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# Loss of BAP1 in Pheochromocytomas and Paragangliomas Seems Unrelated to Genetic Mutations

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## Abstract

Breast cancer–associated protein 1 (BAP1) gene is a broad-spectrum tumor suppressor. Indeed, its loss of expression, due to biallelic inactivating mutations or deletions, has been described in several types of tumors including melanoma, malignant mesothelioma, renal cell carcinoma, and others. There are so far only two reports of *BAP1*-mutated paraganglioma, suggesting the possible involvement of this gene in paraganglioma (PGL) and pheochromocytoma (PCC) pathogenesis. We assessed BAP1 expression by immunohistochemistry (IHC) in a cohort of 56 PCC/PGL patients (and corresponding metastases, when available). Confirmatory Sanger sequencing (exons 1–17) of *BAP1* has been performed in those samples which resulted negative by IHC. BAP1 nuclear expression was lost in 2/22 (9.1%) PGLs and in 12/34 (35.3%) PCCs, five of which harboring a germline mutation predisposing the development of such tumors (*MENIN, MAX, SDHB*, *SDHD*, and *RET* gene). Confirmatory Sanger sequencing revealed the wild-type *BAP1* status of all the analyzed samples. No heterogeneity between primary and metastatic tissue was observed. This study documents that the loss of BAP1 nuclear expression is quite a frequent finding in PCC/PGL, suggesting a possible role of *BAP1* in the pathogenesis of these tumors. Gene mutations do not seem to be involved in this loss of expression, at least in most cases. Other genetic and epigenetic mechanisms need to be further investigated.

**Keywords** BAP1 · Immunohistochemistry · Sanger sequencing · Pheochromocytoma · Paraganglioma

## Introduction

Pheochromocytoma (PCC) and paraganglioma (PGL) are rare non-epithelial neuroendocrine tumors that arise in the adrenal medulla and extra-adrenal paraganglia, respectively, and are considered part of the same disease spectrum [1]. Known genetic alterations in about 30 genes account for the pathogenesis of the majority of PCC/PGL and at least 30% of the overall cases harbors germline mutations [2, 3]. Based on multiple genomic analyses, PCC/PGL-related genes have been clusterized by The Cancer Genome Atlas (TCGA) in four groups (pseudo-hypoxic pathway, WNT signalling cluster, kinase signalling cluster, and cortical admixture subtype), which explain the molecular etiology of the 95% of the TCGA cohort cases [3]. Nevertheless, mutations in some genes have been described only in single patients or families [4]. In this scenario, the pathogenesis of some tumors remains to be disclosed.

Breast cancer associated protein 1 (BAP1) is a deubiquitinating enzyme involved in many cellular functions, such as regulation of cell cycle and DNA transcription, cellular differentiation, cell death, DNA damage response, and others [5, 6]. *BAP1* gene (located on chromosome 3p21.1) is a broad-spectrum tumor suppressor. Indeed, its loss of expression, due to biallelic inactivating somatic or germline alterations [7, 8] (insertions, deletions, frameshift, nonsense and missense mutations) has been described in several tumors as follows: atypical benign melanocytic lesions [7, 9, 10], uveal, and cutaneous melanoma [7, 11], malignant mesothelioma (MM) [5, 7], renal cell carcinoma (RCC) [12], and others [13–15]. Wadt and colleagues identified a patient carrying a

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germline *BAP1* splice mutation causing a premature termination codon who developed a PGL with somatic loss of *BAP1* wild-type allele [11]. More recently, a germline *BAP1* frameshift mutation was described in a 16-year-old male with multiple nevi, all with BAP1 loss at immunohistochemistry (IHC) [10]. The proband's father died of a fatal PGL. Although his germline mutational status was unavailable, the authors hypothesized a relation with the *BAP1* mutation detected in the son [10]. Moreover, in a series of 52 carotid body tumors, a pathogenetic somatic *BAP1* mutation was identified in one case [16], further suggesting the potential involvement of *BAP1* in PCC/PGL pathogenesis. In our study, we tested the hypothesis that BAP1 has a pathogenetic role in PCC/PGL, investigating BAP1 protein expression and its mutational status.

