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PheoSeq

A Targeted Next-Generation Sequencing Assay for Pheochromocytoma and Paraganglioma Diagnostics

Maria Currás-Freixes,[*] Elena Piñeiro-Yañez,[†] Cristina Montero-Conde,[*] María Apellániz-Ruiz,[*] Bruna Calsina,[*] Veronika Mancikova,[*] Laura Remacha,[*] Susan Richter,[‡] Tonino Ercolino,[§] Natalie Rogowski-Lehmann,[¶] Timo Deutschbein,[‖] María Calatayud,[**] Sonsoles Guadalix,[**] Cristina Álvarez-Escolá,[††] Cristina Lamas,[‡‡] Javier Aller,[§§] Julia Sastre-Marcos,[¶¶] Conxi Lázaro,[‖‖] Juan C. Galofré,[***] Ana Patiño-García,[†††] Amparo Meoro-Avilés,[‡‡‡] Judith Balmaña-Gelpi,[§§§] Paz De Miguel-Novoa,[¶¶¶] Milagros Balbín,[‖‖‖] Xavier Matías-Guiu,[****†††††] Rocío León,[*] Lucía Inglada-Pérez,[*‡‡‡‡] Rafael Torres-Pérez,[*] Juan M. Roldán-Romero,[*] Cristina Rodríguez-Antona,[*‡‡‡‡] Stephanie M.J. Fliedner,[§§§§] Giuseppe Opocher,[¶¶¶¶] Karel Pacak,[‖‖‖‖] Esther Korpershoek,[*****] Ronald R. de Krijger,[*****†††††] Laurent Vroonen,[‡‡‡‡‡] Massimo Mannelli,[§] Martin Fassnacht,[‖] Felix Beuschlein,[¶] Graeme Eisenhofer,[‡§§§§§] Alberto Cascón,[*‡‡‡‡] Fátima Al-Shahrour,[†] and Mercedes Robledo[*‡‡‡‡]

From the Hereditary Endocrine Cancer Group and the Translational Bioinformatics Unit,[†] Spanish National Cancer Research Centre, Madrid, Spain; the Institute of Clinical Chemistry and Laboratory Medicine,[‡] University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technische Universitat Dresden, Dresden, Germany; the Department of Experimental and Clinical Biomedical Sciences Mario Serio,[§] University of Florence and Istituto Toscano Tumori, Florence, Italy; the Department of Internal Medicine IV Campus Innenstadt,[¶] University-Hospital, Ludwig-Maximilians-University of Munich, Munich, Germany; the Department of Internal Medicine I,[‖] Division of Endocrinology and Diabetes, University Hospital, University of Würzburg, Würzburg, Germany; the Department of Endocrinology and Nutrition Service,[**] University Hospital 12 de Octubre, Madrid, Spain; the Department of Endocrinology,[††] University Hospital La Paz, Madrid, Spain; the Department of Endocrinology,[‡‡] Albacete University Hospital Complex, Albacete, Spain; the Department of Endocrinology,[§§] University Hospital Puerta de Hierro, Madrid, Spain; the Department of Endocrinology,[¶¶] Virgen de la Salud Hospital–Toledo Hospital Complex, Toledo, Spain; the Molecular Diagnostics Units of the Hereditary Cancer Program at the Catalan Institute of Oncology,[‖‖] Barcelona, Spain; the Department of Endocrinology[***] and the Department of Pediatrics and Clinical Genetics Unit,[†††] University of Navarra Clinic, Navarra, Spain; the Department of Endocrinology,[‡‡‡] Reina Sofia University Hospital, Murcia, Spain; the High Risk and Cancer Prevention Group,[§§§] Medical Oncology Department, Vall d'Hebron University Hospital and Vall d'Hebron Institute of Oncology, Barcelona, Spain; the Department of Endocrinology,[¶¶¶] San Carlos (Clínico) Hospital, Madrid, Spain; the Department of Molecular Oncology,[‖‖‖] Central University Hospital of Asturias and University Institute of Oncology of Asturias, University of Oviedo, Oviedo, Spain; the Department of Endocrinology and Nutrition,[****] University Hospital Arnau de Vilanova, IRBLLEIDA, Lleida, Spain; the Department of Pathology,[††††] Hospital Universitari de Bellvitge, IDIBELL, Barcelona, Spain; Biomedical Research Networking Center on Rare Diseases (CIBERER)[‡‡‡‡] Madrid, Spain; the 1st Department of Medicine,[§§§§] University Medical Center Schleswig-Holstein, Campus Lübeck, Lübeck, Germany; the Department of Endocrinology,[¶¶¶¶] Department of Medical and Surgical Sciences University of Padova, Padova, Italy; the Section on Medical Neuroendocrinology,[‖‖‖‖] Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, Maryland; the Department of Pathology,[*****] Erasmus University Medical Center, Rotterdam, the Netherlands; the Department of Pathology,[†††††] Reinier de Graaf Hospital, Delft, the Netherlands; the Department of Endocrinology,[‡‡‡‡‡] Centre Hospitalier Universitaire de Liège, Liège, Belgium; and the Department of Medicine III,[§§§§§] University Hospital Carl Gustav Carus, Dresden, Germany*

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Address correspondence to Mercedes Robledo, Ph.D., Hereditary Endocrine Cancer Group Leader, Centro Nacional de Investigaciones Oncológicas, C/Melchor Fernández Almagro 3, 28029 Madrid, Spain. E-mail: mrobledo@cnio.es.

Genetic diagnosis is recommended for all pheochromocytoma and paraganglioma (PPGL) cases, as driver mutations are identified in approximately 80% of the cases. As the list of related genes expands, genetic diagnosis becomes more time-consuming, and targeted next-generation sequencing (NGS) has emerged as a cost-effective tool. This study aimed to optimize targeted NGS in PPGL genetic diagnostics. A workflow based on two customized targeted NGS assays was validated to study the 18 main PPGL genes in germline and frozen tumor DNA, with one of them specifically directed toward formalin-fixed paraffin-embedded tissue. The series involved 453 unrelated PPGL patients, of whom 30 had known mutations and were used as controls. Partial screening using Sanger had been performed in 275 patients. NGS results were complemented with the study of gross deletions. NGS assay showed a sensitivity ≥99.4%, regardless of DNA source. We identified 45 variants of unknown significance and 89

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pathogenic mutations, the latter being germline in 29 (7.2%) and somatic in 58 (31.7%) of the 183 tumors studied. In 37 patients previously studied by Sanger sequencing, the causal mutation could be identified. We demonstrated that both assays are an efficient and accurate alternative to conventional sequencing. Their application facilitates the study of minor PPGL genes, and enables genetic diagnoses in patients with incongruent or missing clinical data, who would otherwise be missed. *(J Mol Diagn 2017, 19: 575—588; http://dx.doi.org/10.1016/j.jmoldx.2017.04.009)*

Pheochromocytomas (PCCs) and paragangliomas (PGLs), altogether PPGLs, are rare neuroendocrine tumors arising from adrenal gland (PCCs), sympathetic thoracoabdominal paraganglia, and head and neck parasympathetic paraganglia.

