Immunohistochemical expression of stem cell markers in pheochromocytomas/paragangliomas is associated with SDHx mutations

L Oudijk1,*, C M Neuhofer2,*, U D Lichtenauer2, T G Papatheos3, E Korpershoek1, H Stoop1, J W Oosterhuis1, M Smid3, D F Restuccia1, M Robledo4, AA de Cubas4, M Mannelli5, A P Gimenez-Roqueplo6,7,8, W N M Dinjens1, F Beuschlein2 and RR de Krijger1,9

1Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Postbus 2040, 3000 CA Rotterdam, The Netherlands, 2Endocrine Research Unit, Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Ziemssenstrasse 1, D-80336 Munich, Germany, 3Department of Medical Oncology, Erasmus MC Cancer Institute, Cancer Genomics Netherlands, Rotterdam, The Netherlands, 4Human Cancer Genetics Programme, Spanish National Cancer Research Centre (CNIO) and ISCIII Center for Biomedical Research on Rare Diseases (CIBERER), Madrid, Spain, 5Department of Experimental and Clinical Biomedical Sciences, University of Florence and Istituto Toscano Tumori, Florence, Italy, 6Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Genétique, F-75015 Paris, France, 7INSERM, UMR970, Paris-Cardiovascular Research Center at HEGP, F-75015 Paris, France, 8Université Paris Descartes, Faculté de Médecine, F-75005 Paris, France and 9Department of Pathology, Reinier de Graaf Hospital, Delft, The Netherlands

*(L Oudijk and C M Neuhofer contributed equally to this work)

Correspondence should be addressed to R R de Krijger
Email r.dekrijger@erasmusmc.nl

Abstract

Objective: Pheochromocytomas (PCCs) are neuroendocrine tumors that occur in the adrenal medulla, whereas paragangliomas (PGLs) arise from paraganglia in the head, neck, thorax, or abdomen. In a variety of tumors, cancer cells with stem cell-like properties seem to form the basis of tumor initiation because of their ability to self-renew and proliferate. Specifically targeting this small cell population may lay the foundation for more effective therapeutic approaches. In the present study, we intended to identify stem cells in PCCs/PGLs.

Design: We examined the immunohistochemical expression of 11 stem cell markers (SOX2, LIN28, NGFR, THY1, PREF1, SOX17, NESTIN, CD117, OCT3/4, NANOG, and CD133) on tissue microarrays containing 208 PCCs/PGLs with different genetic backgrounds from five European centers.

Results: SOX2, LIN28, NGFR, and THY1 were expressed in more than 10% of tumors, and PREF1, SOX17, NESTIN, and CD117 were expressed in <10% of the samples. OCT3/4, NANOG, and CD133 were not detectable at all. Double staining for chromogranin A/SOX2 and S100/SOX2 demonstrated SOX2 immunopositivity in both tumor and adjacent sustentacular cells. The expression of SOX2, SOX17, NGFR, LIN28, PREF1, and THY1 was significantly associated with mutations in one of the succinate dehydrogenase (SDH) genes. In addition, NGFR expression was significantly correlated with metastatic disease.

Conclusion: Immunohistochemical expression of stem cell markers was found in a subset of PCCs/PGLs. Further studies are required to validate whether some stem cell-associated markers, such as SOX2, could serve as targets for therapeutic approaches and whether NGFR expression could be utilized as a predictor of malignancy.

Introduction

Pheochromocytomas (PCCs) are catecholamine-producing neural crest-derived tumors of the adrenal medulla. Paragangliomas (PGLs) are closely related to PCCs and arise from paraganglia of the head and neck or of the sympathetic trunk (1).

Although the majority of PCCs/PGLs occur sporadically, about one-third of these tumors develop as a result of germline mutations (2, 3). So far, 16 genes are known to be associated with PCCs/PGLs: SDHA, SDHB, SDHC, SDHD, and SDHAF2 (together SDHx), VHL, RET, NF1, TMEM127, MAX, KIF1B, and PHD2, as well as the recently identified HIF2A (2, 4, 5, 6), HRAS (7), FH (8), and PHD1 (9). The identification of distant metastases is still the only proof of malignancy in PCCs/PGLs, and because treatment options are limited, finding an appropriate strategy poses a clinical challenge (10). A better mechanistic understanding of tumorigenesis, proliferation, and malignant behavior is therefore warranted.

