Somatic mutation profiling in head and neck paragangliomas

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**Context:** Head and neck paragangliomas (HNPGLs) are rare neoplasms with a high degree of heritability. Nevertheless, paragangliomas present as polygenic diseases caused by combined alterations in multiple genes, however, many driver changes remain unknown.

**Objective:** The objective of the study was to analyze somatic mutation profiles in HNPGLs.

**Design:** Whole-exome sequencing of 42 tumors and matched normal tissues obtained from Russian patients with HNPGLs was carried out. Somatic mutation profiling included variant calling and utilizing of MutSig and SigProfiler packages.

**Results:** 57% of patients harbored germline and somatic variants in the PGL susceptibility genes or potentially related genes. Somatic variants in novel genes were found in 17% of patients without mutations in any known PGL-related genes. The studied cohort was characterized by six significantly mutated genes: *SDHD, BCAS4, SLC25A14, RBM3, TP53, and ASCC1*, as well as four COSMIC SBS-96 mutational signatures (SBS5, SBS29, SBS1, and SBS7b). Tumors with germline variants specifically displayed SBS11 and SBS19, when SBS33 specific mutational signature was identified for cases without those. B allele frequency analysis of copy number variations revealed loss of heterozygosity of the wild-type allele in one patient with germline mutation c.287-2A>G in the *SDHB* gene. In patients with germline mutation c.A305G in the *SDHD* gene, frequent potential loss of chromosome 11 was observed.

**Conclusion:** These results give an understanding of somatic changes and the mutational landscape associated with HNPGLs and are important for the identification of molecular mechanisms involved in tumor development.

**Keywords:** head and neck paragangliomas, somatic mutations, significantly mutated genes, mutational signature, whole-exome sequencing, BAF analysis
1. Introduction

Paragangliomas (PGLs)/pheochromocytomas (PCCs) or PPGLs are rare neuroendocrine tumors arising from chromaffin or glomus cells in the ganglia of the autonomic nervous system and the adrenal medulla, respectively. PGLs and PCCs originated from sympathetic ganglia are endocrine active tumors secreting catecholamines (norepinephrine, epinephrine, and dopamine); PGLs of parasympathetic origin located in the head and neck (HN) are predominantly non-secretory. Carotid body tumors account for the majority of HNPGLs (60%), followed by middle ear (29%), vagal (13%), and laryngeal (very rare) paragangliomas. PPGLs are often characterized by slow growth; nonetheless, all these tumors have some metastasis potential, which is defined as the presence of chromaffin tissue in non-chromaffin organs, such as lymph nodes, liver, lungs, and bone. Nowadays, reliable markers predicting the metastasis formation of the primary tumor remain unknown.

PPGLs carry the highest degree of heritability among all human neoplasms. Up to 40% of these tumors are associated with germline mutations and might develop as a part of the syndromes or caused by de novo mutations in the following genes: RET (multiple endocrine neoplasia type 2 [MEN2]), MEN1 (multiple endocrine neoplasia type 1 [MEN1]), NF1 (neurofibromatosis type 1 [NF1]), SDHA (paraganglioma syndrome 5 [PGL5]), SDHB (PGL4), SDHC (PGL3), SDHD (PGL1), SDHAF2 (PGL2), VHL (von Hippel-Lindau syndrome [VHL]), EPAS1/HIF2A (polycythemia-paraganglioma-somatostatinoma syndrome), MAX, MDH2, DLST, TMEM127, KIF1B, PHD1/2, FH, SLC25A11, TP53, DNMT3A, and GOT2. Somatic mutations have been detected in many genes, including SDHD, SDHA, EPAS1/HIF2A, KIF1B, RET, NF1, HRAS, CSDE1, MAML3, TP53, IDH1/2, and others. The reported frequency of germline and somatic mutations in PPGLs vary in different cohorts.
The study of 202 PPGLs has found 37% of patients with germline mutations and 8% of carriers with somatic variants. More recently, L. Fishbein et al. detected 27% of cases with germline mutations and 39% of ones with somatic variants among 173 patients with PPGLs. Importantly, both germline and somatic mutations have been predominantly observed in an exclusive manner. However, due to the rarity of the tumors, the studied cohorts included paragangliomas of different localizations with a significant prevalence of PCCs. Moreover, HNPGLs were not included in most complex molecular-genetics studies because of the necessity of their embolization before surgery. The frequency and co-occurrence of germline and somatic mutations in HNPGLs, as well as driver alterations and molecular mechanisms of tumor development remain poorly investigated.

