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Next-generation sequencing for the diagnosis of hereditary pheochromocytoma and paraganglioma syndromes

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Abstract

Purpose of review—About 40% of the neuroendocrine tumors pheochromocytomas and paragangliomas (PPGLs) are caused by an inherited mutation. Diagnostic genetic screening is recommended for patients and their families. However, the number of susceptibility genes involved is high and continues to grow, making conventional sequencing costly and burdensome. Next-generation sequencing (NGS) enables accurate, thorough, and cost-effective identification of inherited mutations. Here we review recent successes, limitations, and the future of NGS for diagnosis of pheochromocytoma and paraganglioma syndromes.

Recent findings—NGS-based screen of genetic disorders in the clinical setting shows improved diagnostic rates over conventional tests. Both broad, whole-exome sequencing, and targeted NGS approaches have been tested for screening of PPGLs, with accurate mutation detection, higher speed, and reduced costs compared with current assays. Flexibility to expand the targeted gene set is immediate in whole-exome sequencing, and adjustable in targeted NGS, but both methods have limitations.

Summary—The high degree of genetic heterogeneity and heritability of PPGLs make NGS an ideal medium for their diagnostic screening. However, improved detection of large genomic defects and underrepresented gene areas are needed before NGS can fully realize its potential as the premier option for routine genetic testing of these syndromes.

Keywords

diagnostics; genetic screening; next-generation sequencing; paragangliomas; pheochromocytomas

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Conflicts of interest

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INTRODUCTION

Pheochromocytomas and paragangliomas (PPGLs) are neural crest tumors derived from catecholamine secreting cells of the adrenal medulla or extra-adrenal sympathetic paraganglia, respectively [1,2]. Two striking features of these tumors are their genetic heterogeneity and their high degree of heritability (40% of the cases). Recent Clinical Practice Guide lines set forth by the American Endocrine Society recommends that genetic testing be performed for certain groups at high risk for hereditary PPGL, as detailed below, but that it should be considered for all PPGL patients [3]. The American Society of Clinical Oncology directions reach further by suggesting that all patients with a risk of heritability higher than 10% should undergo testing [4]. Therefore, PPGLs fall well into the category of diseases for which genetic screening is advised. However, as the number of susceptibility genes increases, currently spanning over 200 exons, so does the complexity of genetic testing. Next generation sequencing (NGS) methodology has dramatically changed the field of genetics in the past decade. NGS use has broadened widely since its inception, with improvements in the technology and decrease in costs. NGS methods have now been applied in multiple clinical diagnostic settings, including inherited developmental disorders and cancers, in many cases with greater success rate compared with conventional sequencing techniques [5–11]. Over the past few years, a picture of the state of NGS use in the field of PPGL started to emerge. In this review, we will discuss these studies and address the advantages and challenges of distinct NGS approaches for inherited PPGL diagnosis. Although the use of NGS testing for diagnosis of somatic variants is recognizably relevant from a clinical perspective, these studies will not be extensively discussed here.

THE COMPLEX GENETICS OF PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS

Much progress has been made on our understanding of the genetic basis of PPGLs in the past decades and many familial forms of the disease are now recognized (Table 1). Excellent reviews describing unique clinical features of these various inherited disorders have been published recently [12–14]. PPGLs are arguably the most heritable human tumors. Familial PPGL is usually inherited as an autosomal dominant trait, so the offspring of a mutation carrier will have a 50% chance of having inherited the relevant PPGL gene mutation [1,2]. Genetic testing is recommended for individuals at high risk for susceptibility, which includes positive family history, presence of syndromic features, early onset disease, presence of multiple tumors, malignancy, paraganglioma location, or a combination of some of these characteristics, whereby the pretest probability of mutation detection is high [3]. Many diagnostic stepwise algorithms have been proposed to streamline the increasingly burdensome and costly process of genetic screening of PPGLs [12,15–18]. These algorithms incorporate clinical features to guide the prioritization of the gene for screen and are particularly effective for high-risk groups. However, there is a strong argument for extending genetic testing to all PPGL patients, based on the recognition that at least 10% of ‘low risk’ cases may carry predisposing mutations [13]. In these cases, low penetrance of the mutant allele, the existence of parent-of-origin effects on disease penetrance (in SDHD, SDHAF2 and MAX mutations) or de-novo mutations in the index patient can obscure the diagnosis of

inherited PPGL [2]. In nonsyndromic cases, the number of screened genes expands, which makes the process lengthy, if genes are analyzed individually and/or, very costly, if they are tested simultaneously.

