708
10-40 mm	10	55.5	16	61.5		18	60	8	57.1	
>40 mm	3	16.7	1	3.9		2	6.7	2	14.3	
Stage										
I	5	27.8	9	34.6	0.567	10	33.3	5	35.7	0.879
II	6	33.3	5	19.2		7	23.3	4	28.6	
III	7	38.9	12	46.2		13	43.3	5	35.7	
IV	0	0	0	0		0	0	0	0	
Nodal metastasis										
No	24	80	21	84	0.741	18	60	6	42.9	0.342
Yes	6	20	4	16		12	40	8	57.1	

6 Zhu Z, Gandhi M, Nikiforova MN, Fischer AH and Nikiforov YE: Molecular profile and clinical-pathologic features of the follicular variant of papillary thyroid carcinoma. An unusually high prevalence of *ras* mutations. Am J Clin Pathol *120*: 71-77, 2003.

7 Clayman GL and el-Baradie TS: Medullary thyroid cancer. Otolaryngol Clin North Am *36*: 91-105, 2003.

8 Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C, Eng C and Freiburg-Warsaw-Columbus Pheochromocytoma Study Group: Germline mutations in nonsyndromic pheochromocytoma. N Engl J Med *346*: 1459-1466, 2002.

9 Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, Beller U, Westra WH, Ladenson PW and Sidransky D: *BRAF* mutation in papillary thyroid carcinoma. J Natl Cancer Inst *95*: 625-627, 2003.

10 Ciampi R, Knauf JA, Kerler R, Gandhi M, Zhu Z, Nikiforova MN, Rabes HM, Fagin JA and Nikiforov YE: Oncogenic *AKAP9-BRAF* fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. J Clin Invest *115*: 94-101, 2005.

11 Xing M: *BRAF* mutation in thyroid cancer. Endocr Relat Cancer *12*: 245-262, 2005.

12 Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, Zhu Z, Giannini R, Salvatore G, Fusco A, Santoro M, Fagin JA and Nikiforov YE: *BRAF* mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J Clin Endocrinol Metab *88*: 5399-5404, 2003.

13 Kahn SM, Jiang W, Culbertson TA, Weinstein IB, Williams GM, Tomita N and Ronai Z: Rapid and sensitive nonradioactive detection of mutant *K-ras* genes *via* 'enriched' PCR amplification. Oncogene *6*: 1079-1083, 1991.

14 Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, Kambara T, MacPhee DG, Young J, Leggett BA, Jass JR, Tanaka N and Matsubara N: Colorectal cancer with mutation in *BRAF, KRAS,* and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol *22*: 4584-4594, 2004.

15 Karga H, Lee JK, Vickery AL, Thor A, Gaz R and Jameson JL: *RAS* oncogene mutations in benign and malignant thyroid neoplasms. J Clin Endocrinol Metab *73*: 832-836, 1991.

16 Figge L: Molecular pathogenesis of thyroid cancer. *In*: Thyroid Cancer: A Comprehensive Guide to Clinical Management. Wartofsky L. (ed.). Humana Press, Totowa New Jersey, pp. 57-75, 2000.

17 Bockhorn M, Frilling A, Kalinin V, Schrader S and Broelsch CE: Absence of *H-* and *K-ras* oncogene mutations in sporadic medullary thyroid carcinoma. Exp Clin Endocrinol Diabetes *108*: 49-53, 2000.

18 Moley JF, Brother MB, Wells SA, Spengler BA, Biedler JL and Brodeur GM: Low frequency of *ras* gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers. Cancer Res *51*: 1596-1599, 1991.

19 Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE and Fagin JA: High prevalence of *BRAF* mutation in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signalling pathway in papillary thyroid carcinoma. Cancer Res *63*: 1454-1457, 2003.

20 Namba H, Rubin SA and Fagin JA: Point mutation of *ras* oncogenes are an early event in thyroid tumorigenesis. Mol Endocrinol *4*: 1474-1479, 1990.

21 ManentiG, Pilotti S, Re FC, Della Portaa G and Pierotti MA: Selective activation of *ras* oncogenes in follicular and undifferentiated thyroid carcinoma. Eur J Cancer *30A*: 987-993, 1994.

22 Carta C, Moretti S, Passeri L, Barbi F, Avenia N, Cavaliere A, Monacelli M, Macchiarulo A, Santeusanio F, Tartaglia M and Puxeddu E: Genotyping of an Italian papillary thyroid carcinoma cohort revealed high prevalence of *BRAF* mutations, absence of *RAS* mutations and allowed the detection of a new mutation of *BRAF* oncoprotein ($BRAF^{V599Ins}$). Clin Endocrinol *64*: 105-109, 2006.

23 Xing M, Vasko V, Tallini G, Larin A, Wu G, Udelsman R, Ringel MD, Ladenson PW and Sidransky D: *BRAF* T1796A transversion mutation in various thyroid neoplasms. J Clin Endocrin Metab *89*: 1365-1368, 2004.

24 Namba H, Nakashima M, Hayashi T, Hayashida N, Maeda S, Rogounovitch TI, Ohtsuru A, Saenko VA, Kanematsu T and Yamashita S: Clinical implication of hot spot *BRAF* mutation, V599E, in papillary thyroid cancers. J Clin Endocrinol Metab *88*: 4393-4397, 2003.

25 Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A, Pardo J, Wu R, Carcangiu ML, Costa J and Tallini G: Ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. J Clin Oncol *21*: 3226-3235, 2003.

26 Capella G, Matias-Guiu X, Ampudia X, de Leiva A, Perucho M and Prat J: *Ras* oncogene mutations in thyroid tumors: polymerase chain reaction-restriction-fragment-length polymorphism analysis from paraffin-embedded tissues. Diagn Mol Pathol *5*: 45-52, 1996.

Received September 10, 2007
Revised November 16, 2007
Accepted December 14, 2007