Clinical exome next-generation sequencing panel for hereditary pheochromocytoma and paraganglioma diagnosis

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Abstract. Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumors with an annual incidence of ~2 cases per million worldwide. The hereditary form is more likely to present in younger patients. To date, PPGL is considered a complex pathology that is difficult to diagnose. The present study aimed to improve the molecular diagnosis and other driver mutations related to PPGLs using TruSight One clinical exome panel (Illumina, Inc.). The clinical protocol used involved examining 28 patients with suspicion of genetic alterations as the cause of PPGLs. The variants of genes commonly associated with PPGLs (*RET*, *FH*, *VHL*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *NF1*, *MAX*, *HIF2A*, *TMEM127* and *TP53*) were filtered across the panel. The libraries were sequenced on a MiSeq instrument (Illumina, Inc.) and the result was ≥20X coverage on 95% of the target regions in the panel, calculated by averaging the mean coverage for each exon. The results of sequencing detected 7% of pathogenic variants in the 18-40 years age subgroup and 11% in the 41-59 years age subgroup, whereas no pathogenic/likely pathogenic variants were identified in patients ≥60 years old. The identification of a germline mutation in patients with apparently sporadic PPGLs could lead to an early diagnosis of multiple or more aggressive tumors, or other neoplastic syndromes, in patients. Furthermore, this information may improve the development of targeted primary and secondary prevention programs tailored to these high-risk groups.

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Introduction

The World Health Organization defines pheochromocytoma (PCC) and paraganglioma (PGL), together denoted PPGL, as neuroendocrine tumors that arise from neural crest tissue (1). Patients diagnosed with PPGL are typically identified through elevated catecholamine levels and hypertension. PPGLs have the potential to be associated with renal, stromal, and gastrointestinal tumors (GIST) or, more rarely, pituitary tumors.

The annual incidence of these rare tumors is approximately 2 cases per million, with the highest incidence between the third and fifth decade of life, with no significant difference between women and men (2,3). PPGLs are hereditable tumors, with around 30% of patients having a germline variant predisposed to the tumor (4), and the hereditary form is more likely to present in younger patients (5). This is a rare disease but has a high variability, with differing symptoms that are frequently hidden by other clinical conditions (6). While the majority of PPGL cases appear benign, 2-26% may progress to develop metastases (7). Surgery is the preferred choice of therapy whenever possible. In cases of inoperable or metastatic disease, systemic therapy options include chemotherapy, radionuclide therapy, and tyrosine kinase inhibitors (8). However, for all patients with a history of PPGL and asymptomatic mutation carriers, lifelong follow-up is necessary. The approach to follow-up is specific to the individual, depending on their mutation status and disease characteristics (8).

Next-generation sequencing (NGS) technology has revolutionized the collection of genetic information, allowing the identification of germline or somatic gene mutations in 95% of patients (8). The genes associated with susceptibility

to PPGLs and commonly analyzed include *RET, FH, VHL, SDHA, SDHB, SDHC, SDHD, NF1, MAX, HIF2A, TMEM127* and *TP53* (4). Among these, *RET, NF1* and *VHL* are also involved in three distinct clinical syndromes associated with PPGLs: multiple endocrine neoplasia type 2 (MEN2), a syndrome caused by *RET*, neurofibromatosis type I caused by *NF1*, and Von Hippel-Lindau disease caused by *VHL* (4).

To date, PPGL still remains a rather complex pathology, difficult to diagnose, which can lead to a delay in appropriate treatment and pave the way for new diagnostic challenges (9). Our previous NGS analyses allowed us to highlight two rare cases of familial PPGLs: Carney-Stratakis and Chuvash syndromes.

The first case report is a 56-year-old man with a medical history characterized by reflux, anemia, fatigue, fecal occult blood, gastrointestinal stroma tumor (GIST) and co-occurrent PCC. We thus analyzed the patient using NGS TruSight One sequencing panel testing, which led to the identification of a missense mutation (p.Tyr629Phe) in the *SDHA* gene, in accordance with what was described by Carney and Stratakis (10). In another case, a 19-year-old man had been experiencing erythrocytosis, fatigue and severe headaches since an early age. The parents died of unknown causes and the brother was in good health. This patient was also analyzed using NGS and we were able to identify the presence of two heterozygous mutations, one in the *VHL* gene and one in the *TMEM127* gene (Arg200Trp and Val90Met, respectively). The concomitant presence of heterozygous mutations in the VHL and *TMEM127* genes has been shown to cause a Chuvash polycythemia phenotype (11).