NEXT-GENERATION SEQUENCING PLATFORM OF CHOICE

Similar to other hereditary disorders, in particular those in which allelic heterogeneity is extensive, the use of NGS, also referred to as massively parallel sequencing, has increased exponentially over the past decade and has begun to replace conventional (Sanger) sequencing in many clinical contexts [6,19,20]. Methodological details of the techniques are beyond the scope of this review. Instead, here we discuss the NGS approaches that have been applied to PPGLs and how these findings will shape the future of genetic testing in these tumors. Table 2 summarizes the context, study design, results, and limitations of these published studies. Most of the NGS studies of PPGLs, a few preceding the review period but included because of their relevance, were performed for purposes of gene discovery and employed whole exome sequencing (WES) [21,22,23▪,24▪▪,25▪▪,26▪, 28▪▪,30▪,32–34▪▪,35]. Targeted NGS analyses were also reported [27▪▪,29,31▪▪,36▪▪]. Two studies directly compared NGS with conventional sequencing for diagnosis of germline mutations in known PPGL genes [27▪▪,28▪▪].

Whole-exome sequencing

In this method, fragmented DNA samples are hybridized to oligonucleotide probes representing coding regions of the genome, the exome, and high throughput sequenced [6]. Approximately, 85% of disease-causing mutations are expected to occur within the exome, which represents 1–2% of the whole-genome region. As a result, WES has become the NGS method of choice in multiple studies of cancer and other hereditary conditions [20]. The advantages of WES, especially in comparison with whole-genome sequencing (WGS) are multiple: costs are lower; the smaller target sequence greatly simplifies sample processing and analysis, the requirements of sample quantity are not too stringent. Furthermore, the existence of genome-wide coverage facilitates the analysis of novel candidate genes as they are uncovered, without the need to reprocess the sample.

McInerney-Leo et al. [28▪▪] tested the efficiency of two different commercial WES platforms for diagnosis of a small cohort of hereditary PPGLs: one mutation was missed by one of the platforms, but detected in the other. Also, by specifically comparing the coverage of 12 PPGL genes across reference data from five exome enrichment kits, it was noticed that only one of them showed complete coverage of all coding sequences of interest, with SDH genes showing the highest degree of variation in the depth of reads. The poor representation of some PPGL-related exons was in part due to low depth of sequence of the reference dataset used, and may be resolved by increasing the depth in actual samples. However, more problematic is the issue of incomplete coverage of the length of some exons, which should be a consideration when selecting the enrichment platform for WES-based screen. Individual PPGL exon coverage and depth is not available from other WES studies in PPGLs but this information could help in developing future guidelines and standards for WES-based screening, as discussed below.

Overall, WES-based screen is the favored platform for comprehensive, yet analytically manage able genetic screen amenable to entering the mainstream of PPGLs diagnostic testing (Table 3). However, further improvements in the efficiency of exome capture methods are needed to ensure that all target exons are represented through their entire length and at adequate depth of coverage. Enhanced alignment and base calling algorithms are also needed to ensure accuracy of the sequencing. Other technical shortcomings are discussed in a separate section, below.

Targeted next generation sequencing

In this approach, the sequencing analysis is limited to known genes and exons, and next generation sequencing is performed in samples amplified by PCR. Custom primers are designed to target whole or specific areas (often exons) of genes of interest. Barcodes are attached to individual samples during library generation allowing for a high degree of multiplexing, which improves the throughput of sequence processing and reduces costs. Targeted NGS has many valuable features: primers can be individually designed and adjusted to achieve similar efficiency across the gene(s), samples are sequenced at much deeper coverage (200–1000), the instrumentation is simplified and affordable by individual labs, and the analysis pipeline is straight forward and customizable. Furthermore, targeted NGS may be the only viable approach for samples with limited amounts of DNA of suboptimal quality. Rattenberry *et al.* [27▪▪] performed a feasibility study of nine PPGL genes in a large sample cohort and found high degree of diagnostic concordance with conventional sequencing (Table 2). However, several problems were highlighted, including sequence errors in repeat areas (instrument-biased) and the inability to multiplex exons with high GC content, which had to be analyzed separately through Sanger sequencing. Limitations in multiplexing were also noted by Welander *et al.* [31▪▪] using a different platform and instrumental setting, although all mutations in 18 known hereditary PPGLs were identified by their approach. Despite the relative simplicity of targeted NGS, in practice achieving optimal and uniform multiplexing of all desired exons in every sample is not straightforward. Furthermore, the number and speed with which novel genes are identified and then incorporated in the targeted screen design can pose technical and economic challenges for implementation. In targeted NGS, unlike WES, addition of new genes to existing panels requires the generation of new libraries and new sample sequencing (Table 3).

Other NGS platforms

WGS is the most comprehensive genome-wide option, as it includes noncoding regions in addition to exons. These areas are increasingly recognized as relevant for diagnosis of genetic disorders and cancers [19,37]. Additional advantages are the ability to identify gene translocations and copy number gains or losses. Major limitations of WGS are the costs, and importantly, the complexity of the bioinformatic analysis. The NIH-sponsored TCGA (The Cancer Genomic Atlas) effort in PPGLs (https://tcgadata.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=PCPG&diseaseName=Pheochromocytoma%20and%20Paraganglioma), which is at its final stages of completion, includes WGS analysis of a large sample collection. Although not meant as a clinical diagnostic tool, WGS data from TCGA is certain to provide insights into genomic alterations that could not have been

detected by other NGS methodologies and will contribute to gauging the added value of WGS for diagnostic purposes in PPGLs.