Since the first description of a PCC by Felix Fränkel,[1] 34 genes have been involved in PPGL development. Thirteen are considered major PPGL driver genes (*NF1*, *RET*, *VHL*, *SDHA, SDHB, SDHC, SDHD, SDHAF2*, *TMEM127*, *MAX*, *HRAS*, *EPAS1,* and *FH*).[2] According to transcriptomic studies, two main clusters of PPGLs have been established: cluster 1, containing *FH*-, *EPAS1*-, *VHL*-, and SDH gene—mutated tumors; and cluster 2, which includes tumors with mutations in *NF1*, *RET*, *TMEM127*, *MAX*, and *HRAS*.[3] The other 21 genes play a minor role in PPGL, as mutations have been described in isolated families (*KIF1B*, *BAP1, EGLN1*,[2] *EGLN2*,[4] and *MDH2*[5]); described in a few sporadic cases (*IDH1*,[6] *MERTK*, *H3F3A*, *SETD2*, *EZH2*, *FGFR1*,[7] and *BRAF*[8]); or mainly reported in patients with mutations in recognized PPGL driver genes, suggesting their secondary role (*ATRX*,[9] *TP53*,[8] *JMJD1C*, *KDM2B*,[7] *KMT2D/MLL2,* and *MET*[10]). PPGLs appear rarely in multiple endocrine neoplasia type 1 (MEN1) syndrome.[11] Finally, promoter alterations in *TERT*,[12] epimutations in *SDHC*,[13] and germline *MITF* mutations[14] have been recently reported.

The high heritability of PPGL, the highest among human neoplasia (40%), and the presence of somatic mutations in 30% to 40% of the cases[15] make the genetic characterization of all patients essential. The identification of the driver gene, the type of mutation (somatic, germline, or mosaicism), and therefore the dysregulated pathway can optimize clinical management strategies in terms of the following: i) diagnosis of associated syndromic tumors and/or features in the patient and relatives; ii) choice of the most appropriated imaging technique[15—17]; iii) assessment of clinical course and outcome; and iv) treatment selection in metastatic or unresectable PPGL.[3,16,18]

Genetic screening algorithms using conventional Sanger sequencing (SS) are still useful, particularly in syndromic cases. However, PPGLs show a high degree of heterogeneity in clinical presentation, and genotype is not always well predicted by phenotype.[19,20] In addition, as the genetic spectrum increases, with newly described genes having low prevalence (<1% of cases) without distinctive clinical features, the genetic diagnosis has become a time- and resource-consuming process. This is particularly problematic in metastatic cases, for which no clear predictors have been found, the 5-year survival is 50%,[18] and the only curative treatment is surgery. After excluding *SDHB*, half of the remaining patients have no genetic diagnosis,[21—23] and the other genes associated with metastatic risk have either a low prevalence (*FH*[24], *MDH2*,[5] or *MAX*[25]) or are large (*NF1*). Thus, a fast and accurate genetic diagnosis is important so that therapeutic approaches targeting the dysregulated pathway can be chosen.

In this context, next-generation sequencing (NGS) technology emerged as a valuable tool. Because of the relatively high cost and the ethical concerns regarding incidental findings, whole-exome sequencing is mainly used in research settings,[5,7,25—28] whereas targeted gene panels (TGPs) have a greater success rate than SS as a diagnostic tool,[29] and are more sensitive for identifying mosaicisms, such as those described in *EPAS1*,[30,31] *NF1*,[32] and *VHL*.[33]

TGPs enable the screening of genes systematically excluded in SS studies because of their large size and facilitate patient selection for the screening of new genes, large rearrangements, or the use of omics platforms to detect mutations beyond coding regions.[10]

This study aimed to perform genetic screening of all PPGL major genes, as well as *MDH2* and three minor genes classically involved in PPGL (*EGLN1*, *EGLN2,* and *MEN1*), using two customized TGPs in 491 DNA samples [obtained from blood, saliva, formalin-fixed paraffin-embedded (FFPE) tissue, and frozen tumor] from 453 PPGL index patients recruited in an international effort to validate their use in the clinical setting.

Materials and Methods

Patients

The genetic screening was performed in a series of 453 index patients affected by PPGL, recruited between 1997

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and 2016 from 10 PPGL referral centers from the European Network for the Study of Adrenal Tumors-ENS@T and one in Bethesda, Maryland.

PPGL diagnosis was based on pathological study and plasma or urine catecholamine and/or metanephrine assessment, as well as imaging tests.

Clinical data and samples were collected as previously described.[20] In brief, data collected included number and tumor location, biochemical phenotype, presence of metastasis, pathological findings, and personal and family history of PPGL or PPGL-related tumors. If available, FFPE tumor slides were evaluated for succinate dehydrogenase complex iron sulfur subunit B (SDHB)-immunohistochemistry (SDHB-IHC) using anti-SDHB rabbit polyclonal antibody (Sigma-Aldrich Corp., St. Louis, MO), as positive staining suggests the absence of a mutation in SDH genes.[34]

Among included patients, 30 carried pathogenic mutations previously detected by SS and were used as controls to validate the NGS assay. The remaining cases consisted of 423 unrelated index patients without a known mutation [wild type (WT)]. Clinical characteristics are summarized in Table 1.

In 305 (72%) of the WT patients, genetic screening by SS had already been partially performed following the previously proposed genetic testing algorithm[20]; the remaining 118 (28%) of the patients had no previous genetic studies.

All patients provided informed consent for germline testing. In addition, tumor tissues from the Erasmus MC (Rotterdam, the Netherlands) were used according to the code of conduct, Proper Secondary Use of Human Tissue, established by the Dutch Federation of Medical Scientific Societies.[35]

Participating Centers

PPGL referral centers from the European Network for the Study of Adrenal Tumors-ENS@T were located in Madrid, Spain (Hereditary Endocrine Cancer Group, Spanish National Cancer Research Center), Florence, Italy (Department of Experimental and Clinical Biomedical Sciences Mario Serio, University of Florence and Tuscan Tumor Institute), Padova, Italy (Endocrinology Unit, Department of Medical and Surgical Sciences, University of Padova), Rotterdam (Department of Pathology, Erasmus University Medical Center), Delft, the Netherlands (Department of Pathology, Reinier de Graaf Hospital), Liège, Belgium (Department of Endocrinology, University of Liège Hospital), Dresden, Germany (Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technical University of Dresden), Lübeck, Germany (First Department of Medicine, University Medical Center Schleswig-Holstein, Campus Lübeck), Munich, Germany (Department of Internal Medicine IV Campus Innenstadt, University-Hospital, Ludwig-Maximilians-University of Munich), Würzburg, Germany (Department of Internal Medicine I, University Hospital Würzburg), and Bethesda (Section on Medical Neuroendocrinology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH).

DNA Samples

A total of 491 DNA samples from 453 index patients were studied. DNA obtained exclusively from tumor was available for 182 (40%) of the cases, matched tumor-germline DNA for 36 (8%) of the patients, and only germline DNA for 235 (52%) of the cases. In the latter group, two patients had germline DNAs from blood and saliva or GenomiPhi. In only two cases, the germline DNA source was saliva. Of 218 tumor samples, 114 (52%) were frozen and 104 (48%) were FFPE.