Many tumors, including PCCs and PGLs, are known to be composed of a variety of cells with different functional properties that are likely caused by an increasing number of genetic alterations (11). However, according to the cancer stem cell theory, tumor heterogeneity could also result from stem cell-like cancer cells (SCCs), which provide the very basis of cellular tumorigenesis (12, 13). Physiologically, stem cells are defined by three functional properties: i) proliferation, including self-renewal and asymmetric cell division; ii) differentiation to maintain organ function; and iii) homeostatic control to balance between self-renewal and differentiation (14). Adult stem cells are multipotent or designated progenitors and can differentiate into only a limited number of cell types. In contrast to their non-pathogenic counterparts, homeostatic control is lost in SCCs, which results in extensive uncontrolled proliferation and allows self-renewal and the generation of various subtypes of cells. This in turn leads to tumor heterogeneity. To date, cells with stem cell properties have been identified in various tumor types (15, 16, 17). Whether SCCs derive from malignant cancer cells that acquire stem cell characteristics or from somatic progenitor cells that turn malignant is not yet well understood (12). However, tumor hypoxia could contribute to this conversion (18), and both processes might play a role.

Stem cells are believed to account for only ~0.1% of a tumor’s total mass but are thought to be responsible for most of its proliferation and the malignant properties. In addition to identifying SCCs for prognostic purposes, specifically targeting this small cell population may lay the foundation for more effective therapeutic approaches (12, 19). To investigate whether stem cells or stem cell signaling can be found in PCCs/PGLs, we chose candidate genes that were previously reported to be associated with stem cells or SCCs. We examined the immunohistochemical expression of the most promising embryonic, hematopoietic, neural, and mesenchymal stem cell (MSC) markers in a large series of PCCs and PGLs. Finally, we correlated stem cell marker expression with genetic background and tumor behavior.

Subjects and methods

Candidate marker selection

A list of relevant progenitor markers identified in stem cells and SCCs was generated by a literature search and was examined by immunohistochemistry (IHC) on tissue microarrays (TMA). These candidate SCC markers included: the embryonic stem cell (ESC) markers LIN28, OCT3/4, SOX2, and NANOG; the hematopoietic stem cell (HSC) markers SOX17, PROMININ (CD133), c-KIT (CD117), and THY1; the neural progenitor marker NESTIN; and the MSC markers NGFR and PREF1 (DLK1).

Patients and tumor samples

IHC was performed on PCCs/PGLs from 216 patients from five European centers (81 from France, 60 from Italy, 48 from Spain, 20 from Germany, and seven from The Netherlands). Patient characteristics were collected on the basis of the European Network for the Study of Adrenal Tumors (ENS@T) registry (www.ensat.org). Sample and data collection was approved by the local ethical committees of partaking centers, and all of the patients provided written informed consent.

For immunohistochemical analysis, five TMAs were constructed using an ATA-27 Automated Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI, USA). For each case, two areas of tumor tissue were selected and marked on a representative hematoxylin and eosin (H&E)-stained slide. Tissue cylinders with a diameter of 1 mm were punched from the representative areas of the ‘donor’ block and brought into the ‘recipient’ paraffin block at predefined coordinates. Normal liver, kidney, placenta, adrenal cortex, and adrenocortical carcinomas were included in the TMAs as internal controls. Moreover, whole sections of four normal adrenal glands were used as a control.
Eight tumors were excluded from further analyses (because of insufficient clinical data in two cases and six tissue core dropouts on the TMA slides). A summary of the clinicopathological characteristics of the remaining 208 PCC/PGL patients analyzed in the present study is provided in Table 1. For two patients, the primary tumor and corresponding metastasis were included, and for one patient, only metastatic tumor tissue was available. Both the germ-line and somatic DNA of all tumors except nine were genetically analyzed for mutations in \( \text{SDHA} \), \( \text{SDHB} \), \( \text{SDHC} \), \( \text{SDHD} \), \( \text{SDHAF2} \), \( \text{VHL} \), \( \text{RET} \), \( \text{NF1} \), \( \text{MAX} \), TMEM127, and \( \text{HRAS} \).