RNA sequencing (RNAseq) differs from other modalities by utilizing RNA (preferentially from tumor tissue), instead of DNA, for analysis, which limits considerably its use in large scale for diagnostic purposes. However, this approach has important attributes: it provides a combination of sequence data and quantification of gene expression in a single methodology; enables an immediate view of the transcription consequences of mutations that occur at splice sites or those that involve gene fusions resulting from translocations or rearrangements, and identifies preferential allelic expression of coding variants. However, analytical pipelines are more complex than WES and targeted NGS. In PPGL, gene fusions and intrachromosomal breakpoints that may be biologically consequential were recently identified by RNAseq [32▪▪]. Further investigation of the frequency of these events will provide new insights into the biology of PPGLs and whether this approach would be of use in the diagnostic arena.

Currently, effective analysis of coding regions of target genes is likely to encompass the great majority of causative mutations in hereditary PPGLs. However, it is difficult to estimate how much has been missed by confining the analysis to exons and exon-intron boundaries. Data outside of these constraints are essentially nonexistent. As research advances, the real contribution of other defects, including large genomic gains or losses, translocations, fusions, and noncoding mutations, to PPGL pathogenesis will become better known. This information will be relevant to determine the method of choice for comprehensive testing of these disorders.

ANALYTICAL AND TECHNICAL SHORTCOMINGS

Analysis of the sequence data produced by these different NGS modalities is beyond the scope of this review, but this is clearly one of the bottlenecks for rapid implementation of NGS methods in clinical practice. Understandably, the analytical complexity of WES, RNAseq and WGS is higher than that of targeted sequencing [6]. The importance of rigorous bioinformatic analysis and interpretation standards cannot be overstated.

NGS technologies have recognizably higher raw base error rates than Sanger sequencing [5,6]. However, since its inception, technological advances in instrumentation, sample processing, and algorithms, coupled with higher depth of sequencing coverage in most study designs, have led to improved accuracy in base calling. For WES and WGS an average sequencing depth of 50–100 of bidirectional (or paired-end) reads is usually considered sufficient to detect most germline single-nucleotide variants accurately [6,38–40]. However, these conservative numbers can, and should be, increased under specific circumstances. Other technical limitations to NGS methods recognized in the setting of PPGLs, discussed above, and off-target sequencing and misalignment to homologous regions, such as paralogs or pseudogenes, can also lead to reduced sensitivity and specificity of variant detection. The rate of alignment errors have substantially decreased with longer read lengths (~50 vs. 100 bp or more in recent pipelines), higher depth of sequencing, and improved base call algorithms.

NGS exhibits much greater sensitivity and specificity for detection of substitutions than it does for other sequence changes [5,41,42]. For detection of insertions, deletions, larger copy number, and structural changes, specific analytical algorithms are required for accurate calling. Leveraging high depth of coverage of the test samples and targeted NGS designs involving whole genes instead of exon-only enrichments can significantly improve detection of these large genomic defects. Several targeted NGS screening platforms for clinical diagnosis of hereditary breast, ovarian, and colon cancer have been developed [41,43–45], in which such designs were implemented coupled with high depth of coverage and robust analytical pipelines led to successful detection of a wide range of deletion or duplication lengths. The ability to identify larger structural defects should be a goal of NGS-based screens in PPGLs because as much as 10% of the defects involving *SDH* and *VHL* can result from whole or partial gene deletions.

Beyond coding sequence mutations and large structural defects of target genes, gene inactivation mediated by epigenetic, but not genetic events (epimutations), have been recently reported in *SDHC* of Carney triad syndrome patients [46,47], in whom paragangliomas are associated with pulmonary chondroma and gastrointestinal stromal tumors (GIST). In these patients, the *SDHC* gene is hypermethylated and hypoexpressed in tumor tissues, but also in nontumoral tissues, suggesting possible mosaicism. These findings, which were considered to be primary drivers of the tumorigenesis in these patients, have not been examined more generally in other PPGL cases, but if confirmed, may indicate that all-encompassing screening of these tumors may require an expansion of the current techniques to identify hypermethylated areas on target genes.

Hence, no single platform currently fulfills the requirements of an ideal PPGL screening test (Table 3). One reasonable expectation is that an improved version of WES, with more uniform and completecoverage of all targetexons relevant to PPGL should be the more immediate goal for implementing a comprehensive primary platform for genetic testing. A separate test, WGS-based or utilizing specially designedtargeted NGS panelsor high-resolution copy number analysis, may be required for detection of larger structural defects or analysis of noncoding variants in patients for which a mutation is not identified in the first test. Furthermore, tests to detect mosaic epimutations may need to be developed if these events are found more generally in PPGLs.

DATA REPORTING

In the setting of genetic disorders, the availability of a clinical summary on the test order form is often a prerequisite to interpreting the results of NGS testing [10,11]. This is not an absolute requirement in PPGLs as the clinical diagnosis is often straightforward. However, information on family history, tumor location, recurrence, malignancy status, and existence of other conditions known to be related to PPGL-related syndromes can be invaluable to improve accuracy of diagnostic reports.