DNA was extracted from peripheral blood samples following a standard method (FlexiGene DNA Kit; Qiagen, Hilden, Germany). For seven patients, sample material amplified by the Illustra GenomiPhi HY DNA Amplification Kit (GE Health Care Life Sciences, Chicago, IL) was used. DNA samples were obtained from saliva using the Oragene·DNA kit (DNA Genotek, Kanata, ON, Canada), from frozen tumor tissue with the DNeasy Blood & Tissue Kit (Qiagen), and from FFPE tumor tissue with the Covaris S2 System (Covaris, Woburn, MA), in all cases according to the instructions provided by the manufacturer. DNA quality was assessed using the NanoDrop spectrophometer (NanoDrop Technologies, Wilmington, DE), considering an absorbance ratio >1.7 to be acceptable for both 260/280- and 230/260-nm measurements. DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA). The Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA) was used to assess the size and quantity of DNA fragments in FFPE DNA samples.

The selection of representative tumor areas was performed on a paraffin slide stained with hematoxylin and eosin. The study of tumor DNA (if available) was prioritized.

Targeted Gene Panels

Two TGPs were designed using the TruSeq Custom Amplicon 1.5 kit system (Illumina, San Diego, CA), one (P-I) to work with germline and frozen tumor DNA and the other (P-II) to study DNA from FFPE tumor tissues (Table 2). Probes were designed using the online DesignStudio software version 2.2 (Illumina) to capture the coding plus 50-bp intronic flanking regions. Both designs contained *RET, VHL, NF1, MAX, TMEM127, SDHA, SDHB*, *SDHD, SDHC, SDHAF2, MDH2, FH, EPAS1,* and *HRAS*. P-I additionally included *KIF1B, MEN1, EGLN1,* and *EGLN2*. As the involvement of exon 7 in *RET* was not known when TGPs were designed, it was analyzed by SS.[36]

DNA libraries were prepared according to the manufacturer's protocol, and samples were sequenced using the MiSeq platform (Illumina) with a paired-end mode using

Table 1 Clinical Characteristics of the 423 Wild-Type Patients Included in the Study

Characteristics	Value
PPGL patients without a known mutation	$n = 423$
Type of sample available	Only germline DNA, $n = 225$ patients (53%) Germline and tumor DNA, $n = 33$ patients (8%) Only tumor DNA, $n = 165$ patients (39%)
Classification of the patients based on prior analysis of the samples using Sanger sequencing	Yes, $n = 305$ (72%): Only germline DNA, $n = 211$ patients Germline and tumor DNA, $n = 16$ patients Only tumor DNA, $n = 78$ patients No, $n = 118$ (28%): Only germline DNA, $n = 14$ patients Germline and tumor DNA, $n = 17$ patients Only tumor DNA, $n = 87$ patients
Patients with syndromic-related tumors	$n = 13$ 4 medullary thyroid carcinomas or C cell hyperplasia: ID2, D234, ID309, and ID412 3 gastrointestinal stromal tumors: ID79, ID95, and ID450 3 patients with NF1 clinical diagnosis: ID325, ID332, and ID357 3 pituitary adenomas: ID23, ID295, and ID440
Family history	$n = 5$ 1 patient belonging to an MEN2A family: ID 381 2 patients with first-degree relatives diagnosed as having NF1: ID5 and ID91 2 patients with first-degree relatives diagnosed as having PPGL: ID30 and ID106
Sex	Female/male: $n = 243$ (59%)/168 (41%) No data, $n = 12$
Age at onset (years)	Median, 48 (IQR, 38—59) Pediatric cases ($\leq$18 years), $N = 13$
No. and location of tumor	Single, $n = 362$ (88%) PCC, $N = 240$ HN-PGL, $n = 71$ TA-PGL, $n = 49$ Unknown-PGL, $n = 2$ Multiple, $n = 49$ (12%) PCC (bilateral and/or multiple), $n = 17$ PCC and PGL, $n = 10$ PGL, $n = 22$ No data, $n = 12$
Catecholamine phenotype	Adrenergic, $n = 66$ (34%) Noradrenergic, $n = 126$ (65%) Dopaminergic, $n = 1$ (0.5%): ID401 Cosecretion of dopamine and noradrenaline/adrenaline, $n = 10$: ID24, ID107, ID109, ID192, ID284, ID285, ID327, ID402, ID405, and ID 446 Cosecretion of ACTH, $n = 2$: ID108 and ID304 Secretion high, but unspecified, $n = 21$ Nonfunctional, $n = 56$ Not done, $n = 6$ No data, $n = 147$
SDHB immunohistochemistry	Positive, $n = 117$ Negative, $n = 17$ Not evaluable, $n = 2$ No data, $n = 287$
Metastasis	$n = 31$ (7.3%)
Singular pathological features	Black PCC, $n = 2$: ID164 and ID429 Composite tumor with ganglioneuroma, $n = 7$: ID65, ID100, ID209, ID232, ID294, ID306, and ID435 Composite tumor with lymphoma, $n = 1$: ID248 Presence of ACTH in the immunohistochemical study, $n = 3$: ID108, ID304, and ID451

The 30 patients with mutations previously found using Sanger are described in Supplemental Table S4. Composite tumor: tumor with presence of neuroendocrine and neural tumor cells.

ACTH, adrenocorticotropic hormone; HN-PGL, head and neck paraganglioma; IQR, interquartile range; MEN2A, multiple endocrine neoplasia type 2A; NF1, neurofibromatosis type 1; PCC, pheochromocytoma; PGL, paraganglioma; PPGL, pheochromocytoma and/or paraganglioma; SDHB, succinate dehydrogenase complex iron sulfur subunit B; TA-PGL, thoracoabdominal paraganglioma.

Table 2 Characteristics of the Targeted Gene Panels Designed

Characteristics	Panel I	Panel II
Type of sample	Germline and frozen DNA	FFPE DNA
DNA input, ng	150	250
Genes included, *n*	18	14
Common genes included	1. -*RET* (NM_020975.4; ENST00000355710): exon 8 to exon 16; 2. -*VHL* (NM_000551.3; ENST00000256474): promotor to exon 3; 3. -*NF1* (NM_001042492.2; ENST00000358273): all exons; 4. -*MAX* (NM_002382.3; ENST00000358664): exon 1 and exon 3 to exon 5; 5. -*TMEM127* (NM_017849.3; ENST00000258439): exon 2 to exon 4; 6. -*SDHA* (NM_004168.2; ENST00000264932): all exons; 7. -*SDHB* (NM_003000.2; ENST00000375499): all exons;	8. -*SDHD* (NM_003002.2; ENST00000375549): all exons; 9. -*SDHC* (NM_003001.3; ENST00000367975): all exons; 10. -*SDHAF2* (NM_017841.2; ENST00000301761): all exons; 11. -*MDH2* (NM_005918.2; ENST00000315758): all exons; 12. -*FH* (NM_000143.3; ENST00000366560): all exons; 13. -*EPAS1* (NM_001430.4; ENST00000263734): exon 9 and exon 12; 14. -*HRAS* (NM_005343.2; ENST00000451590): exon 2 and exon 3
Additional genes included	15. -*KIF1B* (NM_015074.3; ENST00000263934): all exons; 16. -*MEN1* (NM_000244.3; ENST00000394374): all exons; 17. -*EGLN1* (NM_022051.2; ENST00000366641): all exons; 18. -*EGLN2* (NM_053046; ENST00000406058): exon 2 to exon 6	
Type of design	One strand	Double strand (to avoid deamination artifacts)
Read length, bp	2 × 250	2 × 150
Amplicon length, bp	250	150
Amplicons designed	344	399 (×2)
Samples/flow cell, *n*	96	48

NM and ENST numbers are both available at *http://www.ensembl.org*.
FFPE, formalin-fixed, paraffin-embedded.

