JCEM ONLINE

Advances in Genetics—Endocrine Research

H-RAS Mutations Are Restricted to Sporadic Pheochromocytomas Lacking Specific Clinical or Pathological Features: Data From a Multi-Institutional Series

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Context: Somatic or germline mutations in up to 15 disease-causative genes are detectable in up to 50% of patients with pheochromocytoma (PCC) and paraganglioma (PGL). Very recently, somatic *H-RAS* mutations were identified by exome sequencing in approximately 7% in sporadic PCCs and PGLs, in association with male sex and benign behavior.

Objective: To explore the prevalence of RAS mutations in a cohort of 271 PCC and PGL from a European registry and to compare the genotype with clinical and pathological characteristics of potential clinical interest.

Setting and Design: Genetic screening for hotspot mutations in *H-*, *N-*, and *K-RAS* genes was performed by means of Sanger sequencing or pyrosequencing methods on tumor DNA in a series of patients with (n = 107) or without (n = 164) germline or somatic PCC/PGL-related gene mutations.

Results: Overall, *H-RAS* mutations were detected in 5.2% of cases (14/271), which were confined to sporadic PCCs resulting in a prevalence of 10% (14/140) in this cohort. In contrast, no mutations were found in PCC with PCC/PGL-related gene mutations (0/76) or in PGL (0/55) harboring or not mutations in PCC/PGL susceptibility genes. In this large series, *H-RAS* mutations in PCCs lacked any significant correlation with pathological or basic clinical endpoints.

Conclusions: Somatic *H-RAS* mutations are restricted to a relevant proportion of sporadic PCC. These findings provide the basis to study potential *H-RAS*-dependent correlations with long-term outcome data. **(*J Clin Endocrinol Metab* 99: E1376–E1380, 2014)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received October 23, 2013. Accepted March 19, 2014.
First Published Online March 31, 2014

Abbreviations: PCC, pheochromocytoma; PGL, paraganglioma.

Pheochromocytoma (PCC) and paraganglioma (PGL) are neural crest-derived tumors, arising from chromaffin cells of the adrenal medulla or from extra-adrenal paraganglia (1). Approximately 50% of all PCC/PGL patients harbor mutations, either somatic or germline, in one of the 15 disease-causative genes: *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *RET*, *VHL*, *NF1*, *MAX*, *TMEM127*, *HIF2A, FH*, *KIF1B*, *PHD2*, and *IDH* (2–9). Despite this genetic heterogeneity, the tumors can be divided into two groups based on transcription profiling studies. Cluster 1 includes tumors with mutations in *VHL*, *HIF2A,* and *SDHx* (*SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*) and displays a pseudohypoxic signature; cluster 2 represents tumors with mutations in *RET*, *NF1*, *TMEM127*, and *MAX* and displays an activation of kinase-signaling pathways (PI3K/AKT/mTOR and RAS/RAF/ERK). Sporadic PCC and PGL can cluster within both groups (8, 10–12).

Recently, somatic *H-RAS* mutations in sporadic PCCs and PGLs were identified by exome sequencing, showing a frequency of 6.9% (13), following a previous report by Yoshimoto and coworkers (14). These tumors displayed activation of the RAS/RAF/ERK-signaling pathway and were associated with male predominance and clinically benign behavior.

RAS is a family of related proteins consisting of H-RAS, K-RAS, and N-RAS, which are small GTPases involved in cell growth, proliferation, and survival. *RAS* genes belong to the most common mutated genes in human cancer with oncogenic mutation hotspots found in codons 12, 13, and 61. Whereas RAS GTPase signaling is self limiting due to its intrinsic ability to exchange GTP with GDP-mutant RAS protein is defective for this GTP hydrolysis and remains constitutively active (15). RAS signaling activates the RAF/ERK and PI3K/AKT/mTOR signaling pathways, similar to what has been described in cluster 2 PCCs/PGLs(16).

In the present multi-institutional study, we aimed to establish the prevalence of *H-RAS* mutation in a cohort of PCCs and PGLs already genotyped for the main PCC/PGL susceptibility genes, and correlated the presence of mutations with major clinical and pathological parameters.