Interpreting the results of NGS, especially WES, can be more complex than conventional testing due to the massive amounts of data generated. However, on a diagnostic setting, the analysis can be restricted to the known disease genes by computational selection. Typically,

appropriate filters are applied using similar criteria to those already employed in conventional clinical diagnosis to exclude common variants (for example, those occurring in less than 5 or even 1% of the general population), variants that lead to a synonymous change (exceptions are those that generate or abolish a splice site), intronic variants beyond the canonical 2-bases surrounding exons and other, context-specific filtering. The variants that remain after these filters are then classified as follows: benign, deleterious (previously reported in hereditary PPGLs, as referenced in the literature and/or online mutation databases available for various PPGL genes), potentially pathogenic variants (conserved amino acids, nonsense or frameshift), or variants of unknown clinical significance (VUS, further discussed below). The final report of a diagnostic genetic test should be the result of careful analysis and discussion with geneticists or other PPGL experts and extensive literature searches to determine the classification of variants [10,11,44]. It is important to keep in mind that despite technical improvements in design and analytical algorithms, some variants may still require confirmation by conventional sequencing due to poor coverage or to an alignment-challenging sequence context. In fact, although NGS-based tests are still under development, we believe that positive tests should be validated by Sanger sequencing before results are reported to the patient.

In the current scenario of autosomal dominantly inherited PPGLs, pathogenic mutations are expected to be represented as heterozygous variants, and thus the estimated variant frequency threshold (VFT) for heterozygous mutations should be close to 50%. However, in practice, there are instances of allelic strand sequence preference, when VFTs deviate from this pattern, as reported by Rattenberry *et al.* [27▪▪], and also seen in our own experience. VFT values can be variable in mosaic mutations. This is the case in *EPAS1*, wherein a postzygotic de-novo mutation can lead to increased risk of PPGLs, polycythemia and occasionally somatostatinomas of the duodenum [36▪▪]. Determining the risks of germline transmission of mosaic diseases can be challenging and impractical [48], therefore genetic counseling should play a dominant role, more than the genetic test itself, in the discussion of transmission risks of patients with EPAS1 mutations at tumor level.

VUSs are detected in unprecedented numbers by NGS-based screening and represent a common challenge for test reporting. However, limitations in the ability to distinguish pathogenic from nonpathogenic mutations are likely to gradually decrease, as reference databases become more complete and our knowledge of PPGLs improves, with more mutations being recurrently detected and their functional effects tested. Although there has been some debate as to whether VUSs should be reported due to the uncertainty of their value, the predominant view is that the benefits of reporting outweigh risks of, for example, not revealing variants that may be eventually proven to be pathogenic [10,20]. The approach in PPGL should follow the lead of clinical genetics, in which extensive consultation between laboratory personnel with the attending physician and medical geneticists for cases in which the diagnostic classification is uncertain takes place before the results are disclosed to patients [10,49,50]. Laboratories and attending physicians should regularly review the status of VUS cases as more research data become available. In this realm, new government-sponsored initiatives are being developed to make clinically relevant information publicly searchable [51]. Patients should be clearly informed of the significance and potential change in VUS status and are encouraged to seek regular updates from their attending physicians.

Other challenges of NGS based testing involve interpreting alternative modes of inheritance or co-occurrence of multiple variants with potential pathogenic effects in single samples. Appropriate interpretation of these findings will require further research and functional validation of novel variants. Guidelines for application of NGS to genetic diagnostics are still under development [20,49,50,52]; we believe that a framework of PPGL screening standards should be devised (Fig. 1). These recommendations should attempt to integrate these emerging guidelines with established PPGL testing routines and regulatory requirements unique to different centers and countries. The challenges imposed by the complexity of interpreting results demand the inclusion of a medical geneticist or certified genetic counselor in the testing process [10,41]. Leading organizations in the PPGL field, including the investigator-driven Pheochromocytoma Support Organization, the Endocrine Society, the European Network for the Study of Adrenal Tumors, and other worldwide associations, are encouraged to come together to develop standardized practices and policies for NGS-based tests as they begin to enter the mainstream of clinical practice in many countries. Input from various patient advocacy groups (Pheo Para Troopers, Pheo-Para Alliance, VHL Alliance, and others) should also be sought. Another relevant aspect of NGS-based testing, specifically genome wide approaches, involves ‘incidental findings’, the identification of variants unrelated to the phenotype of interest but which may have clinical significance. Reporting of these findings is an area of broad debate in NGS-related clinical applications [10,20,49,50, 53,54] and should be extended to the PPGL field. The framework devised by the clinical genetics field is an excellent starting point to initiate this discussion.

CONCLUSION

The emerging body of evidence in the field of NGS based genetic screen on PPGLs suggests that multiple sequencing approaches (targeted, WES, and WGS) are likely to find applications in the routine diagnostic setting.