## Materials and Methods

This retrospective study was carried out on formalin-fixed and paraffin-embedded (FFPE) surgical resection samples of PCC/PGL (January 2006–December 2015), retrieved from the archives of the Surgical Pathology and Cytopathology Unit of the University of Padova. Inclusion criteria are the following: PCC/PGL diagnosis, adult age, at least 24 months of clinical follow-up, and known mutational status of hereditary PCC/PGLs-associated genes. All the cases were reviewed, and diagnoses confirmed (V.M. and R.C.), according to the fourth edition World Health Organization (WHO) classification of tumors of endocrine organs [1]. We selected 34 PCC and 22 PGL. All the primary tumors and 7 PCC metastases have been tested (63 FFPE specimens in total). The study was performed according to the 1964 Helsinki declaration and its later amendments; it also adheres to the REporting recommendations for tumor MARKer prognostic studies (REMARK) guidelines [17].

### Immunohistochemical Analysis

IHC was performed on 4-μm–thick FFPE whole sections from each tumor sample with the mouse monoclonal antibody anti-BAP1 (clone C-4; dilution 1:50; SantaCruz Biotechnology, California, USA). IHC was done automatically (BonD-MaX, Leica Biosystems, Newcastle Upon Tyne, UK), using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK), as described elsewhere [18]. Sections were then counterstained with hematoxylin. BAP1 staining was defined positive when any nuclear immunoreaction was observed (both in non-neoplastic and neoplastic cells). Lymphocytes, endothelial, or stromal cells acted as internal positive control. Six non-neoplastic tissues (adrenal medulla and ganglion) were also stained. The slides were blindly assessed by two pathologists (V.M. and R.C.), and cases with discordant evaluation were then jointly reassessed until agreement was reached (V.M, R.C., and A.F.).

### Image Analysis

Whole slides sections stained for BAP1 were digitized with an Aperio CS2 scanner (Leica Microsystem, Wetzlar, Germany) at × 20 magnification. BAP1 positive nuclei were automatically quantified in tumor tissue using a custom-made algorithm created with Visiopharm™ software version 4.5.6.5 (Visiopharm, Hoersholm, Denmark). Briefly, a slide from each case was uploaded in Visiopharm environment. A pathologist (L.N.) manually identified and outlined regions of interest (ROIs) including the whole tumor tissue. Automated quantification of total number of nuclei was obtained within the ROIs and then elaborated as number of positive cells (percentage of positive nuclei on the total number of nuclei). Morphologically, non-neoplastic cells, such as stromal and endothelial cells, represented a small percentage of the tissue. Therefore, we set a threshold of 5% to distinguish between positive and negative cases.

### Molecular Profiling

*BAP1* mutational status was evaluated in the 14 BAP1-IHC negative tumors analyzing the coding region (exons 1-17) of *BAP1* gene by Sanger sequencing. Tumor cell enrichment was performed by manual microdissection of tumor areas from 5 consecutive 10-μm–thick unstained FFPE sections following the selection on the hematoxylin-eosin slide. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) and purified using NEBNext® FFPE DNA Repair Mix (New England BioLabs, Ipswich, MA, USA). Exons 1-17 of *BAP1* were amplified by Polymerase Chain Reaction (PCR) and subsequently sequenced using Big Dye Terminator v1.1 on an Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, Foster, CA, USA), according to the manufacturer's instructions. The data were finally analyzed using SeqScape software (Applied Biosystems, Foster, CA, USA). Sequences of primers are available upon request.

### Statistical Analysis

Correlations between the IHC results and clinicopathologic variables were analyzed using the Pearson chi-square and Fischer exact tests, as appropriate. A $p$ value $< 0.05$ was considered significant. The data analysis was performed by SPSS statistical program (version 20.0, IBM SPSS Statistics, Chicago, IL, USA).