In the present study, we analyzed the somatic mutation landscape in HNPGLs. To date, this is the largest study of somatic mutation profiling in PGLs of the head and neck region (42 samples). All HNPGLs were tested for variants in the PPGL panel of genes. 57% of patients were characterized by germline and somatic variants in the tested genes. Somatic mutations in known PPGLs susceptibility genes were found in 9.5% of patients with sporadic tumors, while germline variants were detected in 38% of inherited cases. In 17% of patients with HNPGLs, we revealed somatic variants in novel genes. For patients with mutations in PPGL-associated tumor suppressor genes (TSGs), B allele frequency analysis revealed changes in copy number variations (CNVs). We first found four COSMIC mutational signatures related to HNPGLs. Additionally, we applied MutSigCV to identify significantly mutated genes (SMGs) in HNPGLs. These genes had higher mutation frequency compared to the background mutation rate and might be involved in the development of the tumor.
2. Patients and Methods

2.1 Patients and samples

A total of 42 tumors and paired normal tissues (peripheral blood or lymph nodes) were derived from 39 patients with HNPGLs at the Vishnevsky Institute of Surgery, Ministry of Health of the Russian Federation. The collection included archrival formaldehyde fixed-paraffin embedded (FFPE) tumor tissues of 27 carotid and 15 vagal paragangliomas, 12 FFPE lymph nodes, and 27 blood tissues. Three patients were characterized by multiple PGLs. Written informed consent was obtained from all patients and the study was approved by the Ethics Committee of Vishnevsky Institute of Surgery. The clinicopathologic characteristics of patients are presented in Table 1.

2.2 DNA isolation, library preparation, and sequencing

Genomic DNA was extracted from the FFPE tumor and paired lymph node tissues using a High Pure FFPE DNA Isolation Kit (Roche, Switzerland) according to the manufacturer’s protocol. DNA isolation from peripheral blood was performed with a MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche) on a MagNA Pure Compact Instrument (Roche). A Qubit 4.0 Fluorometer (Thermo Fisher Scientific, USA) was used to measure DNA concentration. DNA quality was assessed by quantitative PCR (qPCR) using QuantumDNA Kit (Evrogen, Russia). Isolated total DNA (100 ng) was used to construct sequencing libraries with Rapid Capture Exome Kit (Illumina, USA) or TruSeq Exome Library Prep Kit (Illumina) following the manufacturer’s protocols. Sequencing of exome libraries was performed on a NextSeq500 System (Illumina) using a 75 or 150 paired-end (PE) sequencing strategy. The average coverage for each sample was at least 300x. Sequencing data are available at NCBI Sequence Read Archive (SRA) BioProject PRJNA778918.
2.3 **Bioinformatics analysis**

Raw read quality was evaluated using FastQC (v. 0.11.9, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic (v. 0.39) was used for read trimming (Q > 28 at 3’-end), filtering (average Q > 16 for each window of 4 nucleotides), and residual adapter removal. Passed reads were mapped to the human reference genome GRCh37/hg19 (Ensembl, release 75) using bowtie2 (v. 2.4.1). The obtained BAM files were processed using samtools (v. 1.10) and picard-tools (v. 2.21.3, http://broadinstitute.github.io/picard/). Base quality score recalibration and marking duplicated reads were performed using GATK (v. 4.1.2).

Somatic mutation calling was carried out using GATK Mutect2. At first, a panel of norms (PoN) was created for filtering out germline variants. Next, we analyzed the samples in tumor-versus-normal mode supplied with PoN. Further, a list of variants (VCF file) was filtered with GATK FilterMutectCalls to eliminate false positives.

Germline variants were called using GATK HaplotypeCaller. Additionally, StrandBiasBySample, StrandOddsRatio, and BaseQualityRankSumTest annotations were added. The list of SNPs was analyzed with GATK VariantFiltration according to the GATK best practice’s guide. Additionally, we manually excluded variants in polyN motifs and/or those present in most of the samples with low alternate allele (AA) frequency (5-30%), which are most likely false positives.