Diagnostic panels for subsets of PPGL genes can already be found commercially in the USA. In academic centers worldwide, the transition from conventional methods to NGS is advancing at a rapid pace. Current limitations of targeted NGS and WES require that these methods are complemented by independent analysis of poorly covered gene areas and copy number analysis for a comprehensive, all-encompassing screen. Further progress in the methodology with longer sequence reads, higher-depth of sequencing, careful target primer design, barcoding and multiplexing, and the possibility of using whole-genome methods to address deletions will likely aid in overcoming the current limitations and further increasing sensitivity. Furthermore, the ability to incorporate other susceptibility targets as they are discovered and added to the list of PPGL genes offers enormous advantage to NGS-based screens, especially the genome-wide methods. As different design and platform options continue to be perfected, a consensus set of guidelines should be developed, at least in the academic setting, to fulfill basic diagnostic and quality control standards for both technical processing and interpretation of the results. These platforms would also be amenable to use in other clinically relevant applications beyond germline diagnosis, including tumor screening for detection of potentially therapeutically targetable somatic mutations.

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KEY POINTS

- PPGLs are genetically heterogeneous and often inherited (40% carry a germline susceptibility mutation).
- NGS technology, now broadly available and cost effective, has been successfully implemented in clinical diagnosis of multiple inherited disorders.
- Pilot studies have shown feasibility of both WES and targeted NGS for diagnosis of inherited pheochromocytomas and paragangliomas.
- Technical fine-tuning, including improved and uniform coverage of all target exons and detection of large copy number changes will be required to improve sensitivity and specificity of NGS for its use in pheochromocytoma and paraganglioma diagnosis.
- A consensus set of guidelines and standards for NGS-based testing in PPGL should be developed in the near future.

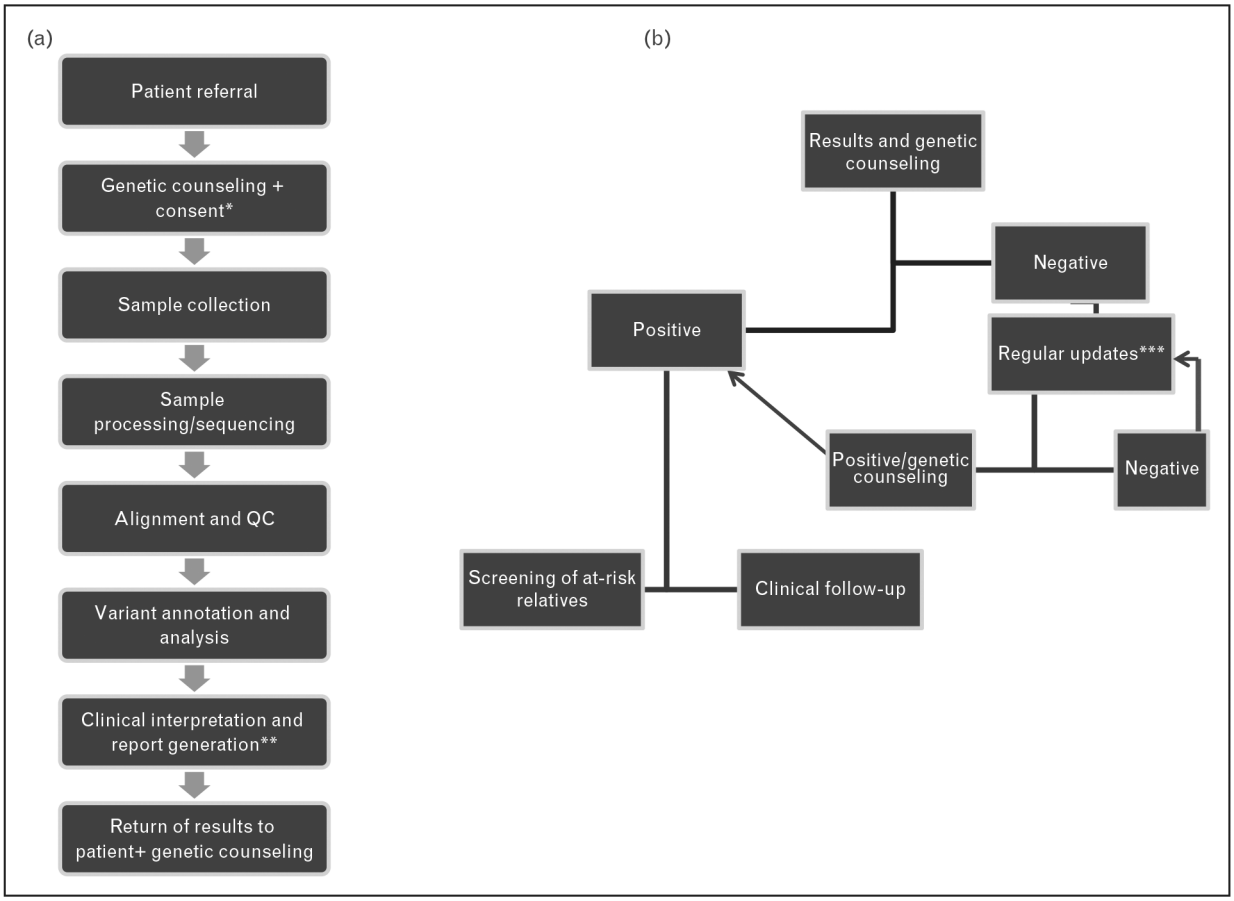

FIGURE 1.