## Results

The study included 23 male and 33 female patients. The mean age was 44.7 ± 17.9 years (range 19–70) for the PGL patients and 49.6 ± 17.5 years (range 18–76) for the PCC patients. The female to male ratio was 1.2:1 and 1.6:1 in PGL and PCC, respectively. Slightly more than half of the PGLs (12 of 22; 54.5%) was located in the head and neck region, while the remaining (10 of 22; 45.5%) were located in the abdominal cavity. Seventeen patients (30.4%) had inherited disease due to germline mutations (as detailed in Table 1). During the follow-up, 10 cases (17.8%; 9 PCCs and 1 PGL) developed progressive disease with metastatic spread; death of disease occurred in one PCC patient (1.8%). The PCCs metastasized to the liver, lymph nodes, bones, small bowel, omentum, and abdominopelvic fibroadipose tissue, while the PGL to multiple cervical vertebrae, eighth rib, and femur. Detailed features of the cases are summarized in Table 1.

### Expression Status of BAP1 by IHC

Adrenal medullary and ganglion cells were diffusely and moderately positive for BAP1 (Fig. 1). Vascular endothelial and stromal cells exhibited heterogeneous and weaker immunoreactivity.

At the first analysis, BAP1 staining was negative in 3 of 22 (13.6%) PGLs and in 14 of 34 (41.2%) PCCs. Remaining cases were strongly and diffusely positive for BAP1 (Fig. 2). Intensity of staining was higher in neoplastic than in non-neoplastic tissues. Several cases had intratumoral heterogenous intensity of BAP1 staining. Image analysis (Fig. 3) was consistent with these results in the majority of cases (91.1%). Discordancy was present in 5 of 56 cases (8.9%), all being evaluated as negative: one PCC showed a weak background positivity with unclear nuclear immunoreaction, so IHC was repeated and then the case was considered positive; two PCC had a brisk diffuse inflammatory infiltrate and a higher stromal component, which explained the 20% and 35% of positive nuclei, respectively; one PGL and one PCC showed 20% of positivity at image analysis and, after revision, we reclassified them as positive. In conclusion, BAP1 was considered lost in 2 of 22 (9.1%) PGLs and in 12 of 34 (35.3%) PCCs (Figure 2), with a slight predominance in male (8/14) compared to female (6/14) ($p > 0.05$).

### Clinicopathological Features of PGL and PCC Lacking BAP1 by IHC

At hematoxylin and eosin, the tumors without nuclear BAP1 staining displayed some similarities with BAP1-inactivated melanocytic tumors [9, 19], being composed of moderate to large epithelioid cells with well-defined cell borders, abundant cytoplasm, varying degrees of nuclear pleomorphism, vesicular chromatin, and focally prominent nucleoli (Fig. 4). The mitotic activity was generally low and tumor-infiltrating lymphocytes were extremely rare (unlike BAP1-inactivated skin tumors). The metastatic PGL was BAP1 positive, while 4 of 9 metastatic PCCs were negative. No relationship between

**Table 1** Clinical, pathological, and molecular features of the series of PCC/PGL

| | PGL ($n$ = 22) | PCC ($n$ = 34) |
|---|---|---|
| F/M | 12/10 | 21/13 |
| Mean age, yr (range) | 44.7 (19–70) | 49.6. (18–76) |
| *VHL* | 1/22 | 4/34 |
| *MENIN* | - | 1/34 |
| *RET* | - | 4/34 |
| *MAX* | - | 1/34 |
| *SDHB* | 2/22 | 1/34 |
| *SDHA* | 1/22 | - |
| *SDHD* | 1/22 | 1/34 |
| Metastatic° | 1/22^ | 9/34 |
| BAP1 loss (IHC) | 2/22[#] | 12/34 |
| BAP1 loss (IHC) in known germline mutation context* | 0/5 | 5/12 |
| BAP1 loss (IHC) in metastatic cases | 0/1 | 4/9 |

°The metastatic PGL harbored a germline *SDHB* mutation, while 2 of 9 metastatic PCC harbored germline *MENIN* or *MAX* mutations

^Primary PGL tumor located in an abdominal ganglion

[#] These PGLs were pre- and para-aortic

*As for PCCs in a context of inherited disease, loss of BAP1 expression was found in cases harboring germline *MENIN*, *MAX*, *SDHB*, *SDHD*, or *RET* mutation. On the contrary, none of the 2 PGLs cases harbored a known germline mutation