### Table 1  Patient characteristics.

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>( n = 208 )</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>91</td>
<td>43.7</td>
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<tr>
<td>Female</td>
<td>117</td>
<td>56.3</td>
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<tr>
<td>Age (median 45 years)</td>
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<td></td>
</tr>
<tr>
<td>&lt;45</td>
<td>97</td>
<td>46.6</td>
</tr>
<tr>
<td>( \geq 45 )</td>
<td>111</td>
<td>53.4</td>
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<tr>
<td>Genotype (pheochromocytoma)</td>
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<td></td>
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<tr>
<td>( \text{VHL} ) germline/somatic</td>
<td>143/3</td>
<td>8.4/1.8</td>
</tr>
<tr>
<td>( \text{RET} ) germline/somatic</td>
<td>29/3</td>
<td>17.5/1.8</td>
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<tr>
<td>( \text{NF1} ) germline/somatic</td>
<td>8/4</td>
<td>4.8/2.4</td>
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<td>( \text{MAX} ) germline/somatic</td>
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<td>2.4/0.6</td>
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</tr>
<tr>
<td>( \text{SDHC} ) germline</td>
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<td>9.5</td>
</tr>
<tr>
<td>( \text{SDHD} ) germline</td>
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<td>14.3</td>
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<tr>
<td>Nonmutated</td>
<td>83</td>
<td>0.5</td>
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<tr>
<td>Not examined</td>
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<tr>
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<tr>
<td>Nonmutated</td>
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<td>Genotype (head and neck paraganglioma)</td>
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<tr>
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<td>Nonmutated</td>
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<td>33.3</td>
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<tr>
<td>Genotype (unknown localization)</td>
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<td></td>
</tr>
<tr>
<td>( \text{RET} ) germline</td>
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</tr>
<tr>
<td>( \text{SDHD} ) germline</td>
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<td>66.7</td>
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<tr>
<td>Metastatic</td>
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Immunohistochemistry

IHC for OCT3/4, SOX2, SOX17, LIN28, NANOG, CD133, CD117, NGFR, NESTIN, THY1, and PREF1 was performed on 4–5 \( \mu \)m sections that were cut from the TMAs. The sections were deparaffinized, rehydrated, and exposed to heat-induced epitope retrieval; they were then incubated in 3% H\(_2\)O\(_2\) in PBS for 20 min. The primary antibody specifications and experimental conditions are shown in Supplementary Table S1, see section on supplementary data given at the end of this article.

For SOX2, SOX17, NANOG, LIN28, and PREF1, a biotinylated rabbit anti-goat secondary antibody was used, and for THY1, a biotinylated goat anti-rabbit antibody was used. After 30 min of incubation with the secondary antibody, the slides were rinsed in PBS. Then, Avidin Biotin Complex solution (ABC, Vectastain ABC Kit, Burlingame, CA, USA, no. PK-6100) was applied for 30 min at room temperature. For CD133, Dako ChemMate Envision HRP rabbit-mouse was applied for 30 min (Dako Envision Kit, Glostrup, Denmark). Slides were again rinsed in PBS, and bound antibody complex was visualized with DAB (3’3’ Diaminobenzidine, Dako Envision Kit), which was applied twice for 5 min each, after which the slides were washed with distilled water, dehydrated, counterstained with hematoxylin, and coverslipped using permanent mounting medium. For PREF1 and THY1, the counterstaining was performed by incubating the slides for

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**Figure 1**

Immunohistochemical staining of human positive control tissue. Representative images showing CD117 staining of mast cells (A), SOX17 staining of normal endothelial cells (B), staining of the fibrovascular network surrounding the tumor cell nests for NGFR (C) and NESTIN (D), and staining of testicular embryonic carcinoma for OCT3/4 (E) and NANOG (F).
13 min at 60 °C with methylgreen (Vector Laboratories, no. H-3402).