Materials and Methods

Patients selection and characteristics

A series of 271 samples was collected from different institutions participating in the European Network for the Study of Adrenal Tumors (ENS@T), www.ensat.org), and included 97 cases from Italy, 39 cases from Spain, 126 from The Netherlands, and nine cases from Germany. All cases were genetically characterized for the presence of germline mutations in the VHL, RET, NF-1, MAX, SDHAF2, SDHA, SDHB, SDHC, and SDHD on either blood or tissue samples, and for the presence of somatic mutations in VHL, RET, EPAS1, and MAX on tumor tissue samples when screening for germline mutations was negative. Genetic screening was performed independently in the enrolling centers as clinical routine work, and methodological conditions are available from the authors upon request.

Among this series, 107 cases, 76 PCCs, and 31 PGLs, harbored somatic or germline mutations in one of the susceptibility genes discussed above (Supplemental Figure 1). The residual 140 PCCs and 24 PGLs lacked germline or somatic mutations in the main PCC and PGL susceptibility genes. All except three patients were diagnosed with a single tumor and none had family history of the disease. Institutional Review Board approval was obtained for the study by each of the centers, and informed consent was obtained from all patients.

Clinical variables collected for this study included sex, age, number of PCC/PGLs, tumor location, tumor size, necrosis, capsular and vascular invasion, mitotic index, as well as presence of metastatic disease. Malignancy was defined as the presence of metastases where chromaffin cells are normally absent. These clinical variables were collected electronically into preformatted forms provided to all contributors and statistically analyzed in a single center.

DNA extraction and *RAS* gene mutations

DNA was extracted from either fresh frozen or formalin-fixed, paraffin-embedded specimens, using standard protocols. Sections of each sample were evaluated by a pathologist and contained at least 80% of tumor cells. All cases, including the series of tumors without known mutations (no. 164) and those with already-known gene mutations (no. 107) were screened for hotspot mutations in *H-*, *N-*, and *K-RAS* genes (exons 2 and 3 for *N-* and *K-* and exons 2, 3, and 4 for *H-RAS*) in three centers (Madrid, Rotterdam, and Turin). Either Sanger sequencing (Madrid and Rotterdam) or pyrosequencing (Turin) methods were employed. For direct sequencing, amplified DNA was purified and directly sequenced using an automatic sequencer ABI PRISM TM 3700 (Applied Biosystems). Pyrosequencing method was applied as previously described (17). PCR and sequencing primers were designed using the PSQ Assay Design Software version 1.0.6 (Biotage AB), and sequencing was performed using a PyroGold reagent Kit (Biotage AB) and analyzed using PSQ-96 MA 2.0.2 software (Biotage AB). Primer sequences not already published for both direct sequencing and pyrosequencing are available as Supplemental Table 1.

Statistical analysis

The correlation between *RAS* mutations and known clinical pathological parameters was assessed by χ^2 and Student *t* tests; $P = .05$ was set as the level of significance. Statistical analysis was performed using GraphPad Prism 4 and SPSS software (IBM).

Results

H-RAS mutations are restricted to sporadic PCCs

H-RAS mutations were detected in 14 cases, all PCCs without germline or somatic mutations in any of the known PCC/PGL susceptibility genes. In contrast, none of

Table 1. Main Clinical and Pathological Features of *H-RAS*-mutated and Wild-Type PCC

Parameter	PCC *H-RAS*	PCC Without Known Mutations	*P* Value
N	14	126	
M/F	5/9	61/65	.27
Median age, y [range]	59 [38–79]	51 [14–81]	.08
Median Size, mm [range]	50 [17–90]	53 [11–200]	.83
Presence of multicentric disease/total	0/14	3/122	1.0
Presence of VI/total	1/13	13/115	1.0
Presence of CI/total	0/14	11/112	.61
Presence of necrosis/total	1/14	19/112	.46
Clinically malignant disease/total	1/13[a]	5/119	.47

Abbreviations: M, male; F, female; VI, vascular invasion; CI, capsular invasion.