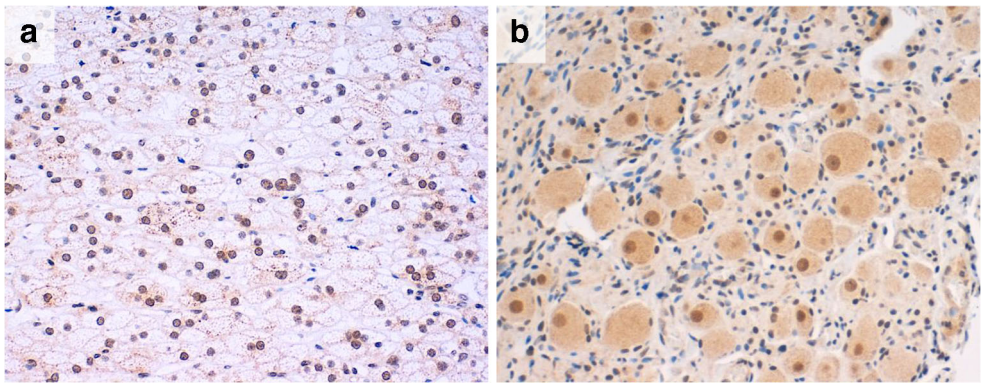


**Fig. 1** BAP1 nuclear immunoreaction in non-neoplastic tissues is diffuse with moderate intensity (original magnification × 20). **a** Adrenal medulla. **b** Ganglion cells

BAP1 status and metastatic disease was identified ($p > 0.05$). All the metastatic samples showed the same immunoprofile of the corresponding primary tumor (7 of 7).

## Mutational Analysis

Sanger sequencing extended to all 17 exons of *BAP1* gene (transcript reference NM_004656.4) did not reveal any mutation in all BAP1-IHC negative samples (2 PGL and 12 PCC). Our sequencing result was concordant with that obtained by consulting the cBioPortal for Cancer Genomics database [20]. Selecting the 173 PCC/PGLs of the TCGA [3] and querying them for presence of *BAP1* alterations, no *BAP1* mutations were observed (data not shown).

## Discussion

This is the first study that specifically investigated BAP1 in a cohort of PCC/PGL patients. At protein level, we found that loss of BAP1 nuclear expression occurs in these tumors, compared with the corresponding tissues of origin, and appears to be more common in PCCs (35.3%) than in PGLs (9.1%).

Although a recent metanalysis reports that loss of BAP1 is more common in women than in men across many tumor types [15], we observed a slight predominance of BAP1 negativity in male PCC/PGL patients. Negative BAP1 by IHC has been found in patients with known germline mutations (5 of 14 negative cases of our cohort), suggesting that BAP1 is not a driver in these tumors. *BAP1* and *VHL* are both located on the short arm of chromosome 3. Point mutations and not loss of