Testicular embryonic carcinoma/CIS was used as a positive control for all of the markers. Normal endothelial cells served as an internal positive control for SOX17 (see Fig. 1), as did human pancreas tissue for CD133.

Staining for CD117, NESTIN, and NGFR was performed with a BenchMark XT automated immunostainer.
(Ventana Medical Systems, Tucson, AZ, USA). The positive control tissues were human kidney for NESTIN and colon for CD117 and NGFR. In PCCs/PGLs, the fibrovascular network surrounding the tumor cell nests served as an internal positive control for NESTIN and NGFR, whereas mast cells served as an internal positive control for CD117 (see Fig. 1).

SOX2/S100 and SOX2/chromogranin A double staining were performed on whole sections of tumors that corresponded to the positive cores to determine if the S100-expressing sustentacular cells co-expressed SOX2. This protocol is available upon request. 3-Amino-9-ethylcarbazole (A5754; Sigma)/H2O2 was used for nuclear red SOX2 staining, and Fast Blue/naphthol AS-MX phosphate (F3378 and N500; Sigma) was used for cytoplasmic blue S100/CgA staining (20).

**Scoring of TMAs**

An overview of the scoring (nuclear, cytoplasmic, or membranous) is provided in Supplementary Table S1, see section on supplementary data given at the end of this article. If positive staining was present, a quantity score of 1–3 was given to each core (1 = ≤10% of cells; 2 = 10–50% of cells, and 3 = >50% of cells). For THY1 and PREF1, staining intensity was scored in three grades (1 = weak, 2 = moderate, and 3 = strong) and multiplied by the quantity score to establish a final score that ranged from 0 to 9. The resultant score was classified as negative (0), weak (1–2, or 1+), moderate (3–4, or 2+), or strong (6–9, or 3+). The scoring system was established with an expert endocrine pathologist (RdK) and carried out by three observers (LO, CN, and TP) for each marker. A consensus score was reached in cases of discrepancy. The highest score of the paired cores was taken into consideration.

**Statistical analysis**

All immunohistochemical quantity scores of 1–3 were considered as positive for the statistical analysis. Because of background staining, negative and weak

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**Figure 3**

The expression of stem cell markers in human PCCs/PGLs. Representative images showing the expression of nuclear SOX17 (A), nuclear (B) and cytoplasmic (C) LIN28, membranous CD117 (D), cytoplasmic NESTIN (E), nuclear NGFR (F), cytoplasmic THY1 (G), and cytoplasmic PREF1 (H). Magnification 40×.
scores of THY1 and PREF1 were considered negative, whereas moderate and strong THY1 and PREF1 staining was considered positive for the statistical analysis. Associations of immunohistochemical expression between markers as well as those of marker expression vs clinical and pathological parameters were analyzed using Fisher’s exact test in Stata version 11 (Stata Corp., College Station, TX, USA). FDR values and two-sided P values of <0.05 were considered statistically significant.

Results

Immunohistochemistry

TMAs containing 208 cases of PCC/PGL (including three metastases) were analyzed for the ESC markers LIN28, OCT3/4, SOX2, and NANOG, the HSC markers SOX17, CD133, CD117, and THY1, the neural progenitor marker NESTIN, the neural crest stem cell (NCSC), and the MSC markers NGFR and PREF1/DLK1. No OCT3/4, CD133, or NANOG expression was seen in any of the cores. The scores and percentages of the other investigated markers are displayed in Table 2, and they are exemplified in Fig. 2 for SOX2. Figure 3 provides an overview of the investigated markers. SOX2-positive cells were found in 12% (25/210) of samples, and in most cases in <10% of the cells. SOX2-positive nuclei were seen in both sustentacular cells as well as in PCC/PGL cells, and this was confirmed by double staining with S100 and chromogranin A (CgA) (Figs 2 and 4). Nuclear expression of the ESC marker LIN28 was seen in 15% (31/209) of cases, whereas cytoplasmic LIN28 expression was seen in 24% (51/209) of samples, with co-occurrence in 24 cases (40% of all LIN28 positive cores). NGFR expression was shown in 19% (39/210), with an equal distribution of the weak, moderate, and strong expression subgroups. In one patient, from whom both primary and metastatic tumor tissue was included in the TMA, the primary tumor showed nuclear NGFR staining in 10–50% of the tumor cells, whereas NGFR was negative in the metastatic lesion. Moderate PREF1 staining and NESTIN staining was demonstrated in 5% (10/207) and 3% (7/208) of cases respectively. The lowest frequencies of expression were seen for the HSC markers SOX17, which was expressed in 2% (5/210) of cases, and CD117, which was expressed in 3% (6/210) of cases. The HSC marker THY1 was expressed at a much higher frequency, with positivity in 16% (33/208) of samples. It is important to mention that no positive stem cell marker expression could be seen in the chromaffin cells of any of the four normal adrenal medullary tissues analyzed. However, we did see some SOX2-positive sustentacular cells in the normal adrenals.