The annotation of germline and somatic variants was performed using ANNOVAR, including data on population allele frequency (gnomAD, Kaviar, and Exome Sequencing Project), clinical significance (ClinVar, dbSNP, and COSMIC [v.70 and v.90]), localization of variants in protein domains (InterPro), genomic region conservation score (PhastCons),
pathogenicity prediction score (SIFT, PolyPhen2, MutationTaster, LRT, InterVar, PROVEAN, MetaSVM, and MetaLR, FATHMM, VEST3, CADD, and DANN). Only variants with a maximum population frequency of less than 1% were passed to further analysis.

2.4 Analysis of copy number variations

Analysis of CNVs was performed on tumors with mutations in the SDHx, NF1, and FH genes (corresponding chromosomes) based on the beta allele frequency (BAF) method. Using exome-sequencing data, we first filtered heterozygous single nucleotide polymorphisms (SNPs) identified in normal tissues with total read depth > 25 and variant allele frequency (VAF) ranged from 0.35 to 0.65. Also, only SNPs with annotations in dbSNP (v. 150) were included in the further analysis. Filtered SNPs were plotted according to their VAF values on the diagram with the same SNPs found in the tumor simultaneously (VAF plot). Then, VAF values for each SNPs were compared between tumor and normal tissues using the exact Fisher’s. The difference between VAF values (delta-VAF) greater than 0.15 or less than -0.15, which passed Fisher’s test p < 0.05 threshold, was considered significant (delta-VAF plot).

2.5 Identification of significantly mutated genes (SMGs)

To detect significantly somatic mutated genes, we used MutSigCV (v. 1.3.4) with the mutation annotation format (.maf) file. Input file includes a list of somatic variants obtained using Mutect2 (described above) with additional filtering with gnomAD. MutSigCV estimates SMGs based on the background mutation rate quantified by silent mutations in the gene and noncoding mutations in the surrounding regions. This algorithm additionally allows for such gene properties, as replication time and expression level, and generates p-
values (significance levels) and \( q \)-values (False Discovery Rates). The genes with \( p \)-values \( \leq 0.01 \) were considered significantly mutated.

### 2.6 Analysis of mutational signatures

The mutational signature analysis was performed using SigProfiler \(^{23} \). At first, we created mutational matrices for somatic single nucleotide variants (SNVs) filtered with GATK Mutect2 and annotated as PASS. The SigProfilerExtractor tool was applied to identify de novo signatures from the matrix. We used single base substitution (SBS) mode for six variants of mutated base (C>A, C>G, C>T, T>A, T>C, and T>G) including their 5’ or 3’ sequence context generating 96 possible mutation types. Extracted mutational signatures were annotated based on the COSMIC mutational signatures (v. 3.2, [https://cancer.sanger.ac.uk/signatures/sbs/](https://cancer.sanger.ac.uk/signatures/sbs/)). The mutation burden for each sample within COSMIC signatures was evaluated.

## 3. Results

### 3.1. Significantly mutated genes

We performed the analysis of exome sequencing data of 42 HNPGLs and matched normal tissues, as well as separate analysis for those from 27 CPGLs and 15 VPGLs. A total of 1310 somatic nonsilent variants (1059 missense, 78 nonsense, 34 splicing, 29 indels, 109 frameshifts, and 1 nonstop) were found. Interestingly, approximately twice as many nonsilent mutations were identified for VPGLs compared to CPGLs (836 vs 474 variants), indicating higher mutation frequency for paragangliomas of vagal localization. However, the percentages of mutation types in carotid and vagal PGLs are approximately equal (Figure 1).
Using the MutSigCV, we identified six genes, *SDHD*, *BCAS4*, *SLC25A14*, *RBM3*, *TP53*, and *ASCC1*, that were mutated above the background mutation rate among 42 samples of HNPGLs. These genes were characterized by *p*-values ≤ 0.01, but *q*-values couldn’t reach ≤ 0.01 because of the low mutation rate in HNPGLs and the limited number of samples. Two pairs of genes from this set were significantly mutated in CPGLs (*SDHD* and *SLC25A14*, *p* ≤ 0.01) and VPGLs (*BCAS4* and *RBM3*, *p* ≤ 0.01).