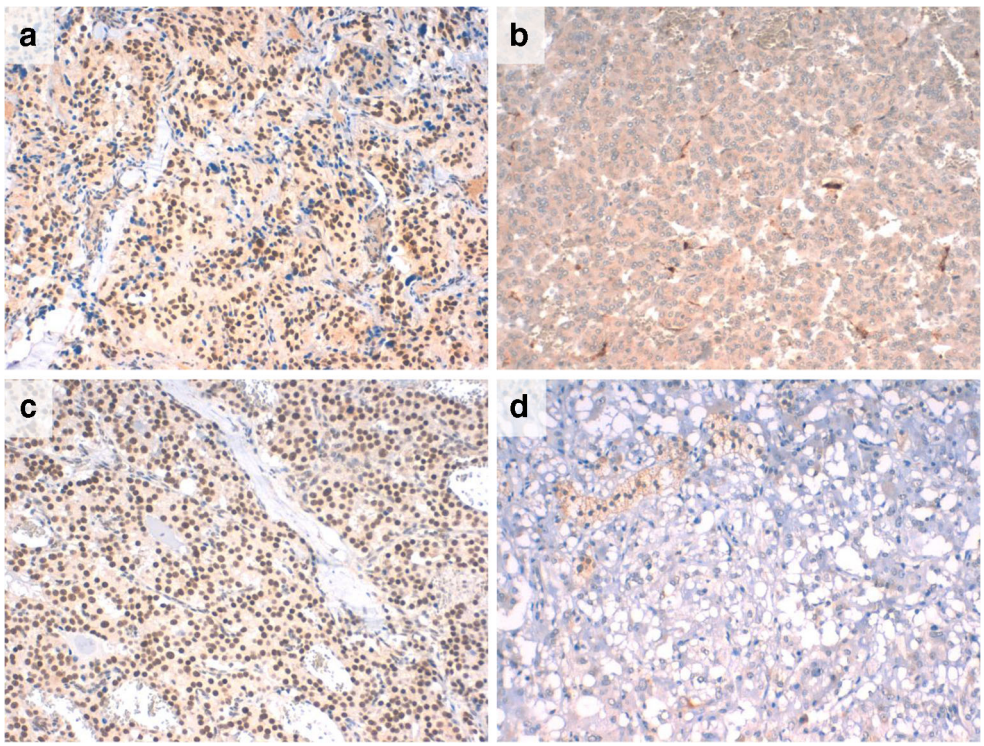


**Fig. 2** BAP1 staining by IHC, representative cases (original magnification × 20). BAP1 immunoreaction is stronger in neoplastic cells than in non-neoplastic cells. **a** A PGL (case #25) diffusely positive for BAP1. **b** A PGL (case #11) negative for BAP1. **c** A PCC (case #7) diffusely positive for BAP1. **d** A PCC (case #23) negative for BAP1

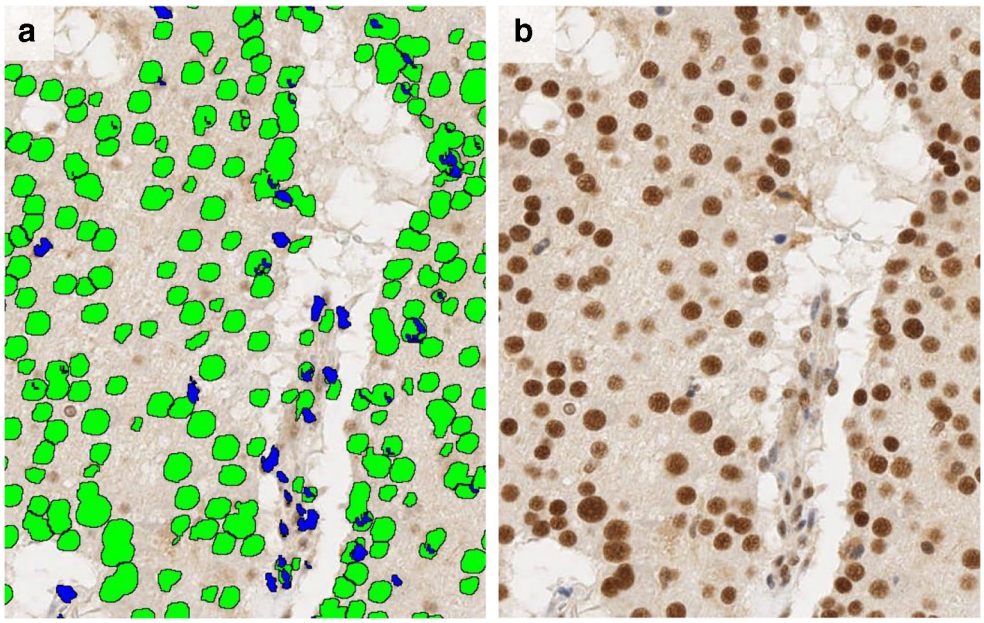


**Fig. 3** **a** The selected region of interest (ROI) of a representative positive PCC (case #4). Negative nuclei are labelled in blue, positive in green. **b** The corresponding BAP1 IHC

chromosome 3p were the defect harbored by *VHL*, excluding the latter mechanism as responsible for BAP1 loss.