In a further study, NGS analysis revealed to be useful in the diagnosis of a difficult case. The patient diagnosed for the first time with an invasive tumor of the posterior mediastinum and after other findings as a GIST, which had a rapid clinical course with the development of lung metastasis and death after 8 months. This rapid clinical course made it difficult to obtain a final diagnosis. Therefore, during the autopsy, a molecular analysis was carried out using NGS which allowed us to highlight the presence of somatic, germline and copy number variation (CNV) mutations in genes related to PGL, such as *RET*, *NF1* and *FH*. This suggests that the tumor was probably not a GIST but a PPGL (12).

For these reasons, it is recommended to perform genetic testing for certain groups at high risk of hereditary PPGLs, which have a positive family history, the presence of multiple syndromic features, early onset, multiple primary PPGLs, malignancy, extra-adrenal locations, or a combination of these features (13). This study aimed to identify the molecular diagnosis of enrolled participants and other driver mutations related to PPGL. Furthermore, the clinical protocol used in this study aimed to improve the detection rate of rare genetic variants in our cohort and analyze the clinical characteristics of the patients. Gaining knowledge about the presence of pathogenic/likely pathogenic (PLP) variants has a significant impact on targeted therapies, but also on the involvement of the relatives in genetic counseling.

Materials and methods

Study cohort. In this study, 28 patients were recruited between 2016/01/01 and 2023/10/31 from the Endocrinology and Hypertension Center of the AUSL-IRCCS of Reggio Emilia. The inclusion criteria for the suspicion of PPGLs and admission to germline genetic testing were patients with: A family history of PPGLs; or the presence of symptoms of spontaneous PPGLs caused by using drugs or other triggers such as cardiovascular events; or adrenal incidentalomas (with or without hypertension) with density ≥10 HU; or thin patients (BMI <25 kg/m^2) with type 2 diabetes mellitus with or without signs of catecholamine excess. The written informed consent was obtained from all participants, in accordance with the guidelines on good clinical practice (DL 06/11/2007) and the European General Data Protection Regulation (EU GDPR 2016/679). The protocol of this study was approved by the competent ethics committee of the Area Vasta Emilia Nord (approval no. 98/2015/TESS/AUSLRE).

NGS testing. DNA for sequencing analysis was extracted from a peripheral blood sample of selected patients using the Maxwell® 16 LEV Blood DNA Kit (AS1290; Promega Corporation) according to the manufacturer's protocol on the Maxwell® RSC instrument (Promega Corporation) according to the manufacturer's specifications.

The TruSight One clinical exome panel (Illumina), a targeted sequencing panel, was employed to evaluate germline mutations in PPGL-related genes filtered across 4,813 genes with known associated clinical phenotypes. Library preparation was performed using Illumina DNA Prep with Enrichment, a fast hybrid capture-based target enrichment that combines on-bead tagmentation with a single hybridization protocol. The chemistry integrates the DNA extraction, fragmentation, library preparation, and library normalization steps according to the manufacturer's protocol (M-GL-02149; Illumina). The libraries were subjected to sequencing on a MiSeq instrument (Illumina) using the 600-cycle (2x150 paired ends) MiSeq v3 Reagent Kit v3 (Illumina) to analyze 3 samples simultaneously (14). The result was ≥20X coverage on 95% of the target regions in the panel, calculated by averaging the mean coverage for each exon.

Variant filtering, interpretation of clinical significance, and statistical significance. The BaseSpace pipeline and Illumina VariantStudio v3.0 were used for the variant calling and annotation in the analysis. The data were aligned to the GRCh37/hg19 human reference genome (14).

Variants were annotated according to the Human Genome Variation Society (HGVS) and the analysis was filtered and performed only for genes related to PPGLs as reported in Table I. The interpretation of variants was performed based on available data in the scientific literature, from the ClinVar (15), Franklin by Genoox (16), and COSMIC (17) databases.

The association between age and variant classification was assessed by boxplot of the Kruskal-Wallis test. $P<0.05$ was considered to indicate a statistically significant difference. The analysis was performed with R 4.3.0.