MiSeq Reagent Kit V3 (Illumina) and 500 cycles in P-I and 300 cycles in P-II.

NGS Data Analysis

We analyzed sequence data using an in-house pipeline. The workflow is depicted in Figure 1.[37–39]

Sequencing reads were demultiplexed using MiSeq Reporter (Illumina). For raw variant calling, we used Genome Analysis Toolkit v2 in P-I and Somatic Variant Caller in P-II. Variant calling format was annotated using the version 83 of Ensembl Variant Effect Predictor[40] and assembly GRCh37/hg19 of the human reference genome.

In P-II, we doubled checked variants annotated as having a biased prevalence in one of the pools (pool bias), and recovered those previously filtered out because of low coverage in one of the pools if they were detected in at least 20 reads and in 10% of reads. In addition, short insertions/deletions were detected considering a variation cutoff of 15% in the number of reads in consecutive nucleotides. All filtered variants were validated by SS, and the somatic nature was confirmed using constitutional DNA. To avoid false negatives, exons with <50-fold coverage were analyzed by SS in samples without mutations. In addition, as gross deletions cannot be accurately detected by this platform,[29] multiplex ligation-dependent probe amplification (MRC-Holland, Amsterdam, the Netherlands) and/or multiplex PCR were applied to germline DNA if no mutation was found for *VHL*/SDH genes*/FH/MAX/TMEM127/MDH2*.[5,25,29,41–43] In addition, to assess the pathogenicity of variants of unknown significance (VUSs), we performed the immunohistochemistry (in *MAX*,[25] *FH*,[24] and SDH gene[34] variants) and quantitative PCR (in *MDH2* variants),[5] if available.

Results

Technical Assessment and Validation of TGPs

Good amplification quality was obtained for 466 (95%) of the DNA samples, corresponding to 428 (95%) of the patients. The NGS assay failed for the remaining 25 samples, despite libraries being generated twice. Because germline DNA was also available for four of the tumor samples that failed, they were still included in the study (ID47, ID71, ID101, and ID123). Supplemental Table S1 details the number of patients and samples for each step in this study. Supplemental Table S2 details the clinical characteristics of the 21 remaining patients.

The sensitivity of NGS P-I and P-II was assessed based on variants previously found by SS: 534 (73 unique) and 337 (56 unique) for each panel, respectively, and reached

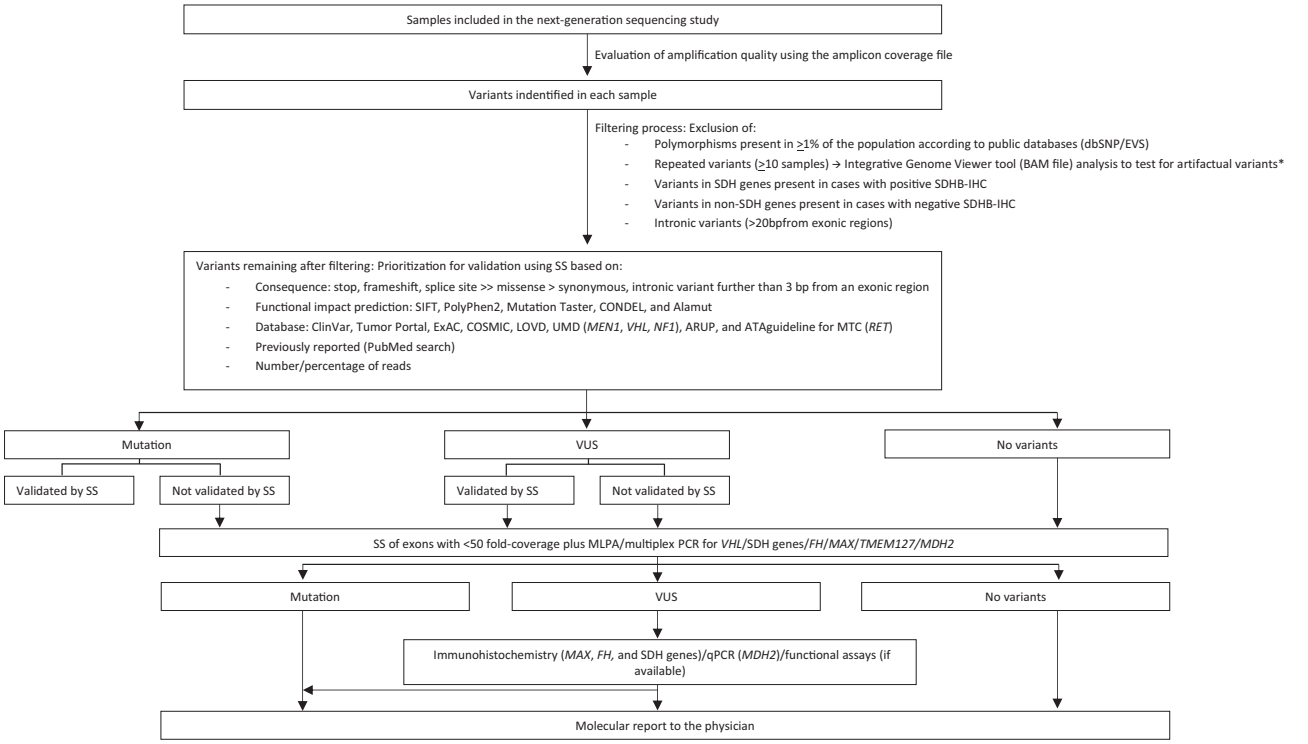

Figure 1 Workflow for next-generation sequencing—based diagnostic testing. *Artifactual variants are those located in GC-rich regions and/or homopolymeric tracts. Alamut Visual software version 2.7 (Interactive Biosoftware, Rouen, France) was used. ClinVar is available at http://www.ncbi.nlm.nih.gov/clinvar; Mutation Taster, http://www.mutationtaster.org; Tumor Portal, http://www.tumorportal.org. All databases were last accessed on October 25, 2016. ARUP, ARUP Scientific Resource for Research and Education (MEN2) *RET* database (http://www.arup.utah.edu/database/MEN2/MEN2_display.php); ATA, American Thyroid Association-Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma; CONDEL, CONsensus DELeteriousness score (http://bg.upf.edu/blog/2012/12/condel-for-prioritization-of-variants-involved-in-hereditary-diseases-and-transfic-for-cancer); COSMIC, Catalog of Somatic Mutations in Cancer (http://cancer.sanger.ac.uk/cosmic); dbSNP, Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/snp); EVS, exome variant server (http://evs.gs.washington.edu/EVS); ExAC, Exome Aggregation Consortium (http://exac.broadinstitute.org); IHC, immunohistochemistry; LOVD, Leiden Open (source) Variation Database (http://www.lovd.nl); MLPA, multiplex ligation-dependent probe amplification assay (MRC-Holland); PolyPhen2, Polymorphism Phenotyping 2 (http://genetics.bwh.harvard.edu/pph2); qPCR, real-time quantitative PCR; SDHB, succinate dehydrogenase complex iron sulfur subunit B; SIFT, Sorting Intolerant From Tolerant (http://sift.jcvi.org); SS, Sanger sequencing; UMD, Universal Mutation Database (http://www.umd.be); VUS, variant of unknown significance.[37–39]