BAP1 protein is normally expressed in the nuclei (2 wild-type copies of the gene), while tumor cells with biallelic inactivation of *BAP1* gene will demonstrate complete loss of nuclear staining with a maintained expression in the non-neoplastic cells (2 wild-type copies of the gene) [21]. In the case of homozygous deletion of the gene, all the cells will have a complete loss of nuclear staining. Indeed, IHC is an excellent surrogate of *BAP1* mutational status (positive and negative predictive value of 100% and 98.6%, respectively [8]) in multiple tumors [9, 12–14, 21–23], also described in two PGLs [10, 11]. In our cohort, we can indirectly exclude a homozygous deletion due to the maintained positive internal control at IHC in all the cases. Concerning the loss of nuclear protein expression, we need to remember that germline or somatic *BAP1* mutations and the loss of chromosome 3p21 have been reported in the literature in the large majority of cases [5, 7–14, 22–26]. Sanger sequencing (the gold standard technique to detect single point mutations in many tumors) revealed the wild-type status of all the cases with BAP1 lost at IHC; this is in line with the lack of PCC/PGL spontaneous development in *BAP1*-mutant mice [27]. Anyway, discrepancy between IHC and molecular testing has already been reported [28]. There could be two possible explanations for this genotype-phenotype discordancy: (i) misinterpretation of BAP1 immunostaining; (ii) loss of protein expression might be caused by other genetic alterations as structural chromosomal and epigenetic modifications. Concerning the interpretation of IHC, we used three precautions. First, we applied a dichotomous approach (positive versus negative), rather than a semiquantitative approach (based on intensity and/or percentage of expression). This is the dominant assessment method used by surgical pathologists in routine practice, with excellent interobserver concordance and concurrence with genotype in many tumor types. Moreover, it allows avoiding ambiguity that could be related to intratumor heterogeneity and/or polyclonality [29]. Second, since heterogeneity in the intensity of BAP1 staining was observed in some cases, we used image analysis to avoid a human-related potential source of error, obtaining a more objective evaluation. Anyway, at first analysis, there was a 91.1% concordance between pathologist and software assessment. Third, regarding the cytoplasmic BAP1 pattern of expression reported in uveal melanomas [22, 30], none of our cases showed this type of staining. Concerning the loss of nuclear protein expression, we cannot exclude the presence of intronic splicing mutations of *BAP1* [24, 31], which may generate altered epitopes not recognized by the IHC antibody or unstable protein. Rarely, it has been

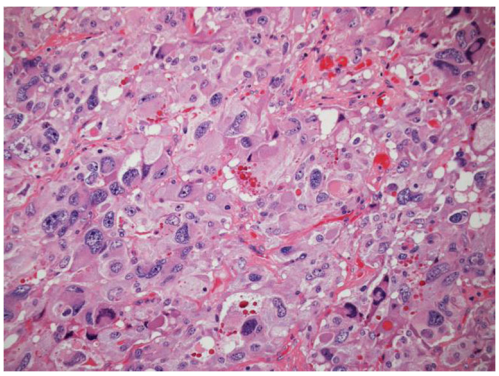

**Fig. 4** Hematoxylin and eosin of a PCC (case #42) with BAP1 loss by IHC (original magnification × 20). It shows that the tumor is composed of large epithelioid cells with well-defined borders, diffuse nuclear atypia, and some prominent nucleoli. These cytological features are similar to those of the *BAP1*-inactivated melanocytic tumor of the skin

reported that other molecular mechanisms than *BAP1* genetic mutations could be involved [22, 30, 32–35]. In uveal melanoma 6 of 26 cases with nuclear BAP1 lost at IHC showed wild-type status of the gene. One of these cases had a focal perinuclear immunopositivity [30] which has been hypothesized to be due to a post-translational mechanism [36]. The discordancy in the remaining cases was unexplained [30]. Similar phenotype–genotype has been previously described by two independent groups in 5 and 3 cases, respectively [34, 35]. Another group reported one uveal melanoma (74 cases in total) with a heterogenous staining for BAP1 (50% negative cells), even though no *BAP1* mutation was detected by exome sequencing [22]. The authors detected loss of heterozygosity of chromosome 3 by fluorescent in situ hybridization (FISH) and somatic single nucleotide polymorphism (SNP)-array, hypothesizing that intronic variants, or more complex genetic rearrangements could explain the immunoprofile of the tumor [22]. Concerning epigenetic *BAP1* regulation, its methylation was specifically analyzed in gallbladder carcinoma, MM, RCC, and melanoma, but almost no decrease in BAP1 expression due to methylation was found [24, 33, 37, 38]. More recently, a novel hypermethylated site within the *BAP1* locus was discovered in uveal melanoma, suggesting that *BAP1* itself is epigenetically regulated [39]. Could be argued that the use of a single molecular technique is insufficient to detect all types of *BAP1* alterations [24]. Sanger sequencing cannot detect large DNA deletions (or insertion) [24]. Moreover, some abnormal splicing forms of *BAP1* have been detected by RNA sequencing, but not by Sanger sequencing in the same tumor sample [24]. But recently, an epithelioid melanocytic lesion with BAP1 lost at IHC demonstrated no chromosome 3 abnormalities by *BAP1* Sanger sequencing or somatic SNP-array, further not only supporting that there might be several mechanisms responsible for BAP1 loss [40] but also that we are not able to predict the underlying mechanism in a minority of BAP1-immunonegative cases. *BAP1* mutation-specific micro-RNA (miRNA) signature has been reported [41], and miRNAs seem to play regulatory functions in PCC/PGL [3, 42]. Interestingly, in lung cancer [43] and intrahepatic cholangiocarcinoma [18] miR-31 is a direct regulator of BAP1, and its overexpression contributes to tumorigenesis through the suppression of BAP1 [41]. Taken together, these studies highlight that, in a minority of cases, *BAP1* alterations are more complex than initially expected. Given the above literature, we believe that intronic splicing mutations, followed by the methylation status of the gene and miRNA are the most likely mechanisms that deserve to be further investigated in PCC/PGL.