[a] One patient died of unknown causes and was considered not informative.

the familial PCCs (n = 76) nor any of PGLs (n = 55) tested positive for *H-RAS* mutations in the present series. Thereby, the overall prevalence of *H-RAS* mutations in this cohort of PCCs/PGLs was 5% (14/271) with 8% (14/164) considering cases without known mutations only, and 10% (14/140) considering PCCs without known mutations, only. The most common mutation was *H-RAS* p.Q61R (12 cases), the remaining two being one *H-RAS* p.Q61K and one *H-RAS* p.G13R mutation. No mutations were found in *H-RAS* exon 4. The corresponding normal tissue of the 14 mutated cases tested in parallel for exons 2 and 3 was negative, thus showing the somatic nature of *H-RAS* mutations. The prevalence of mutations did not significantly differ among centers performing mutation analysis (9.7, 9.1, and 12% in Madrid, Turin, and Rotterdam, respectively, $P = .92$), thus indicating that both methods employed have a similar sensitivity. *N*- and *K-RAS* sequencing did not reveal any disease-causing mutation.

H-RAS mutations in PCCs lack significant clinical or pathological correlations.

The major pathological and clinical features of *H-RAS*-mutated PCC were compared with cases without known mutations (Table 1). *H-RAS*-mutated PCCs showed heterogeneous morphology, without specific growth patterns or cytological features (Figure 1). A female predominance was observed in our series, and the median age was slightly older than PCC cases without known mutations, although without reaching statistical significance. With regard to morphological parameters potentially associated with clinical aggressiveness (such as size, multicentricity, presence of vascular and/or capsular invasion and necrosis), *H-RAS*-mutated PCCs were not significantly different from PCCs without known mutations. Although most cases were clinically benign, one patient died after local recurrence, another patient presented vascular invasion, and another a single necrotic focus, thus not excluding that, as for sporadic PCCs in general, a small proportion of *H-RAS*-mutated PCCs might have a potential malignant biological behavior.

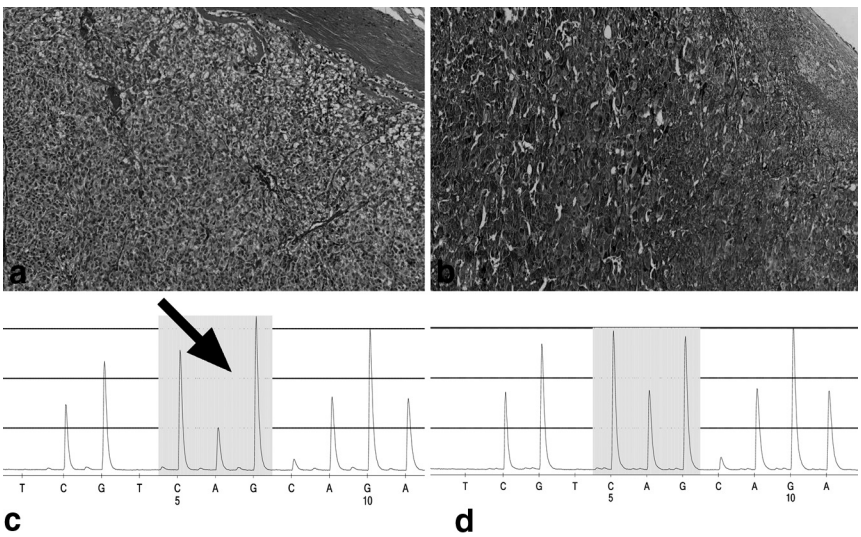

Figure 1. Heterogeneous morphological features of *H-RAS*-mutated PCCs. *H-RAS*-mutated PCC showed heterogeneous cytological and architectural features, with small (A) to large (B) nests of cells having either scant clear/eosinophilic (A) or abundant basophilic (B) cytoplasm. Representative pyrograms showing a mutation in *H-RAS* (C) as compared with wild-type (D) normal adrenal tissue: *H-RAS* p.Q61R mutation, c.182A>G substitution, is demonstrated in (C) by a lower peak corresponding to (A) and a higher peak corresponding to G in the sequence (arrow) as compared with the wild type sequence in (D).