Results

Patient characteristics. In this study, a total of 28 patients were included, of which 12 were male and 16 were female. The age at the time of diagnosis ranged from 18 to 78 years (median 57), with a mean of 53.7 years. There was no

Table I. Genes filtered from Trusight One Clinical Exome Gene Panel to analyze patients with PPGL (Illumina).

Gene	Gene function	Correlated pathologies
RET	Encodes a single-pass transmembrane receptor tyrosine kinase	Papillary thyroid carcinoma, medullary thyroid carcinoma, multiple endocrine neoplasia type 2 syndromes
FH	Encodes the enzyme fumarate hydratase, which helps convert a molecule called fumarate to a molecule called malate	Hereditary leiomyomatosis and renal cell cancer, primary macronodular adrenal hyperplasia
VHL	VHL protein is classified as a tumor suppressor	Von Hippel-Lindau syndrome, familial erythrocytosis, paragangliomas or pheochromocytomas, kidney cancer
SDHA	One of four subunits of the SDH enzyme	Hereditary paraganglioma-pheochromocytoma, Leigh syndrome, gastrointestinal stromal tumor
SDHB	One of four subunits of the SDH enzyme	Cowden syndrome, renal cell carcinoma, non-syndromic paraganglioma or pheochromocytoma, hereditary paraganglioma-pheochromocytoma, gastrointestinal stromal tumor
SDHC	One of four subunits of the SDH enzyme	Cowden syndrome, hereditary paraganglioma pheochromocytoma, gastrointestinal stromal tumor
SDHD	One of four subunits of the SDH enzyme	Cowden syndrome, hereditary paraganglioma-pheochromocytoma, gastrointestinal stromal tumor, non-syndromic paraganglioma or pheochromocytoma
NF1	Encodes the neurofibromin 1 protein that is implicated in negative regulation of RAS transduction.	Neurofibromatosis type 1, cholangiocarcinoma, lung cancer, juvenile myelomonocytic leukemia
TMEM127	This gene encodes a transmembrane protein with four predicted transmembrane domains	Paraganglioma or pheochromocytoma
MAX	Involved in regulating cellular proliferation, differentiation and apoptosis	Paraganglioma or pheochromocytoma
HIF2A	This gene encodes a transcription factor involved in the induction of genes regulated by oxygen	Erythrocytosis familial type 4

SDH, succinate dehydrogenase.

significant difference in age between male and female patients. Table II contains the clinical information for all 28 participants. Among them, 23 had unilateral pheochromocytoma (PCC), while two had bilateral and one had multifocal tumors. None of the patients had head and neck paraganglioma (HNPGL), but 3 had paraganglioma (PGL). Although one patient had a family history of PCC or PGL, 7 patients had a history of familiarity with other types of tumors. Only one patient had a metastatic lesion, and 2 patients experienced recurrence during the follow-up period. One patient underwent chemotherapy, while 26 patients received surgical intervention.

Results of genetic testing in genes related to PPGL. Of the 28 patients analyzed, 10 patients had at least one significant variant of the 11 genes (*RET, FH, VHL, SDHA, SDHB, SDHC, SDHD, NF1, MAX, HIF2A, TMEM127* and *TP53*) related to PPGLs. NGS analysis revealed 6 germline PLP variants in 5 of the 28 participants, while 6 variants of unknown significance (VUS) were found in 5 other patients. All variants of interest are reported in Table III. Most PLP variants were in the *VHL* gene. The pathogenic variants of this gene are frequently associated with Von Hippel-Lindau disease (18), a multisite tumor predisposing syndrome, including PCC (19) and PGL. All *VHL* variants were detected in patients with no mutations in other genes. The information in the literature about the 3 *VHL* variants found correlates perfectly with the clinical characteristics of each patient. As in the literature, *VHL* c. 203C>T (p. Ser68Leu) was observed in a patient with unilateral PCC (uPCC). Following the American College of Medical Genetics and Genomics (ACMG) classification criteria, the *SDHA*, *SDHB*, and *SDHAF2* variants were submitted as VUS. These *SDH* gene family variants make up half of the VUS identified by analysis. A benign variant was found in *TP53* (p.Pro72Arg), and the literature rarely associates *TP53* with PPGLs. Since *TP53* mutations are known to cause aggressive malignancies, discovering a pathogenic *TP53* variant could significantly impact treatment decisions and serve as a potential additional risk factor for the malignant development of tumors (20,21). The NGS panel had an overall detection rate (DR) of 18% for both pathogenic variants and VUS (Table SI).