The BAP1 nuclear expression is also an adverse prognostic marker in many tumor types, except MM, in which it is considered protective [15, 18, 44]. In MM, BAP1 IHC loss strongly support the diagnosis of MM compared with reactive mesothelial proliferations [45]. In PCC/PGL reliable clinical, biochemical, histopathologic, or molecular markers of malignant potential are lacking, being therefore highly advisable [1]. Our findings did not reveal a role for BAP1 in differentiating metastatic from non-metastatic PCC/PGLs. The major risk factor for metastasis is the presence of a *SDHB* mutation [1], which was harbored by our metastatic PGL. To assess the possibility of BAP1 heterogeneity in primary versus metastatic tissues, we also stained all the available metastatic samples. No discrepancy was found in the immunoprofile with the primary tumor.

From a clinical and genetic-counselling perspective, the identification of BAP1-deficient tumors has an increasing importance. Indeed, *BAP1* germline mutations have been implicated in an autosomal dominant hereditary tumor predisposition syndrome (BAP1-TPDS) [5, 25, 46]. These patients have an increased risk to develop several tumor types, especially uveal and cutaneous melanoma, MM, and RCC [5, 8, 26, 46]. Just one proved PGL has been described so far in a Danish multi-cancer family harboring germline inactivating *BAP1* mutation [11]. Interestingly, in that family, there were some patients who developed other tumors which did not display a germline mutation accompanied by loss of the wild-type allele, suggesting a specific cancer-set related to *BAP1*, potentially including PGL [11]. The occurrence of PGL in patients harboring BAP1-TPDS appears very rare, with only this case proved in the approximately 181 families described so far [46]. Anyway, the full spectrum of tumors associated to BAP1-TPDS and the penetrance are not fully understood [46]. It is plausible that epigenetic and/or environmental mechanisms are implied.

Taken together, our data in PCC/PGL compared with other tumor types support a cell type-specific role for BAP1, as already suggested [5, 44]. The main weaknesses of this study are the retrospective design, the relatively small number of cases, and the use of just one molecular technique to investigate *BAP1* status along with IHC.

## Conclusions

This study documents that loss of BAP1 protein expression is quite frequent in PCC/PGL. Gene point mutations do not seem to be the molecular mechanism underlying BAP1 loss in our series. Nevertheless, in the literature two *BAP1*-mutant PGLs have been described. Therefore, the mechanism of BAP1 protein loss in PCC/PGL need to be further investigated.

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**Authors' contributions** VM and RC: conception and design of the study, acquisition, analysis and interpretation of data, writing and revision of the manuscript. LN: acquisition and analysis of data. VG: development of methodology, technical and material contribution. CM and LE: development of methodology, technical and material contribution, revision of the manuscript. FS: collection of data. MG: reagents, material, and analysis tool contribution. AF: conception and design of the study, revision of the manuscript, supervision of the study. All of the authors confirm that each of them qualifies for all the criteria of authorship.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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