Proposed workflow of a genome-wide next-generation sequencing-based screen of patients with pheochromocytoma or paraganglioma. (a) Initial steps of the screening process involve genetic counseling and informed consent ([a]patient must opt in or out of 'incidental finding' reporting and decisions of future evaluation of the collected sequence data for future updates – see text for additional details). [b]It is suggested that the final report be the consensus interpretation of physicians, researchers and clinical geneticists. (b) Results are returned to the patient at a genetic counseling session. Unquestionably, positive results follow the current route of clinical follow-up for index patient and screening of at-risk relatives. Negative results may include lack of a clearly pathogenic mutation in a known susceptibility gene or detection of variants of unknown significance (VUS). Regular updates on the status of VUS or evaluation of novel susceptibility genes from collected data are performed. If a new pathogenic variant is detected or pathogenic status of VUS is established, based on new research data, the patient will be offered genetic counseling and follow procedures for a 'positive' mutation carrier. If there are no changes in the genetic screening status, the process of regular updates may continue.

Table 1.

Main clinical, biological and genetic features of known pheochromocytoma and paraganglioma susceptibility genes

Category	Parameters	*NF1*	*RET*	*VHL*	*TMEM127*	*MAX*	*SDHA*	*SDHB*	*SDHC*	*SDHD*	*SDHAF2*	*FH*	*HIF2/EPAS1*	*KIF1B*	*PHD2/EGLN1*
Clinicol	Inherited syndrome designation	Neurofibromatosis type 1	MEN 2A, MEN 2B, Familial MTC, Hirschsprung disease	von Hippel-Lindau disease, Chuvash polycythemia	TMEM127-related pheochromocytoma	MAX-related pheochromocytoma	F-PGL, Mitochondrial complex II deficiency	F-PGL type 4, familial RCC, Carney–Stratakis syndrome, complex II deficiency	FPGL type 3, Carney–Stratakis syndrome, complex II deficiency	F-PGL type 1, Carney–Stratakis syndrome, complex II deficiency	F-PGL type 2, complex II deficiency	Hereditary leiomyoma renal cell carcinoma	Familial erythrocytosis type 4	Charcot Marie Tooth Disease 2A1	Familial erythrocytosis type 3
	Prototypical presentation	Single pheo, café au lait spots, neurofibromas, family history of neurofibromatosis	Bilateral pheo, Medullary thyroid carcinoma, family history	Young age, bilateral pheo, renal cell carcinoma and CNS heman-gioblastoma, family history	>35 years old, pheo, family history less frequent	Pheo, family history less frequent, paternal transmission	Pheo or PGL	Single PGL, malignant features, occasional family history	Head and neck PGL, family history less frequent	Multiple PGLs, predominantly head and neck, family history, paternal transmission	Multiple PGLs, predominantly head and neck, family history, paternal transmission	Multiple pheo or PGLs, malignant features frequent	PGLs, polycythemia, somato-statinoma	2 cases only (one familial, bilateral pheo, second single, sporadic pheo)	1 case only (PGL, polycythemia)
	Other manifestations or other conditions associated with mutations ir these genes	Neurofibromas, malignant peripheral nervous sheath tumors, gliomas	MTC, hyperparathyroidism, marfanoid habitus	RCCs, CNS hemangioblastomas	RCC	None reported	GISTs	GISTs, RCCs	GISTs	GISTs	None reported	Uterine leiomyoma	Polycythemia, somatostatinomas	Neuroblastoma, lung cancer[a]	Polycythemia
Biological	Transcription cluster[a]	2	2	1	2	2	1	1	1	1	1	1	1	2	U
	Methylation cluster[b]	3	3	2	3	3	1	1	1	1	U	1	U	U	U
Genetic	Mutation type	S>G	G>S	G>S	G	G>S	G	G	G	G	G	G	S + M	G	G
	Inheritance (autosomal dominant = AD, *P* = parent of origin effect,U = unknown, N/A = not applicable) **<delete?>**	AD	AD	AD	AD	AD-P	AD	AD	AD	AD-P	AD-P	AD	U	AD	AD

HRAS mutations were only detected somatically, not in the germline and have therefore not been included in this table. AD, autosomal dominant; CNS, central nervous system; FH, fumarate hydratase; G, germline; GIST, gastrointestinal stromal tumor; M, mosaic; MTC, medullary thyroid carcinoma; P, parent of origin effect (paternal transmission); PGL, paraganglioma; PHEO, pheochromocytoma; RCC, renal cell carcinoma; S, somatic; U, unknown.

[a]Mutant tumors belong to one of two main transcriptional clusters: 1, pseudohypoxia; 2, kinase-related signaling.