Table II. Clinical characteristics of the patients included in the study.

Code	Sex	AoD, years	uPCC	Bil	HNPGL	PGL (others)	Maximum tumor size, cm^3	Meta	Multi focal	Hormone type	S	C	R	Fam history of PPGL	Fam history of other cancer	Variant of interest
1	M	62	Y	N	N	N	5.4	N	N	NE/NM	Y	N	N	N	Y	
2	F	27	Y	N	N	N	9.5	N	N	NA	Y	N	N	N	N	SDHBp.Cys191Tyr, FH p.Arg101Leu
3	M	56	Y	N	N	N	1	N	N	NA	Y	N	N	Y	N	
4	F	45	Y	N	N	N	10.5	N	N	E/M	Y	N	N	N	N	
5	M	20	N	Y	N	Y	1	N	Y	NA	Y	N	Y	N	N	VHL p.Arg200Trp
6	F	57	Y	N	N	N	3	N	N	E/M	Y	N	N	N	Y	
7	F	42	Y	N	N	N	3.9	N	N	E/M	Y	N	N	N	N	
8	F	33	Y	N	N	N	7	N	N	E/M	Y	N	N	N	N	
9	F	59	Y	N	N	N	4.5	N	N	NE/NM	Y	N	N	N	N	VHL p.Val84Leu
10	M	18	Y	N	N	N	3.5	N	N	NE/NM	Y	N	N	N	N	SDHAF2 p.Tyr105Cys, NF1 p.Ile1482Thr
11	M	74	Y	N	N	N	10	Y	N	NE/NM	Y	Y	N	N	N	FH p.Ile116Phe
12	M	65	Y	N	N	N	3	N	N	NA	Y	N	N	N	Y	
13	M	51	Y	N	N	N	3.5	N	N	E/M	Y	N	N	N	N	
14	F	72	Y	N	N	N	2.9	N	N	NE/NM	Y	N	Y	N	N	
15	F	57	N	N	N	Y	4.7	N	N	NE/NM	Y	N	N	N	Y	SDHA p.Ala655 HisfsTer51
16	F	75	Y	N	N	N	5	N	N	E/M	Y	N	N	N	N	
17	M	78	Y	N	N	N	2.4	N	N	NE/NM	Y	N	N	N	Y	
18	F	77	Y	N	N	N	3	N	N	E/M	Y	N	N	N	Y	
19	M	58	Y	N	N	N	3.2	N	N	NE/NM	N	N	N	N	N	SDHB p.Ala25Pro
20	M	50	Y	N	NA	NA	40	N	N	NE	Y	N	N	N	NA	
21	F	76	Y	N	NA	NA	12	N	N	NA	Y	NA	N	N	NA	
22	F	48	Y	N	NA	NA	15	N	N	NE	Y	NA	NA	NA	NA	VHL p.Ser68Leu
23	M	60	Y	NA	NA	NA	28	N	N	NE/NM	Y	NA	N	N	NA	
24	F	59	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	RET p.Lys722Glu
25	F	61	Y	N	N	N	19	N	N	NE	Y	NA	N	N	Y	
26	F	34	Y	N	NA	NA	40	N	N	NE	Y	N	N	N	NA	
27	M	59	NA	NA	N	Y	10	N	NA	NA	Y	NA	NA	N	N	
28	F	53	N	Y	N	N	20	N	N	NA	Y	NA	NA	NA	NA	RET p.Cys618Ser

AoD, age of diagnosis; uPCC, unilateral pheochromocytoma; Bil, bilateral; HNPGL, head and neck paraganglioma; PGL, paraganglioma; Meta, metastasis; NA, not available; N, not present; Y, present; E/M, epinephrine/metanephrine; NE/NM, norepinephrine/normetanephrine; S, surgery; C, chemotherapy; R, recurrence.

Table III. Pathogenic and likely pathogenic variants in high- and medium-risk genes for PPGLs were identified according to the ACMG classification criteria.