Correlations among the investigated stem cell markers by immunohistochemistry

The correlation of marker expression was assessed by the Fisher’s exact test (see Supplementary Table S2, see section on supplementary data given at the end of this article). Significant relationships were observed between SOX2 and cytoplasmic/nuclear LIN28 (P=0.006/P<0.001), CD117 (P=0.002), and THY1 (P=0.02). Cytoplasmic/nuclear LIN28 expression correlated with SOX17, PREF1 (P<0.001), and NGFR (P=0.001) expression. Furthermore, NGFR expression correlated with PREF1 (P=0.01) and THY1 (P=0.001) expression. THY1 therefore in turn was synergistically expressed with SOX2 (P=0.02), nuclear LIN28 (P<0.001), NGFR (P=0.001), and PREF1 (P=0.048). PREF1 expression correlated significantly with all other markers except for NESTIN and CD117, and SOX17 correlated significantly with all markers except for NESTIN and NGFR.

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Associations between stem cell marker expression and clinical pathological features

The correlation of the expression of each stem cell marker was tested with metastatic behavior, tumor size, and genotype. The expression of the stem cell markers SOX2, SOX17, NGFR, LIN28, PREF1 and THY1 positively correlated with an SDHx mutation status (all $P < 0.01$; Table 3). In RET-mutated tumors, there was an association with the absence of THY1 expression ($P = 0.033$). Overall, tumor size did not correlate with stem cell marker expression. Detailed analyses revealed a significant inverse relation between stem cell marker expression and tumor size for CD117 ($P < 0.003$) and a tendency for an inverse relation for SOX2 and nuclear LIN28 ($P = 0.08$ each; Fig. 5A, B, and C). NGFR expression was significantly associated with malignancy ($P = 0.039$; Fig. 5D), whereas a tendency was observed for THY1 expression ($P = 0.09$).

Discussion

Stem cell markers have not yet been systematically studied in PCCs/PGLs. Given the accumulating evidence to support the presence of SCCs in other endocrine tumors (21), we investigated stem cell marker expression in a large series of PCCs/PGLs by IHC analysis for the potential association between stem cell phenotype, genotype, and/or metastatic behavior.

Whereas OCT3/4 and NANOG, which take part in a molecular network that has been shown to induce pluripotency in somatic cells (22), were not expressed in any PCCs/PGLs, all other markers were detectable at variable frequencies. The present findings are consistent with those of Looijenga et al. (23), who demonstrated OCT3/4 immunonegativity in the 36 PCCs/PGLs that they examined, whereas a study by Alexander et al. (24) showed strong diffuse cytoplasmic OCT4 expression in 30 PCCs. Although this discrepancy might be attributable to the employment of different antibodies or IHC techniques, the present findings in our large, multicenter cohort argue against the assertion that the cytoplasmic OCT4 staining pattern can be regarded as a stem cell marker in PCCs/PGLs.

