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

Lindsey Oudijk, Ronald R. de Krijger, Ida Rapa, Felix Beuschlein, Aguirre A. de Cubas, Angelo P. Dei Tos, Winand N. M. Dinjens, Esther Korpershoek, Veronika Mancikova, Massimo Mannelli, Mauro Papotti, Simona Vatrano, Mercedes Robledo, and Marco Volante

Department of Pathology (L.O., R.R.d.K., W.N.M.D., E.K.), Erasmus MC University Medical Center, 3015 CN Rotterdam, The Netherlands; Department of Pathology (R.R.d.K.), Reinier de Graaf Hospital, 2625 AD Delft, The Netherlands; Department of Oncology (I.R., M.P., S.V., M.V.), University of Turin at San Luigi Hospital, 10043 Orbassano, Turin, Italy; Endocrine Research Unit (F.B), Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, D-81675 Munich, Germany; Hereditary Endocrine Cancer Group (A.A.d.C., V.M., M.R.), Spanish National Cancer Research Centre (CNIO), 28029 Madrid, Spain; Department of Pathology and Molecular Genetics (A.P.D.T.), General Hospital, 31100 Treviso, Italy; Department of Clinical Pathophysiology (M.M.), University of Florence, 50121 Florence Italy; and Centre for Biomedical Network Research on Rare Diseases (CIBERER) (M.R.), 28029 Madrid, Spain

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)
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The presence of germline mutations in the VHL gene is 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 χ2 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

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.
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.

**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

![Figure 1](https://example.com/figure1.png)

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCC H-RAS</th>
<th>PCC Without Known Mutations</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>5/9</td>
<td>61/65</td>
<td>.27</td>
</tr>
<tr>
<td>Median age, y [range]</td>
<td>59 [38–79]</td>
<td>51 [14–81]</td>
<td>.08</td>
</tr>
<tr>
<td>Presence of multicentric disease/total</td>
<td>0/14</td>
<td>3/122</td>
<td>1.0</td>
</tr>
<tr>
<td>Presence of VI/total</td>
<td>1/13</td>
<td>13/115</td>
<td>1.0</td>
</tr>
<tr>
<td>Presence of CI/total</td>
<td>0/14</td>
<td>11/112</td>
<td>.61</td>
</tr>
<tr>
<td>Presence of necrosis/total</td>
<td>1/14</td>
<td>19/112</td>
<td>.46</td>
</tr>
<tr>
<td>Clinically malignant disease/total</td>
<td>1/13*</td>
<td>5/119</td>
<td>.47</td>
</tr>
</tbody>
</table>

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

* One patient died of unknown causes and was considered not informative.
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.

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Address all correspondence and requests for reprints to: Marco Volante, Pathology Unit, Department of Oncology, University of Turin at San Luigi Hospital, Regione Gonzole 10, 10043, Orbassano, Turin, Italy. E-mail: marco.volante@unito.it.

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Disclosure Summary: The authors have nothing to disclose.

References


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