Code	Gene	Transcript variant	Protein variant	dbSNP ID	ACMG
2	*SDHB*	NM_003000.2:c.572G>A	NP_002991.2:p.Cys191Tyr	rs2077978456	Likely pathogenic
	FH	NM_000143.3:c.302G>T	NP_000134.2:p.Arg101Leu	NA	Likely pathogenic
5	*VHL*	NM_000551.3:c.598C>T	NP_000542.1:p.Arg200Trp	rs28940298	Pathogenic
9	*VHL*	NM_000551.3:c.250G>T	NP_000542.1:p.Val84Leu	rs5030827	Pathogenic
22	*VHL*	NM_000551.3:c.203C>T	NP_000542.1:p.Ser68Leu	rs869025617	Pathogenic
28	*RET*	NM_020975.6:c.1852T>A	NP_066124.1:p.Cys618Ser	rs76262710	Pathogenic
10	*SDHAF2*	NM_017841.2:c.314A>G	NP_060311.1:p.Tyr105Cys	rs1402726087	VUS
	NF1	NM_001042492.2:c.4445T>C	NP_001035957.1:p.Ile1482Thr	rs746994734	VUS
11	*FH*	NM_000143.3:c.346A>T	NP_000134.2:p.Ile116Phe	rs201532589	VUS
15	*SDHA*	NM_004168.2:c.1962_1963delTG	NP_004159.2:p.Ala655Hisfs Ter51	rs2075912	VUS
19	*SDHB*	NM_003000.2:c.73G>C	NP_002991.2:p.Ala25Pro	rs768101924	VUS
24	*RET*	NM_020975.4:c.2164A>G	NP_066124.1:p.Lys722Glu	rs1262183810	VUS

PPGLs, pheochromocytoma and paraganglioma; dbSNP, single nucleotide polymorphism database; ACMG, American College of Medical Genetics and Genomics; VUS, variant of unknown significance; NA, not available.

Data validation of NGS has also been carried out using Sanger sequencing (Fig. S1). In Sanger sequencing results it was possible see the order of nucleotide bases in a specific amplified segment of DNA sample. The principle of this technology use a light detectors that collect the fluorophore nucleotide information from the DNA passing through the capillary. This information is translated into a chromatogram visualized using Sequencher Software. Sanger Sequencing were performed on variant classified as PLP or VUS in NGS to validate gene variants calling. The Sanger allows to validate with an IVD device complies with the European In-Vitro Diagnostic Devices Directive (IVDD 98/79/EC). In Fig. S1 is possible to see following variants confirmed: SDHBp. Cys191Tyr (Fig. S1A), FH p.Arg101Leu (Fig. S1B), VHL p.Arg200Trp (Fig. S1C), VHL p.Val84Leu (Fig. S1D), NF1 p.Ile1482Thr (Fig. S1E), FH p.Ile116Phe (Fig. S1F), SDHB p.Ala25Pro (Fig. S1H), VHL p.Ser68Leu (Fig. S1I), RET p.Lys722Glu (Fig. S1J), RET p.Cys618Ser (Fig. S1K). The overlap of two peaks show in the position of nucleotide variant in heterozygosis.

Instead, the variant SDHA p.Ala655HisfsTer51 is a frameshift variant due to a deletion of two bases (c.1962_1963del CTG>C). In the chromatogram (Fig. S1G) is possible observe the deletion of the bases that cause the overlap in the sequence.

Correlation between age and pathogenic variants. Patients were divided into three age subgroups: 18-40 years (n=5), 41-59 years (n=14), and over 60 years (n=9). The PLP variants were 7% in the 18-40 age subgroup, 11% in the 41-59 age subgroup, while no PLP variants were identified in patients over 60 years old. Despite the distribution, statistical analysis revealed no significant association between the presence of pathogenic variants and age (P=0.197). The absence of significance may be attributed to the limited number of enrolled patients (Figs. 1 and S2).

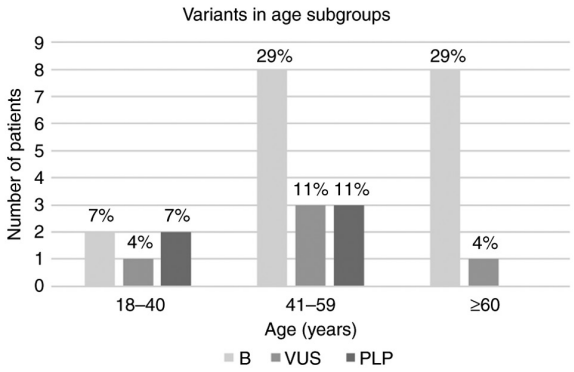

Figure 1. Identification of variants in age subgroups. For each age subgroups the histogram represent the number of the different variant. Furthermore, the percentage of the total is reported. B, benign; PLP, pathogenic/likely pathogenic; VUS, variant of unknown significance.