[b]Mutant tumors belong to three main methylation clusters: 1: *SDH/FH-mutant tumors*; 2: *predominantly VHL-mutant tumors*; 3: *NF1/RET/MAX/TMEM127*-mutant and some sporadic tumors.

Table 2.

Summary of the results of published next generation sequencing studies in pheochromocytomas and paragangliomas

Study	Reference	*n*	Phenotype	Mutation in known susceptibility gene ?	Sample type	Method	Enrichment platform/ library generation	Read direction/ fragment length	Average depth of coverage	Sequencing platform	Genetic analysis approach	Main genetic outcomes/ discoveries	Accuracy	Notes
1	Comino-Mendez *et al.* [21]	3	Transcriptionally clustered pheochromocytomas with no known mutation	No	Blood	WES	Agilent SureSelect Human All Exon	2X75PE	PPGL genes were covered at a minimum of 10X (94–96%), overall average not available	Illumina Genome Analyzer II	Broad discovery analysis	*MAX* identified as a novel PPGL susceptibility gene	n/a	
2	Qi *et al.* [22]	6 relatives	MEN2 and FMTC family	No	Blood	WES	Agilent SureSelect Biotinylated RNA Library	2X90PE	min 5OX (RET)	Illumina HiSeq2000	Analysis focused on *RET*	compound *RET* mutations associated with modified phenotypes	NA	
3	Toledo *et al.* [23▪]	6	PPGLs of unknown genetic cause	No	Paired blood and tumor [4] and two paired tumors from the same patient	WES	Agilent Sure Select 44Mbp	2X54PE (2 tumors), 2X90PE (4 pairs)	52X (tumor-tumor pair); 52–55X(blood-tumor pairs)	Illumina HiSeq2000	Broad discovery analysis	*EPAS1* mutation discovery in one sample and 3 additional *EPAS1* mutations in 167 sporadic PPGLs screened by Sanger	NA	
4	Crona *et al.* [24▪▪]	4	Benign and sporadic PPGLs	No	Tumor	WES	SureSelect Human All Exon 50 Mb kit	2XPE, size?		Illumina HiSeq2000	Broad discovery analysis	*HRAS* somatic mutations identified in PPGLs	NA	
5	Letouze *et al.* [25▪▪]	1	*SDH*-like pheochromocytoma, but without a germline mutation in SDH genes	No	Paired blood and tumor	WES	Agilent SureSelect Human All Exon Kit v4+UTR	2X75PE	8OX (sample is included in Castro-Vega *et al.*	Illumina HiSeq2000	Broad discovery analysis	*FH* identified as a novel PPGL susceptibility gene	NA	
6	Cron a *et al.* [26▪]	3	Sporadic	No	Tumor	WES	Agilent SureSelect	?	?	Illumina HiSeq2000	Analysis focused on *SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, EPAS1, RET, NF1, TMEM127 and MAX*	One *NF1* variant and one *RET* variant	NA	
7	Ratten berry *et al.* [27▪▪]	205	Familial and sporadic PPGLs	85	Blood	PCR + NGS sequencing	48.48 Access Array system (Fluidigm)	310–460bp amplicon size range	min 3OX	GS Junior NGS sequencer Roche 454	Analysis focused on *MAX, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2J-MEM127, VHL*	Multiple novel and known mutations of PPGL genes	98.5% NGS assay sensitivity	454 sequencing technology is prone to false positives and sequence context-based errors (e.g., repeat regions)
8	Mclnerney-Leo *et al.* [28▪▪]	11	Familial (germline mutations in *VHL, RET, SDHB, SDHC or SDHD*)	Yes	Blood	WES	HluminaTruSeq (7 cases); NimbleGen-SeqCap EZ v3.0 (5 cases) + computa-computational analysis of PPGL genes in WES from reference samples of five capture kits: Agilent SureSelectXT; Nimblegen-SeqCap EZ v3.0; HluminaTruSeqT-MExome Enrichment Kit v2.0; NexteraTM Rapid Capture Expanded Exome; Illumina-NexteraTM Rapid Capture Exome	2X100PE	?	Illumina HiSeq2000	Analysis focused on *RET, NF1, VHL, SDHx, SDHAF2,-KIF1B, TMEM127, EGLN1, MAX*	At least one exon was not captured in *SDHA/D* (1/5 platforms), *MAX/KIF1B* (2/5), *NF1/SDHC* (3/5)	6/7 (85.7%) using HluminaTruSeq; 5/5 (100%) mutations detected using Nim-bleGenSeqCap EZ v3.0	Appropriate choice of capture platform is critical to ensure adequate coverage of all exons of known PPGL genes, especially *SDHA/C/D*
9	Casey *et al.* [29]	31	Mostly sporadic PPGLs (28/31]	No	Blood	PCR + NGS sequencing	information unavailable	?	?	Illumina sequencer ?	*VHL, SDHA, SDHB, SDHC, SDHD,*	WES NGS allows PPGL screen to include less frequently studied PPGL	NA	