In our TMA series, SOX2, LIN28, SOX17, NGFR, and THY1 appeared to be frequently co-expressed and were all significantly associated with SDHx mutation status, which possibly suggests interdependence or a common regulatory mechanism. In fact, SOX2 and LIN28 co-expression is in line with the known function of SOX2 as a direct binding partner of LIN28A in a nuclear protein–protein
complex that thereby modulates LIN28A activity in ESCs and induced pluripotent stem cells (iPSCs) (25). Nuclear SOX2 expression was the highest and was found in 12% of the PCC/PGL samples. Double staining for S100/SOX2 (sustentacular cells) and for chromogranin A/SOX2 (tumor cells) identified SOX2 expression not only in sustentacular cells within normal adrenal glands but also in tumor cells of PCCs. So far, sustentacular cells have been considered to be non-neoplastic cells (26, 27), but the precise origin and nature of this cell type in PCCs/PGLs has not yet been fully clarified. Histological studies have described varying ratios of sustentacular cells in metastatic PCCs as compared to locally growing PCCs (28, 29), which suggests their importance for tissue homeostasis in the normal adrenals and their possible role in PCC neoplastic/metastatic potential. Interestingly, in the anterior pituitary gland, S100/SOX2 co-expressing folliculo-stellate cells have been identified and have been proposed to be pluripotent adult stem cells (21). There are rare reports of sellar neoplasms that are assumed to originate from folliculo-stellate cells (30) and of a distinctive neoplasm with a suggestive derivation from sustentacular cells of the adrenal (26), both of which support the notion that folliculo-stellate cells may become neoplastic. However, in the present cohort, a correlation between SOX2 expression and clinical features or metastatic behavior could not be found. This could be a result of the relatively low numbers of malignant tumors in our TMAs.

Although PCCs and PGLs originate from the neural crest, we could not identify a significant expression of CD133, which has previously been described as a hematopoietic, neural, and cancer stem cell marker (31). The absence of NESTIN expression in the TMA samples is not unexpected, seeing as NESTIN is commonly found in stem cells of the central nervous system and is not routinely expressed in tissues of the sympathetic nervous system.

Stem cell markers of the hematopoietic system are well established. Because these markers are widely used to screen for progenitor cells in other tissues, we utilized SOX17, CD117, and THY1 in our screening. Of these, we found only THY1 to be expressed in PCCs/PGLs. THY1 (CD90) is commonly used in cell sorting protocols to enrich for hematopoietic (32) and other stem cells, such as progenitor cells of the liver (33). Its role in oncogenesis is still unclear, but it is found in a variety of cell types and seems to play a role in a large number of cellular processes (34). Because of the complex functional background of THY1, the interpretation of THY1 positivity in PCCs/PGLs is difficult, and further research is warranted.

PREF1 is considered to be an MSC marker because of its inhibitory role on adipose tissue differentiation through MEK/ERK signaling (35). PREF1 was detectable only at very low levels, so it therefore does not seem to be a major player. In contrast, NGFR (p75 low affinity) displayed the second highest expression levels of all of the markers tested. NGFR has been described as being a potent MSC marker (36) and has been found to be expressed, for example, in the progenitor cells of human salivary glands (37). In the present study, NGFR expression was significantly more often associated with malignant PCCs/PGLs (S/12, or 42%) as compared to non-metastatic PCCs/PGLs (31/194, or 16%). Of interest, Loriot et al. (38) described how activation of the epithelial–mesenchymal transition process might play a critical role in SDHB-metastatic PCCs/PGLs, which further addresses the mesenchymal marker NGFR as a marker of interest. The fact that NGFR was also expressed in apparently benign tumor cores could account for the limited specificity of the marker, but on the other hand, it could also highlight the general problem of defining non-metastatic disease in PCC/PGL patients. Certainly, taking into account the limited sample size of metastatic cases in the present series, further studies are needed in order to properly assess NGFR before it can

![Figure 5](https://www.eje-online.org)
be claimed that it is a potential diagnostic or therapeutic molecular marker that indicates malignancy in these tumors in any given genetic context.

In conclusion, we performed IHC on TMAs from 208 tumors, and we found stem cell marker–positive cells in a subset of PCCs/PGLs. Interestingly, stem cell marker expression was associated with mutations in one of the succinate dehydrogenase (SDH) genes. In addition, NGFR expression was significantly correlated with metastatic disease. Further studies are required to validate if any of these markers could serve as targets for future therapies or as predictors of malignancy.

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