99.6% (P-I) and 99.4% (P-II). The only four variants not detected by TGPs were located in amplicons showing low coverage (≤50 reads): one VUS in *TMEM127* (exon 2) and one single-nucleotide polymorphism in *MDH2* (exon 1) in P-I, and two single-nucleotide polymorphisms in exon 1 of *MDH2* in P-II. The assay was still informative, as 17 single-nucleotide polymorphisms located there were validated (Supplemental Tables S3 and S4).

Considering both panels, 7% of exons included in the design (16/224 of P-I and 11/157 in P-II) showed low coverage, 38% affecting exon 1 of different genes. SS of low-coverage regions did not detect any variant.

In addition, cross-amplification of *SDHA* and *NF1* pseudogenes was ruled out in both panels because 29 *SDHA* and three *NF1* previously known variants were validated by P-I, and 25 *SDHA* variants by P-II. Similarly, 19 *NF1* variants were found using P-II, and validated by SS.

The longest duplication detected was 6 bp in length (*SDHB:* c.424-19_424-14dupTTCTTC) in both panels. The largest deletions identified by P-I and P-II spanned 6 bp (*SDHB*: c.424-19_424-14delTTCTTC) and 22 bp (*NF1*: c.2364_2385delAAAGCTAATCCTTAACTATCCA) in length, respectively. *SDHB* gross deletions were not detected by the NGS assay in a positive control and a new positive case (ID 152).

Genetic Characterization

Detection of Variants in WT Patients

NGS analysis of the properly amplified 403 WT patients revealed 89 pathogenic mutations (71 unique), 29 germline mutations (GMs), 58 somatic mutations (SMs), and two mutations in tumor DNA of patients without germline DNA available. Figures 2 and 3[44] detail mutated cases.

The most frequent germline mutated genes were *SDHB* (2.2%, 9/403) and *SDHD* (1.2%, 5/403), followed by *SDHC, FH, NF1* (0.7%; three mutations in each gene), *SDHA* (0.5%, 2/403), and *SDHAF2, VHL, RET,* and *MAX* (0.25%; one mutation in each gene).

Among the 183 tumor samples, *NF1* was the gene most frequently mutated (14%). SMs in *VHL, HRAS,* and *RET* were found in a similar percentage (6.6%, 5.5%, and 4.4%,

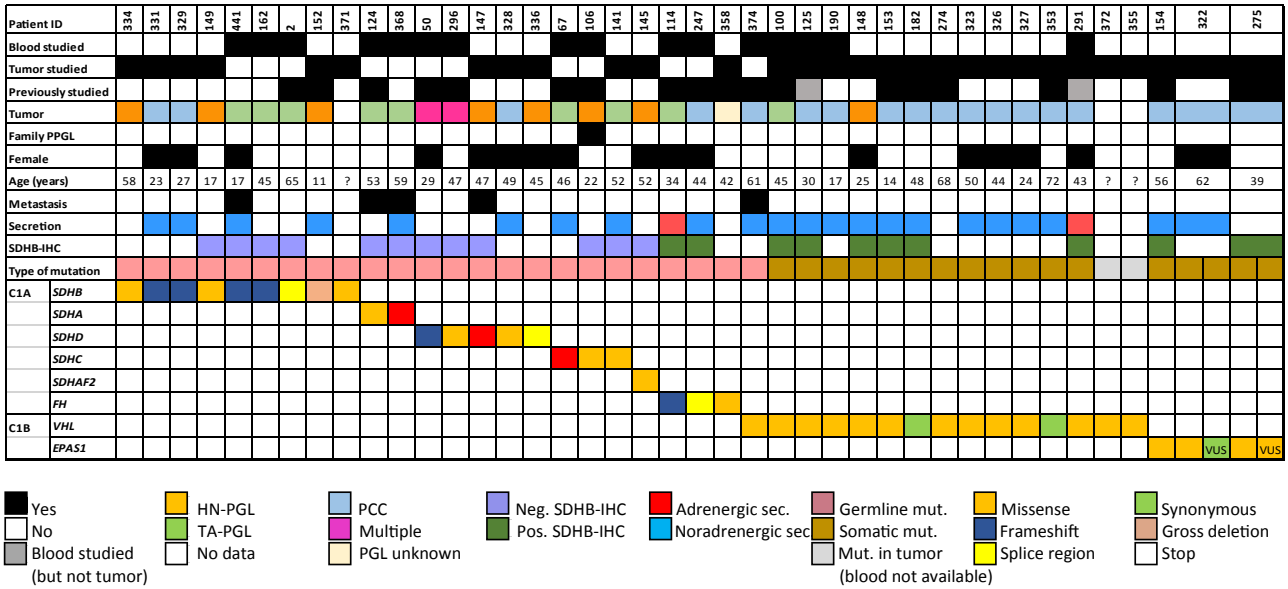

Figure 2 Cluster 1 mutations. Cases categorized as probably germline or somatic are represented as germline and somatic mutations, respectively. ?, data not available; Adrenergic sec., predominantly adrenergic secretion; HN-PGL, head and neck paraganglioma; IHC, immunohistochemistry; mut., mutation; Neg., negative; Noradrenergic sec., predominantly noradrenergic secretion; PCC, pheochromocytoma; Pos., positive; PPGL, pheochromocytoma and/or paraganglioma; SDHB, succinate dehydrogenase complex iron sulfur subunit B; TA-PGL, thoracoabdominal paraganglioma; VUS, variant of unknown significance.

respectively), and *EPAS1* was involved in three (1.6%) of cases. Of note, one germline DNA and five tumors apparently negative by SS showed mutations with a low percentage of reads (<15%) by NGS. A review of the chromatograms and/or second tumor selection confirmed the NGS findings by SS (Supplemental Figure S1).

GMs were more prevalent in cluster 1 genes (83%), whereas SMs predominantly affected cluster 2 genes (74%).

In addition, 45 VUSs (42 as the unique finding) were found, 35 germline and 10 in tumor DNA (two of them SMs). Three VUSs were found in patients carrying pathogenic mutations. Twelve VUSs involved SDH genes, but SDHB-IHC could only be performed in two, strongly arguing against pathogenicity, as the SDHB-IHC result was positive. Other VUSs involved *NF1* ($n = 7$), *FH* ($n = 5$), *MEN1* ($n = 2$), and *RET* ($n = 1$); none of these patients presented with syndromic features. VUSs were also found in *EPAS1* ($n = 4$), *MDH2* ($n = 6$), *KIF1B* ($n = 3$), and *TMEM127* ($n = 2$). A summary of mutations and VUSs is shown in Supplemental Tables S5[24,25,27,28,34,38,44–66] and S6,[11,61,67,68] respectively.