Study	Reference	*n*	Phenotype	Mutation in known susceptibility gene ?	Sample type	Method	Enrichment platform/ library generation	Read direction/ fragment length	Average depth of coverage	Sequencing platform	Genetic analysis approach	Main genetic outcomes/ discoveries	Accuracy	Notes
											TMEM127, MAX, and RET	genes (e.g., *SDHA* and *KIF1B*), improving detection of rarer mutations		
10	Clark *et al.* [30▪]	1	Child pheochromocytoma of unknown genetic cause	No	Blood	WES	Agilent SureSelect All Exon 50Mb Target Enrichment System	2X76PE	?	Illumina GA Analyser-IIx	Broad discovery analysis	*FH* mutation discovery in proband and one additional mutation in 71 samples screened by Sanger	NA	
11	Welander *et al.* [31▪▪]	86	18 familial/syndromic; 56 apparently sporadic	18/86	Tumor	*PCR + NGS* sequencing	IlluminaTruSeq custom amplicon	2X150PE; 272 amplcons	i-915X	IlluminaMiSeq	Analysis focused on *EGLNI, EPASI, KIF1B, MAX, MENl, NFl, RET, SDHA, SDHB,SDHC, SDHD, SDHAF2, TMEM127, VHL*	265/272 amplicons (97%) yieldec sequence reads, with a mean depth of 915 per amplicon and sample.	100% sensitivity and specificity in known cases	Target sequencing of PPGLs genes carries high sensitivity and specificity although some exons could not be multiplexed
12	Flynn *et al.* [32▪▪]	40	PPGLs of both known and unknown genetic cause	Yes, in 14 cases	Paired blood and tumor (40 for WES and 39 tumors for RNAseq)	WES and RNAseq	Nimblegen V2 (Nim-blegen, Roche, WI, USA) or the Agilent SureSelect V5 exome capture	2X100PE	120X (WES); 60–80M reads (RNAseq)	Illumina HiSeq2000	Broad discovery analysis	Novel somatic mutations in multiple genes, major structural defects, gene fusions	all 14 previously known mutations were confirmed by WES and/or RNAseq	
13	Fishbein *et al.* [33▪▪]	21 (+ 103]	PPGLs of unknown genetic cause	No	Paired blood and tumor	WES + targeted NGS for validation	Agilent SureSelect All Exon v3	2X100PE	84X(tumor); 85X (germline); unknown depth of validation cohort	Illumina HiSeq2000; IlluminaMiSeq for targeted sequencing	Broad discovery analysis	somatic *ATRX* mutations associated with malignant PPGLs	NA	
14	Castro-Vega *et al.* [34▪▪]	31	PPGLs of both known and unknown genetic cause	Yes, in 17 cases	30 paired blood and tumor, 1 trio (blood, primary tumor and metastasis)	WES	SureSelect Human All Exon Kit v4 + UTR, Human All Exon v4 + UTR-70Mb	2X75PE	80X	Illumina HiSeq2000	Broad discovery analysis	Novel mutations in multiple genes and structural defects, few recurrent mutations	NA	

FH, fumarate hydratase; FMTC, familial medullary thyroid carcinoma; MEN2A, multiple endocrine neoplasia type 2A; NGS, next-generation sequencing; PE, paired-end; PGL, paraganglioma; PHEO, pheochromocytoma; PPGL, pheochromocytoma and paraganglioma; RNAseq, RNA sequencing; WES, whole-exome sequencing; ?, information not provided. Next-generation sequencin

Table 3.

Summary of distinguishing features of whole xome sequencing, targeted next generation sequencing, and conventional Sanger sequencing in pheochromocytomas and paragangliomas

Feature	WES	Targeted NGS panel	Conventional testing (Sanger or MLPA)
Detection of known PPGL genes	Yes[a]	Yes	Yes
Need to process some PPGL exons separately (by conventional sequencing)	High	High	NA
Detection of novel genes	Yes	No[b]	No[b]
Detection of large genomic or copy number defects	Low	Yes[b]	Yes[c]
Fast turnaround time	Yes	Yes	No[d]
Low costs	Yes	Yes	No[e]
Complexity of bioinformatic analysis	High	Low	NA
Sequencing error rates	High	High	Low
Incidental findings	Yes	No	NA
VUS	High	High	Low
Performed in a stepwise manner	No	No	Yes
Individual lab autonomy for sequencing	No	Yes	Yes
Scalability	Low[f]	High	NA

MLPA, multiplex ligation-dependent probe amplification, method used to detect copy number changes in PPGL genes; NA, not applicable; PPGL, pheochromocytomas and paragangliomas; VUS, variants of unknown significance; WES, whole-exome sequencing.

[a] Detection of some PPGL gene exons may be incomplete in current platforms.

[b] New assay design required or use of a broad targeted panel.

[c] By MLPA assay.

[d] Exception when first clinically driven test identifies mutated gene.

[e] High costs if multistep gene analysis is required.

[f] Increase in WES scale can only occur at the expense of reduced sequencing depth per sample – not recommended.