Among identified genes, *SDHD* is a known driver gene associated with PPGLs. In the studied cohort, *SDHD* had the highest frequency (13%) of somatic nonsilent mutations (Figure 1). Interestingly, somatic mutations in the *TP53* gene were shown to be rare events in PPGLs, however, we found 5% of *TP53* mutation frequency in HNPGLs. Other genes were not previously reported concerning these tumors but demonstrated a surprisingly high frequency of nonsilent somatic mutations: *BCAS4* (8%), *SLC25A14* (8%), *RBM3* (5%), and *ASCC1* (5%).

### 3.2 Landscape of germline and somatic variants

All HNPGLs were subjected to the analysis of the mutations in the PPGL panel of genes. This panel has been recently proposed by Gieldon et al. and consists of 20 PPGL susceptibility genes (*EGLN1*, *EGLN2*, *MDH2*, *FH*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MAX*, *RET*, *TMEM127*, *VHL*, *EPAS1*, *NFL*, *H3F3A*, *IDH1*, *IDH2*, *ATRX*, and *HRAS*) and 64 candidate genes, such as well-known tumor-associated genes, as well as genes participating in energy metabolism and epigenetic regulation. We included four additional genes in this panel: *SDHAF3*, *SDHAF4*, *CSDE1*, and *SLC25A11*. The *SDHAF3* and *SDHAF4* genes encode for proteins that participate in the assembly of the succinate dehydrogenase complex and are important for its normal activity. *CSDE1* was supposed as a somatically-mutated driver
Gene in PPGLs. Germline mutations and loss-of-heterozygosity (LOH) in the SLC25A11 gene have been found in PPGLs with the association of metastatic disease.

Part of the studied samples was previously tested, but herein we first revealed germline and somatic status of identified mutations updating the sample set by merged norms. The pathogenicity of variants was estimated based on the criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) using the InterVar suite. In the list of variants, we also included those with low population frequency (<1%) and predicted as pathogenic by multiple prediction algorithms but classified them as “uncertain significance” according to the ACMG-AMP. These variants are the potential candidates for the likely pathogenic/pathogenic mutations. Some of these variants were described as pathogenic/likely pathogenic in the ClinVar database. A whole list of germline and somatic variants identified in PPGL susceptibility genes and candidate genes are presented in Table S1.

Germline and somatic variants in PPGL susceptibility genes and candidate genes were found in 57% (24/42) of patients with HNPGLs. 38% (16/42) of patients carried germline variants in known PPGLs-related genes, namely: SDHB, SDHC, SDHD, NF1, FH, and IDH2 (Figure 2). Among PPGLs susceptibility genes, somatic variants were detected only in the SDHD gene in 9.5% (4/42) of cases; somatic missense variant identified in patient 18tv (Figure 1) was defined as likely benign according to ACMG-AMP and was excluded from the list of deleterious variants. Notably, the majority of patients harbored either germline or somatic variants in PPGL susceptibility genes. In two patients (102tc and 40tv), we have seen germline variants co-occurring in two genes, SDHB/SDHC and SDHB/IDH2, respectively (Figure 2). One of four patients with SDHD somatic mutation (103tc) had additional somatic variants in one gene from the tested panel of genes, TP53. Another one
(117tv) had germline variant in the *NF1* gene. Somatic variants in novel genes were found in 17% (7/42) of patients who carried no germline or somatic mutations in any genes studied. Profiles of somatic mutations were different in groups of patients with germline mutations and without ones (Figure 2).

3.3 Copy number variations

We performed BAF-analysis for estimation of CNVs in tumors with mutations in PPGLs-related TSGs (*SDHx*, *NF1*, and *FH*). We estimated differences in VAF values between germline heterozygous SNPs in tumors and matched normal tissues. VAF of heterozygous two-copy SNPs in tumors nears 0.5 (taking into account sequencing coverage, errors, and percentage of tumor cells in the sample) and should be the same in the matched normal tissues. VAF values near 0 or 1 correspond to homozygous SNPs and can indicate deletion, chromosomal loss, or trisomy. We found deletion of the short arm (p) of chromosome 1 in patient 106tc with germline splicing mutation c.287-2A>G in the *SDHB* gene (Figure 3). Potential loss of chromosome 11 (or trisomy) were revealed in all patients (1tc2, 67tc, 68tc, 100tc, 120tc, and 143tc) with *SDHD* germline missense variant c.A305G, as well as in a patient 62tc with somatic frameshift variant in the *SDHD* gene (Figure S1 33).