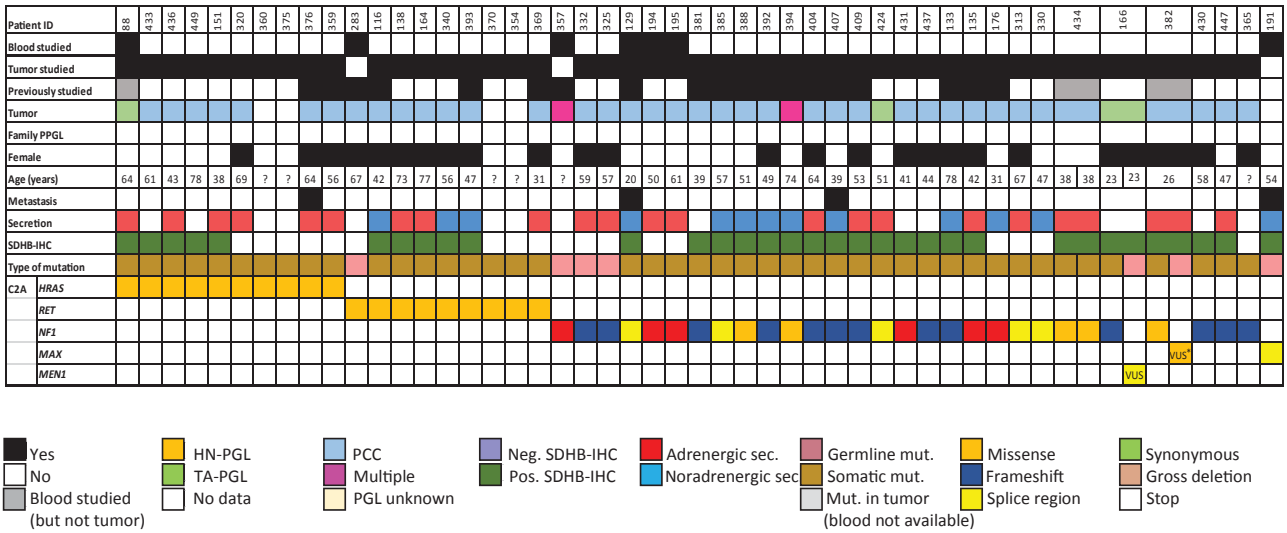

Figure 3 Cluster 2 mutations. Cases categorized as probably germline or somatic are represented as germline and somatic mutations, respectively. One variant of unknown significance (VUS) in *MAX* had been previously reported (marked with an **asterisk**), and functional assays found that it was not pathogenic.[25,44] ?, data not available; Adrenergic sec., predominantly adrenergic secretion; HN-PGL, head and neck paraganglioma; IHC, immunohistochemistry; Mut., mutation; Neg., negative; Noradrenergic sec., predominantly noradrenergic secretion; PCC, pheochromocytoma; Pos., positive; PPGL, pheochromocytoma and/or paraganglioma; SDHB, succinate dehydrogenase complex iron sulfur subunit B; TA-PGL, thoracoabdominal paraganglioma.

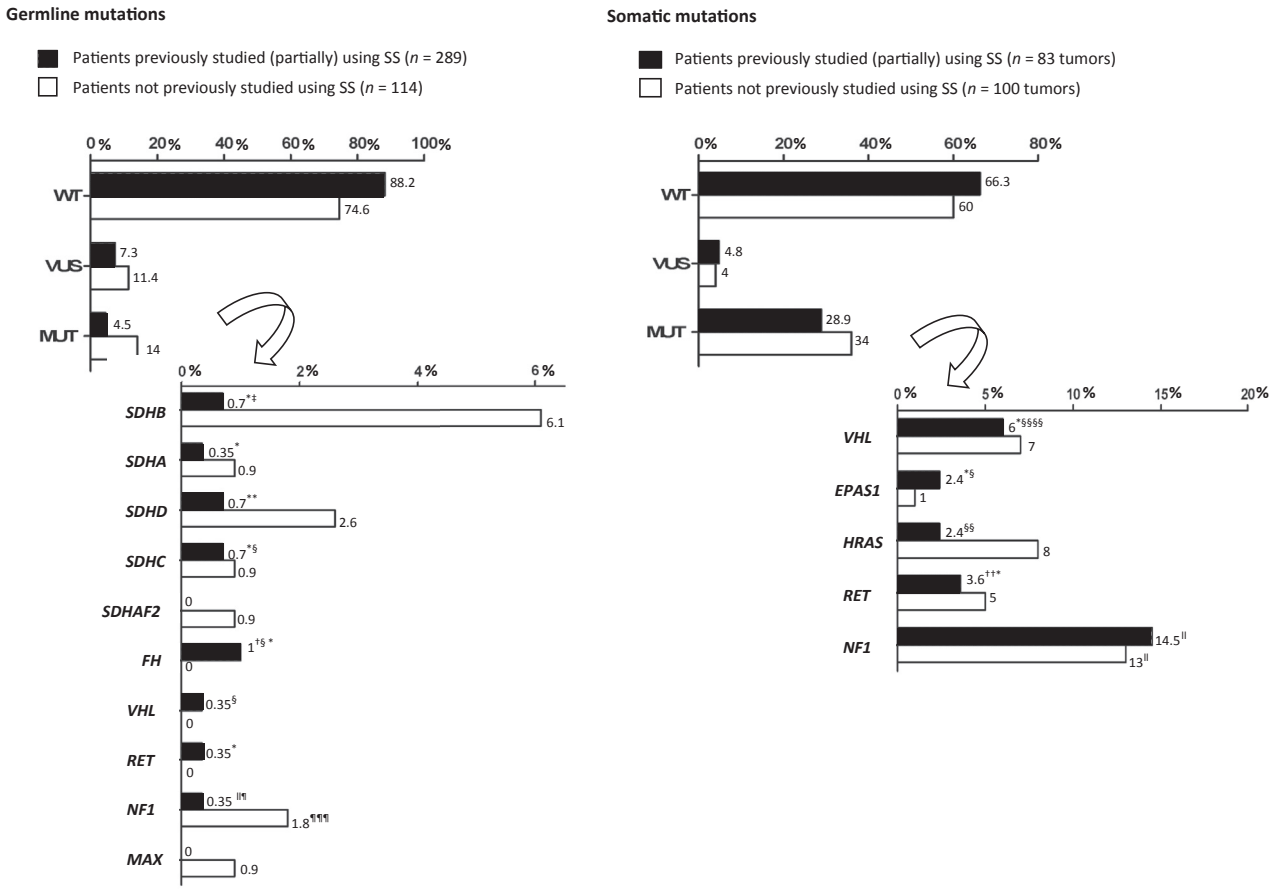

Figure 4 Distribution of mutations according to whether they had been previously studied (partially) using Sanger sequencing. Reason for having not considered the study of the gene found mutated in the cases previously studied using SS: *no predominant biochemical secretion data available; †opposite biochemical secretion data; ‡no blood available previously to perform gross deletions, only frozen tumor; §data from secretion received between SS-Miseq; ¶syndromic features; ‖not previously studied using SS. Each symbol represents a single case within the listed criteria; multiple symbols represent the number of patients with the listed criteria. MUT, mutation; SS, Sanger sequencing; VUS, variant of unknown significance; WT, wild type.