Discussion

The increasing use of NGS in the routine clinical setting greatly facilitates genetic diagnosis and improved personalized management of hereditary cancer syndromes. Knowledge of heritable mutations has important implications for surveillance and monitoring of probands and their family members, and multi-gene screening should become part of routine care for patients with tumors. Although uncommon, PPGLs are often associated with germline mutations. Given the availability of rapid genetic sequencing, screening of both the patient and, subsequently, their relatives becomes a crucial step in ensuring lifelong vigilance against these potentially life-threatening tumors.

Advancements in genetic profiling have accelerated the discovery of distinct clinical, biochemical, and imaging hallmarks for PPGL diagnosis, management, and long-term follow-up (22,23).

This study was started in 2015, with the aim of using a clinical exome panel to efficiently explore genetic variants associated with various diseases by selecting different genes by means of a single panel.

Among the 28 patients, only 5 were found positive for PLP variants (5/28=18%). This outcome allows for two considerations. First, the low frequency of significant and pathogenic variants in our cohort underlines the importance of expanding the list of genes analyzed in order to understand the hereditary correlation of tumors (4).

Furthermore, younger age at tumor manifestation was associated with the presence of germline mutations as reported in the literature (5,24). However, in our results there is no significant P-value between age and the presence of germline PLP mutations, the germline test is recommended when PPGLs are diagnosed at a young age (25).

Considering this information, it might be appropriate to re-evaluate the inclusion criteria for hereditary genetic testing, and it is crucial to perform NGS testing on DNA extracted from tissue biopsies to check for the presence of somatic variants that may have caused the disease and understand its etiology in PPGL cases.

Our data present similarities with other studies in the literature. Several studies have used NGS approaches such as whole exome sequencing (WES) and targeted gene panels to study PPGL patients (3,13,26).

Sarkadi *et al* (13) in their study considered debated the use of WES in analysis of PPGLs. They applied in clinical practice the target gene panel (ENDOGENE) and WES for discovering mutations of PPGLs associated genes. They compared different pipeline analysis to find an optimization of bioinformatics pipeline. Variants were correctly identified by all methods, but the different accuracy of results and data were affected by methods of libraries preparation, sequencing platform and bioinformatical settings. WES highlights mutations in SDHs gene family with already known *SDHB* p.Cys196Gly and p.Cys196Arg mutations. Instead, ENDOGENE targeted gene panel was evaluated for cost effect analysis in comparison with WES. For the first time, they tested the panel effectiveness on samples with known mutations and diagnosis. All known mutations were successfully identified. Following, they used the panel for prospective cohort. In this cohort, they detected and confirmed in sanger sequencing mutations in *SDHs* family, *FH, NF1, TMEME127* and *VHL* genes. Variants p.Thr88Ile and p.Arg90Gly on *SDHB* gene were detected in a patient with another PLP variant p.Arg91del (13).

In another interesting study (26), authors analyzed 23 patients carrying germline mutation of NF1 using a targeted genes panel for PPGLs. Mellid *et al* (26) used a TruSight oligo probes panel designed on 33 PPGL related-genes (*CSDE1, KIF1B, SDHA, SDHB, SDHC, SDHD, SDHAF1, SDHAF2, EGLN1, EGLN2, FH, MDH2, DLST, IDH2, IDH1, TMEM127, VHL, MET, RET, PTEN, HRAS, MEN1, KRAS, MAX, GOT2, NF1, PRKAR1A, ATRX, BRAF, EPAS1, SLC25A11, DNMT3A, H3F3A*). The analysis showed three germline variants, one in *DLST* (p.Gly374Glu) and two in the *MDH2* (p.Lys314Met) gene. In addition, in the cohort of 23 patients analyzed, two somatic mutations were identified in *H3-3A* (p.Gly35Trp) and *PRKAR1A* (p.Arg16Ter).

Furthermore, they analyzed other 3 patients, not included in the initial cohort, with *NF1* somatic mutations. Each of these patients presented a different mutation. One patient carrying a *SDHB* germline mutation (c.423+1 G>A), one patient a germline p.Gly374Glu mutation on *DLST* gene, and one patient an *ATRX* somatic truncating mutation (p.Arg808Ter).