3.4 Mutational signatures

Using SigProfilerExtractor, we extracted two *de novo* SBS mutational signatures based on the SBS-96 classification that were then decomposed into four COSMIC reference signatures: SBS1, SBS5, SBS7b, and SBS29 (Figure 4). Among identified signatures, most mutations were related to SBS5 (50.2%) followed by SBS29 (24.8%), SBS1 (15%), and SBS7b (10%). SBS7b and SBS29 signatures were enriched by mutations from seven samples each, whereas SBS1 and SBS5 included variants from 35 and 40 samples, respectively. The highest
mean mutation burden was revealed for the SBS29 mutational signature (~0.5 somatic mutations per megabase). Etiology has been previously proposed for SBS1 (spontaneous deamination of 5-methylcytosine to thymine) 34, SBS7b (exposure to ultraviolet light) 35, and SBS29 (tobacco chewing) 36. SBS5 signature is characterized by unknown etiology but has been found in association with tobacco smoking and correlated with the age of individuals 37.

Further, we performed separate analysis of mutational signatures for tumors with germline mutations identified and without these mutations. Three mutational signature (SBS1, SBS5, and SBS29) were common for both groups and were enriched by a large number of somatic variants. Additionally, two mutational signatures, SBS11 and SBS19, were found in tumors with germline mutations, and SBS33 was detected in cases without those. SBS19 and SBS33 are related to have unknown mutation processes 37,38; SBS11 was proposed to be associated with exposure to alkylating agents 37. These specific signatures were enriched by somatic variants from a few number of samples: five samples for SBS19 (125tv, 66tc, 106tc, 128tc, and 157tc), and a sample for each SBS33 (18tv) and SBS19 (125tv).

4. Discussion

The genetics of PPGLs has made significant progress over the last 20 years. Currently, it is well known that about one-third of PPGLs have a genetic predisposition 39. SDHx-related PPGLs (PGL syndromes) are inherited in an autosomal dominant manner and caused by heterozygous mutations 40. SDHx encode for the four subunits of succinate dehydrogenase (mitochondrial complex II) and act as tumor suppressor genes 41. According to the classical two-hit hypothesis, TSGs inactivation requires simultaneous loss of both alleles resulting from mutations or epi-mutations. In hereditary tumors, individuals have inherited the first
mutation in one allele from their parent and gained another mutation in the wild-type allele during their life. In PPGLs, the somatic second hit of SDHx genes is predominantly associated with loss-of-heterozygosity of the wild-type allele and, to a lesser extent, with point mutations or promoter hypermethylation. In the studied cohort, we found germline variants in SDHB, SDHC, and SDHD, as well as two other TSGs, NF1 and FH, in tumors with no somatic point mutations in these genes.