Five VUSs might be pathogenic: an *SDHB* missense variant predicted by *in silico* tools to be deleterious or possibly damaging, a *FH* missense variant associated with positive SDHB-IHC and negative 5-hydroxymethylcytosine IHC, a *RET* synonymous variant described to affect splicing,[69] and two candidate second hit *EPAS1* variants, located close to the hydroxylation site in patients carrying pathogenic *EPAS1* mutations. Further functional assays are required to determine their pathogenicity.

Twenty-four variants reported by NGS were not validated by SS; two were located in homopolymeric tracts in *KIF1B*, and 22 showed low coverage of the variant (<12% and <13 reads), suggesting they were artifacts (Supplemental Table S7).

For 272 patients, no variants were found; tumor DNA was available and correctly amplified for 87 (32%) of these patients.

Mutational Event Distribution according to Previous SS Study

The sample set was divided into two groups: cases previously studied by SS according to our genetic testing algorithm[20] (WT^{ps}; $n = 289$) and patients not previously studied (WT^{notps}; $n = 114$). The distribution of the variants in each group is represented in Figure 4 and Supplemental Figure S2.

As expected, mutations were more frequently found in WT^{notps} (52/114, 46%) than in WT^{ps} (37/289, 13%). Although WT^{notps} had more GM (14%, 16/114) than WT^{ps} (4.5%, 13/289), the percentage of SMs was similar: 34 (34%) of the 100 tumors available in WT^{notps} and 24 (29%) of the 83 WT^{ps} tumors.

Three *FH* mutations (3/289, 1%), two mutations in each of *SDHB*, *SDHD,* and *SDHC* (2/289, 0.7%), and one mutation in each of *SDHA*, *RET,* and *VHL* (1/289, 0.35%) were found among the WT^{ps}. Among WT^{notps}, SDH genes were the major players.

All *NF1* GMs were found in NF1 syndromic patients, and SMs were found in a similar percentage in WT^{ps} (15%) and WT^{notps} (13%), as *NF1* was not previously studied by SS.

Genetic Variants Found in Cases with Singular Features

Among the four non-*RET* cases with medullary thyroid carcinoma, one had an *SDHB* GM (ID2). No mutations were found in patients with gastrointestinal stromal tumors or pituitary adenomas.

An *SDHC*-GM was identified in one (ID30) of the two PPGL familial cases. In the two patients with NF1-affected relatives, no *NF1* GMs were found, suggesting they might be phenocopies. We could not assess this hypothesis, because tumor DNA was not available. Patient ID381, from a MEN2A family, appeared to be a phenocopy because of a *NF1* SM.

One SM in *VHL* (ID327) was found among the 11 dopamine-secreting cases, and in one of the eight composite tumors (ID100). The two black PCCs harbored *RET* variants, one (ID164) a RET^{M918T} SM and the other (ID429) a germline VUS. No mutations were found in the three adrenocorticotropic hormone−immunostaining positive cases.

Regarding pediatric cases, a driver mutation was found in 41.7% (5/12): three *SDHB* GMs and two *VHL* SMs (of the seven with tumors available). In cases with multiple tumors, mutations were identified in 13% (6/47): one *NF1* GM in a clinically diagnosed NF1 case, three *SDHD* and one *SDHAF2* GM in multiple head and neck paraganglioma patients, and one *NF1* SM in a reported double PCC (of 15 available tumors).

Finally, a driver mutation was detected in 30% (9/30) of metastatic cases. Six harbored GM in *SDHA* ($n = 2$), *SDHB, SDHD, MAX,* or *VHL* ($n = 1$). Of the 16 available tumors, 3 (18.8%) harbored SMs in *NF1* ($n = 2$) and *HRAS* ($n = 1$). No mutations were found in *MDH2* or *FH*.

Discussion

Nowadays, TGPs are broadly used because of their cost-effectiveness and ease of management. Several groups have already used this technology for PPGL genetic testing.[11,29,57,70] Although it is difficult to compare these studies, mainly because of their different design,[57] it is clear that an optimal and uniform multiplexing of all regions of interest is yet to be established.

Herein, we designed a comprehensive TGP for PPGL, including, for the first time, *EGLN1*, *EGLN2, MEN1,* and *MDH2*, and screened a large international multicenter series of patients using germline and tumor DNA. In addition, we performed a stringent process of validation and a multistep workflow analysis to confirm this platform as an efficient and accurate alternative to conventional sequencing.

Consistent with previous reports, the sensitivity of the TGPs was extremely high (99.5%).[29] We used the variant filtering threshold to prioritize variants for validation, instead of using it for filtering them out. Applying fixed thresholds can significantly reduce the detection sensitivity for heterozygous variants because of normal tissue contamination,[10] intratumor heterogeneity,[71] and mosaicism.[30−33] Three cases showed potential mosaicism, as the variants were detected in 20% of reads, two affecting *VHL* (ID153 and ID190) and one involving *SDHD* (ID296), the latter not previously described. As *VHL* was somatically mutated in pediatric cases, this mechanism should be taken into account. Even applying a stringent threshold of 50-fold coverage, we did not find any additional variants in the SS of these regions, suggesting that the 30-fold coverage threshold is appropriate.[29]

Our pipeline allowed us to rescue pool biased variants, as well as insertions/deletions, such as the *NF1* frameshift variant (c.7269_7270delCA) in ID445. As our workflow included the multiplex ligation-dependent probe amplification/multiplex analysis of TGP-negative patients, an additional case was diagnosed (0.3%, 1/291 germline DNA available), highlighting that this is a rare event (<1%).[3] Furthermore, performing a multiplex ligation-dependent probe amplification/multiplex study on selected cases as a second step reduces cost and processing time, and the protocol can be even more focused using SDHB and MYC associated factor X (MAX)-IHC.[34,72]

Genetic Screening

TGPs detected GMs in 7.2% of cases, considering the whole series. This proportion is expected, because 95% of the patients were not syndromic and had no family history. In fact, in WT^{notps}, the prevalence was similar to that previously reported in nonsyndromic cases (14%).[20]

The prevalence of GMs involving the minor genes was similar to that reported (<1%)[2,3]: 0.7% in *FH* and *SDHC*, 0.5% in *SDHA*, and 0.25% in *MAX* and *SDHAF2,* with no mutations found in *TMEM127*. Thus, genetic screening of these genes (comprising 42 exons) by conventional methods would have delayed the diagnosis of these cases, which is especially critical for *MAX, FH, SDHA,* and *SDHC*[24,25,34,73] mutation carriers, as they have been associated with metastasis and poor prognosis.