They underlined how the co-occurrence of an alteration in *NF1* (present in the cohort of patients under investigation) with mutations present in other genes involved in different signaling cascades (e.g. pseudohypoxic signaling), as identified by the authors in their study (*SDHB, ATRX, DLST , MDH2;H3-3A, PRKAR1A*), could contribute to the development of PPGL. It could explain incomplete penetrance of variants observed in some cases.

Our results and the results presented in papers of Sarkadi *et al* (13) and Mellid *et al* (26) are obtained by targeted sequencing approach. Between one study and another, change the number of genes included in the panel. The number of genes included could be different cause different needs of laboratories. The focus for PPGLs related genes, genes selected for the analysis, are in general similar (*KIF1B, SDHA, SDHB, SDHC, SDHD, SDHAF1, SDHAF2, EGLN1, EGLN2, FH, IDH2, IDH1, TMEM127, VHL, MET, RET, PTEN, HRAS, MEN1, KRAS, MAX, NF1).* All studies are conducted to be applied in clinical practice. Authors of the two studies cited above, like our laboratory, sequenced the targeted panel using MiSeq sequencer (Illumina). Furthermore, Mellid *et al* (26) and we have aligned the sequences in Variant Studio (Illumina). Variants were filtered for good quality and depth criteria and variants annotation was carried out with ClinVar, Polyphen and other applications. Sanger sequencing was performed to confirm variants in our study but also in the study of Sarkadi *et al* (13). All three studies highlighted that the principal mutated genes in PPGLs patients belong to *SDH* gene family, *NF1, FH* and *VHL*. The mean of the number of patients in other studies (3,4,26) are comparable with our study. Sarkadi *et al* (13) analyzed in total 37 patients, Mellid *et al* (26), 23 patients, in our study we evaluated 28 patients. There is a difference in the patient cohort used in the study by Gómez *et al* (3), who studied 4 families with PPGLs focusing on the germline mutations in *SDH* family genes, in order to construct a family clinical picture. They lead a retrospective study involved families diagnosed with PPGLs, collecting clinical phenotype and genetic data of the patients. They analyzed the DNA extracted from peripheral blood with a NGS panel including the *SDH* genes family. In the families involved in this study the authors found *SDHB* and *SDHD* germline mutations. In our patients, we found different pathogenic variant in *SDH* genes family (4/28=14.3%) and it underline the importance and the role of this gene in the pathology. Gómez *et al* highlight also that the early detection of variants was useful for treatment and surveillance but also for reducing the morbidity and mortality related to the disease (3).

Our study has several limitations, the principal one being the relatively small sample size, in addition to the high cost of this type of NGS test. Also, the collected data currently lack sufficiency for robust statistical analyses. However, we believe that the results can be improved through the careful selection of patients who are candidates for genetic testing and with

continuous collaboration and communication of the results between all specialists and healthcare workers involved.

The identification of a germline mutation in patients with apparently sporadic PPGLs could lead to an early diagnosis of multiple or more aggressive tumors or other neoplastic syndromes in patients. A germline molecular diagnosis not only facilitates an accurate risk assessment for PPGLs and related malignancies in both the probands and their relatives, but it also enhances the development of targeted primary and secondary prevention programs tailored to these high-risk groups.

Genetic testing panels proved to be an important tool for the identification of pathogenic variants in patients that required the exploration of multiple genes to identify the cause of their disease. Our study underlines the importance of collaborative efforts among clinicians, physicians, geneticists, laboratory staff, bioinformatics specialists, and molecular biologists that are essential for the accurate assessment and interpretation of genetic testing results in the field of hereditary cancer syndromes. It is important to implement a pathway for molecular identification of inherited cancer syndromes in collaboration among the facility's departments.

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Availability of data and materials

The sequencing data generated in the present study may be found in the SRA database (National Library of Medicine-NCBI) under accession number PRJNA1179733 or at the following URL: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1179733. All other data (including. vcf files) generated in the present study may be requested from the corresponding author.

Authors' contributions

BM performed the experiments and wrote the manuscript. DN, AN, AF, CG, MB and EF conceived the present study. VYC, AP, AN, CG and SR supervised the manuscript and contributed to data interpretation. LF, FC and SM performed some experiments. AP, AN, AF, MB and CG enrolled the patients in the study and collected the informed consent. LB performed statistical analysis. BM, DN and VYC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The protocol of this study was approved by the competent ethics committee of the Area Vasta Emilia Nord (approval no. 98/2015/TESS/AUSLRE). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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