Six patients with identical germline SDHD mutation displayed loss of chromosome 11 (or trisomy). Notably, the BAF method used in the study to estimate CNVs does not allow differentiation exactly the loss of chromosome or trisomy. Theoretically, in the case of trisomy, VAF for SNPs should have 0.33 and 0.67 but these values vary depending on the percentage of tumor cells in the sample and sequencing errors. However, there was most probably chromosome loss rather than trisomy that is often observed in SDHD-mutated tumors. For SDHD-mutated tumors, it has been shown a parent-of-origin effect when the mutation is transmitted only from the father. However, despite maternal genomic imprinting, the allele of the SDHD gene from the mother is also active in normal tissues and PPGLs. The paternal transmission of the mutation can be explained by the study of Hensen et al. that revealed selective loss of entire maternal chromosome 11 in SDHD mutation carriers. Based on the Hensen model, locus 11p15 with paternal imprinting can include additional TSG, therefore the loss of maternal chromosome (but not only 11p or 11q region) is a preferred mechanism for the second hit. A more recent study demonstrated loss of maternal chromosome 11 in SDHD, SDHAF2, and VHL-related PPGLs and confirmed the important role of this event in tumor development. Moreover, Pigny et al. reported maternal transmission of the SDHD mutation that is associated with abnormal
hypermethylation of the regulating region upstream H19 gene on the maternal allele. H19 gene is located 11p15 locus and was shown to be maternally imprinted. We also observed loss of chromosome 11 in the genome of the patient 62tc with somatic frameshift mutation NM_003002: c.295_298del, p.L99fs (chr11: 111959715) in the SDHD gene. Somatic mutations in the SDHx genes rarely occur in PPGLs, however, cases with simultaneous SDHB and SDHD somatic point mutations and LOH of the remaining wild-type allele in apparently sporadic PPGLs were previously reported. Three more patients (149tc, 103tc, and 117tv) displayed somatic variants in the SDHD gene: novel frameshift variant NM_003002: c.354_355del, p.D118fs (chr11: 111965567), startloss variant NM_003002: c.A1T, p.M1L (chr11: 111957632, rs104894307), which was previously submitted as pathogenic in ClinVar database, and novel splicing variant c.170-2A>T (chr11: 111959589). However, no any rearrangements or CNVs in chromosome 11 were found in these patients indicating that the second allele inactivation can be caused by alternative molecular mechanisms. Notably, in patients 62tc and 149tc with frameshift SDHD variants, we did not detect germline mutations in PPGL susceptibility genes or somatic potentially deleterious mutations in novel genes. Thus, identified somatic alterations in SDHD seems to be driver events for PPGL development in these patients. In patient 103tc, we also revealed multiple somatic mutation in the TP53 gene apparently leading to its bi-allelic inactivation. Interestingly, the same somatic TP53 mutations were found in another patient with germline SDHD (1tc2). In these cases, bi-allelic TP53 inactivation can be a possible mechanism to escape cell cycle arrest and apoptosis that are stimulated by pseudohypoxic state due to the SDH complex deficiency.
LOH was detected only in one (106tc) in six patients with a germline mutation in the SDHB gene. No LOH was also revealed in tumors with germline variants in the SDHC, NF1, and FH genes. Consequently, we can hypothesize that either inactivation of the TSG wild-type allele was caused by an alternative molecular mechanism, such as promotor hypermethylation or haploinsufficiency of these genes alone may have contributed to the tumor development. However, there is no clear understanding of the role of TSG haploinsufficiency in PPGLs. Heterozygous Sdhb deletion in rats leads to a decrease in mRNA and protein expression, as well as reduced enzyme activity. Moreover, several irradiated rats harboring Sdhb deletion developed PCCs and CPGL. On the other hand, Sdhd+/− mice with decreased gene mRNA expression and SDH activity did not develop PPGLs. Mice with heterozygous Nf1 mutation were characterized by a predisposition to PCCs, however, no LOH of wild-type Nf1 allele was observed in their tumor DNA. Haploinsufficiency of TSGs increased the chance of tumorigenesis but required additional oncogenic changes for tumor initiation. For example, in the case of irradiated Sdhd+/− rats, radiation damage provided unpredictable genetic effects giving advantages for the development of paragangliomas. Also, irradiation of heterozygous Nf1 knockout mice increased the frequency of PCCs. Promoter hypermethylation of the SDHx genes as a mechanism of wild-type allele inactivation has not been reported to date in PPGLs. However, SDH-mutated PPGLs displayed a hypermethylation phenotype associated with severe alterations in gene expression that can potentially lead to the inactivation of SDHx. Hypermethylation phenotype like SDH-related tumors was also found in paragangliomas with FH mutations.