Discussion

Somatic *H-RAS* gene mutations have been reported recently in PCCs and PGLs, thus increasing the number of genetic alterations associated with these tumors. In the article by Crona et al (13), 82 cases were analyzed, including 25 positive for known PCC/PGL susceptibility genes. Four mutated cases were described, one PCCs and one PGL, all sporadic and devoid of mutations in other susceptibility genes. The patients were all males with a benign clinical course. This prompted us to explore the prevalence of RAS mutations in a cohort of 271 PCCs and PGLs from the

ENS@T registry and to compare the genotype with clinical and pathological characteristics of potential clinical interest. In our series, the *H-RAS* gene was found to be mutated exclusively among the *RAS* genes in 5% of PCC/PGLs. All mutated cases were PCCs, clinically sporadic and without mutations in other known PCC/PGL susceptibility genes. Thus, we could confirm the sporadic and the wild-type (for other genes) characteristics of *H-RAS*-mutated tumors, but not the presence of mutations in PGL, because none of the 55 PGLs tested (24 without known mutations and 31 mutated in other genes) was positive. Therefore, the prevalence of *H-RAS* mutations in sporadic PCC without known mutations in other genes should approximate 10% of cases, whereas it seems to be extremely rare (0% in our series) in PGL with the same characteristics.

Clinical and pathological correlations were limited by the small number of *H-RAS*-mutated tumors. However, our series showed, differently from the article by Crona et al, a female predominance and a higher median age (49.5 y in the article by Crona et al, 59 y in our series). These features, however, were not significantly different from sporadic PCCs without *H-RAS* mutation. Moreover, as described in the four *H-RAS* mutant cases reported so far, most the cases in our series did not show an aggressive clinical course. However, one patient died with local recurrence after 9 y; in addition, in two cases, morphological features suspected of potentially malignant behavior (presence of vascular invasion or necrosis) were recorded; a third case had moderate mitotic (three mitoses in 10 high-power fields) index. Moreover, none of the parameters considered was significantly different in the group of *H-RAS*-mutated as compared with wild-type PCCs. Therefore, although the occurrence of metastases at the time of diagnosis or during follow-up has not been described in these tumors, a long-term follow-up is required, especially for patients with clinical predictors of malignancy. Interestingly, the single case associated with local recurrence in our series had a large size, in agreement with previous data in the literature (18).

In contrast, our data confirm that *H-RAS* mutations are among the driver pathogenetic alterations in sporadic PCC. It is worth noticing that, among the different types of cancers harboring somatic *RAS* mutations, a relevant prevalence of *H-RAS* mutations has been documented in sporadic medullary carcinoma (19), although without a specific association with prognosis. Therefore, both PCC and medullary thyroid carcinoma share a similar genetic background but, different from medullary carcinoma, germline or somatic *RET* mutations in PCC are not usually associated with aggressive behavior (20, 21).

All the above observations suggest that *H-RAS*-mutation testing is of potential impact in sporadic PCC and must be validated as a clinically meaningful routine test. In fact, although not significantly associated with a specific clinical behavior, the value of *H-RAS* genotyping to predict therapeutic responsiveness cannot be excluded until its capability to guide systemic therapeutic approaches will be tested in metastatic *H-RAS* positive PCCs.

In conclusion, *H-RAS* mutations are causative for the 10% of PCCs without mutations in other known PCC-related genes and should be considered as part of routine genetic screening when tumor tissue is available to validate its diagnostic, prognostic, or predictive role as a molecular biomarker.

Acknowledgments

We thank Manuel Morente and María Jesús Artiga of the Spanish National Tumor Bank Network (CNIO) for their hard work collecting part of the tumor samples used in this study.

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This work was supported by grants from the University of Turin (ex 60% to M.V. and M.P.) and the Fondo de Investigaciones Sanitarias (project PI11/01359 to M.R.); The research leading to these results has also received funding from the Seventh Framework Programme (FP7/2007–2013) under Grant Agreement No. 259735. L.O. received a support from the European Science Foundation (ESF) within the framework of the ESF activity European Network for the Study of Adrenal Tumors (ENSAT) (Exchange Grant 4202).

I.R. and S.V. are PhD fellows at the University of Turin, Doctorate School of Biomedical Sciences and Oncology. V.M. and A.A.d.C. are predoctoral fellows of "la Caixa"/CNIO international PhD program.

Disclosure Summary: The authors have nothing to disclose.

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