In this study, SMs were detected in 32% of the tumors. The relatively lower prevalence of *NF1* (14%) and *EPAS1* (1.6%) SMs is probably because of the fact that previous studies were performed in selected cases.[49,74]

The mutation detection rate is dependent on the extent of previous conventional genetic screening using algorithms based on available clinical data. In a study by Rattenberry et al,[29] NGS was shown to successfully detect mutations in previously unstudied cases; our data additionally demonstrate that TGPs can detect mutations in genes that have been previously disregarded because of discordant or missing clinical data (4.5% of WT^{ps} patients presented a GM and 28.9% an SM). This finding highlights the risk of relying excessively on phenotypic features to guide mutation testing. For instance, two patients >60 years with a single PCC were found to be carriers of a GM in *VHL* (ID374) or *RET* (ID283). These mutations would probably have been overlooked if methods other than TGPs had been applied. These results are crucial for the management of both index cases and their relatives. This approach also allowed us to detect *NF1* SM in two thoracoabdominal paraganglioma cases, despite this gene being mainly associated with PCC.[49,74]

Another confounding factor could be the catecholamine phenotype.[75,76] In this regard, these data will help to guide screening, but there are incongruous values because of variation in sample collection procedures or interfering drugs or foods.[77] Of note, in this study, one *VHL* and one *FH* mutation were detected in adrenergic-secreting tumors, and three *RET*-mutated cases were noradrenergic. Furthermore, nine cases with an *NF1* SM presented noradrenergic secretion. Although *NF1* has been classically associated with an adrenergic secretion, the heterogeneous profile of *NF1* tumors had been pointed out before.[76]

A remarkable finding was an *NF1* SM in a patient with multiple PCC (ID357). After reviewing the pathological report, the tumor was reclassified as a single multilobulated PCC. NGS allowed us to detect mutations in *SDHA*, *VHL*, *NF1,* and *HRAS* among metastatic cases in which *SDHB* involvement had been ruled out. These genes would likely have been ignored and the diagnosis delayed because of the low prevalence of metastatic cases reported with mutations in these genes, as well as the large size of some of them. Of note, this is the second malignant case related to an *HRAS* mutation (ID376).[20]

Surprisingly, despite black PPGL being rare, the two cases in our series were related to *RET* variants. Patient ID164 had been previously reported,[78] and case ID429 harbored a germline synonymous *RET* variant in exon 11 previously demonstrated to alter the splicing of the gene in Hirschsprung disease.[69] The co-occurrence of MEN2 and Hirschsprung disease is intriguing, because the latter is caused by *RET* inactivating mutations, and MEN2 by activating ones. However, medullary thyroid carcinoma incidence among Hirschsprung disease patients varies between 2.5% and 5%, with all activating mutations located in exon 10.[79] As it was not possible to perform functional studies to assess the pathogenicity of this specific variant, it was classified as a VUS and the recommendation for this case would be to follow it as a potential MEN2 case.

A comprehensive clinical record is especially useful when performing genetic diagnosis, as demonstrated by findings for case ID79. This patient was diagnosed as having a gastrointestinal stromal tumor and multiple noradrenergic PGLs. The tumor showed negative SDHB-IHC, TGP did not detect any variant, and gross deletions were also ruled out. Our workflow led us to select this case to be further studied using multiomics platforms, to finally detect a functional epimutation in *SDHC,* which is an event previously described as causing the disease.[13]

As the list of new PPGL genes is growing constantly, their inclusion to already designed panels is not a cost-effective process, as it requires the generation of new libraries and their validation. Our workflow allowed us to select the specific cases that would benefit from further genetic screening. Examples of this point are the implementation of the study of *MERTK*[7] and exon 7 of *RET*[36] in patients with PCC and medullary thyroid carcinoma, despite no mutation being found, or the selection of WT composite tumors to further study *ATRX* (35 exons), which has not only been related to composite PCC, but also to metastatic PPGL.[41]

In conclusion, SS of the appropriated gene in syndromic cases, as well as *SDHB* in pediatric and metastatic cases, is still an effective first-step approach, with TGPs as the most reasonable second step.

Source and Quality of DNA Samples

Frozen and blood DNA samples have optimal quality for molecular diagnosis. However, their use is not always feasible, as saliva, GenomiPhi, or FFPE tumor samples are sometimes the only available DNA source. Saliva DNA samples performed well, as the germline mosaicism in *SDHD* was detected in a similar percentage of reads to that in the blood DNA of the same patient (ID296). In addition, samples amplified by GenomiPhi were found to be useful for diagnostic purposes as our panel detected all single-nucleotide polymorphisms previously identified by SS.

A common problem with FFPE samples is the high number of false-positive variants resulting from deamination (C:G>T:A); this was resolved by applying double-stranded TGP. In addition, use of the Covaris system improved the DNA extraction efficiency and the percentage of cases diagnosed. In fact, the amplification failed in fewer FFPE samples than in blood or frozen tissue DNA. Thus, we were able to study all types of DNA samples with similar performance.

We therefore consider critical the access to the tumor sample for a complete PPGL genetic screening and diagnosis. Studying tumors allows the diagnosis of germline, somatic, and mosaic variants. In our series, the frequency of SMs (32%) was in agreement with previous reports, even in cases highly likely to carry a GM.[15] Thus, an SM was found in 29% of pediatric, 19% of malignant, and 7% of multiple tumor cases studied. Furthermore, studying the tumor DNA of apparently familial cases can reveal phenocopies. Finally, FFPE samples can be used to perform IHC, which is used not only in the filtering process, but also to test the pathogenicity of VUS.

Variants of Unknown Significance

The use of NGS platforms also leads to the finding of numerous VUSs, which are a challenge for clinical diagnosis. To further characterize the pathogenicity of VUS, an optimal communication with treating physicians is required to obtain updated clinical information, as shown by the study of Burnichon et al,[42] in which the reexamination and review of family history led to the classification of *NF1* germline variants as pathogenic. Current knowledge suggests that mutations in driver genes in PPGL are mutually exclusive. Thus, multiplexing different genes in parallel aids VUS classification,[29] as shown by the finding of an *NF1* SM (ID166) in a case

with a germline VUS in *MEN1* (c.-10G>A), suggesting the latter is not pathogenic.

Other VUSs could be more challenging to classify, as shown previously with co-occurring *NF1*[42] and *EPAS1*[11,43] variants. In our series, one patient harbored a double SM in *NF1* (ID434) and two cases double *EPAS1* variants (ID275: p.Pro531Thr and p.Leu400Pro; ID322: p.Asp539His and p.Gly537Gly). In the latter, it was not possible to rule out that these second variants were acting as modifiers.

The pathogenicity of a variant can also be tested by appropriated functional assays.[44] VUS classification is a resource- and time-demanding task, and an international cooperative effort is required to update existing databases.[20]

In summary, before applying TGPs in the clinical setting, it is critical to ensure adequate library preparation, high accuracy, and avoidance of false-positive and false-negative results through the implementation of alternative techniques. Thus, this technology should be performed in specialized and accredited laboratories with expertise in PPGL.[17]

Herein, we have demonstrated the effectiveness and feasibility of this diagnostic tool, able to detect low-coverage, pool-biased, and insertion/deletion variants. We conclude that our TGP workflow enables the study of the main driver PPGL genes in different DNA sources and improves the clinical management of index cases and their relatives at risk. In addition, TGP is the optimal method to select cases that will benefit from further investigation in a research setting, as the etiology of one third of PPGL cases remains unknown.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2017.04.009*.

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