We also found six potential driver genes that were significantly somatically mutated in HNPGLs. Two of six genes (SDHD and TP53) were previously reported in association with
PPGLs. Mutations in the SDHD gene play a driver role in the PPGL development. Alterations in TP53 are a well-known mechanism contributing to cell proliferation and tumor progression, however, this is a rare event in PPGLs. We revealed several likely pathogenic somatic variants in the TP53 gene presenting in two patients. In the study of Fishbein et al., MutSigCV was used for the analysis of PPGLs but HNPGLs were not included. They identified five SMGs, such as HRAS, NF1, EPAS1, RET, and CSDE1, that were not significantly mutated in HNPGLs according to our results. However, somatic mutations in the TP53 gene were found in both cohorts. Among other SMGs identified in our cohort, SLC25A14 is seemed to be important in HNPGL pathogenesis because it encodes for the protein involved in the mitochondrial proton leak and is widely expressed in the brain. This gene is related to the mitochondrial carrier family (MCF) together with SLC25A11 that was recently suggested as the PPGL susceptibility gene. SLC25A14 also participates in the regulation of reactive oxygen species (ROS) generation. Somatic mutations in this gene might contribute to mitochondrial dysfunction, which is essential in the PPGL pathogenesis. Another SMG, RBM3, encodes for the protein induced by cold shock and hypoxia. RBM3 can bind with both RNA and DNA and participate in translation regulation enhancing protein synthesis, as well as response to DNA damage. Deregulation of RBM3 was shown in many cancers indicating its important role in cell survival; the nuclear protein downregulation was correlated with more aggressive and advanced tumors in prostate, esophageal, and gastrointestinal cancer. Slow-growing brain tumors also demonstrated lower nuclear RBM3 expression. Generally, the role of RBM3 in tumorigenesis has not been fully elucidated. Possibly, the lower RBM3 expression associated with tumor progression and high mutation frequency identified in our study may relate to its tumor suppressor function. The BCAS4 and ASCC1 genes, which were also identified as somatically mutated in HNPGLs,
were also shown to be implemented in cancer, however, little is known about their function in tumorigenesis\textsuperscript{66,67}.

Additionally, four COSMIC SBS-96 mutational signatures were identified in the studied cohort of HNPGLs. SBS5 and SBS1 signatures have been highly enriched by mutations, and, notably, are both associated with aging. However, the SBS1 signature can be caused by the reparation of FFPE artifacts during library preparation for sequencing\textsuperscript{68}. SBS29 signature was enriched by a quarter of all mutations while being characterized by the highest mutation burden. This signature has a putative etiology of tobacco chewing but we could not estimate this association in the studied cohort because the information on tobacco chewing/smoking was not collected. SBS7 signature associated with the UV light exposure was less enriched in HNPGL samples. Separate analysis of tumors carrying germline variants and those characterized by absence of germline mutations revealed three common mutational signatures (SBS1, SBS5, and SBS29). Possibly, these mutational processes does not depend on initial genetic predisposition to paragangliomas, and this finding does not contradict the assumption that SBS1 can be associated with technical artefact of sample preparation. We found two specific mutational signatures for tumors with germline mutations (SBS11 and SBS19) and a signature (SBS33) for cases without germline variants, however, these signatures were enriched by somatic variants from only several samples Among identified specific mutational signatures, only SBS11 has been annotated by mutational processes associated with exposure to the alkylating chemotherapeutic agent temozolomide\textsuperscript{37}. SBS11 was supported by approximately quarter of somatic variants from a patient with SDHB-mutated vagal paragangliomas undergoing surgical resection and no any treatment. DNA alkylation damage can also be caused by
exposure to agents from the environment as well as generated from different endogenous sources \(^6^9\). One of the important mechanism for the repair of alkylated lesions is direct reversal of DNA damage that involves O6-methylguanine-DNA methyltransferase (MGMT) and ALKBH family Fe(II)/α-ketoglutarate dioxygenases. Importantly, the activity of latter is regulated by intermediary metabolites of the TCA cycle, such as succinate, fumarate, and 2-oxoglutarate \(^7^0,7^1\). Thus, germline mutations in genes encoding for TCA cycle enzymes can result in the accumulation of oncometabolites and disruption of the repair mechanism of DNA alkylation damage. Moreover, \(SDHB\)-mutated paragangliomas were characterized by hypermethylation of the \(MGMT\) gene promoter and its decreased expression, and had the better response to treatment with temozolomide \(^7^2,7^3\). Also, association of \(MGMT\) promoter methylation and alkylation-related mutational signature was shown for colorectal tumors \(^7^4\). Thus, presence the alkylating-like signature in a cases with \(SDHB\)-mutated hereditary HNPGL indicate the potential involvement of alkylation repair mechanism in tumor development.

5. Conclusions

Our results demonstrated that most of HNPGLs carried germline and somatic mutations in PPGLs-related genes, and only about one-fifth of tumors were characterized by somatic variants in novel genes. Importantly, most HNPGLs with mutations in TSGs did not harbor LOH or somatic variants of the wild-type allele, as well as chromosomal loss. Thus, the second hit for TSGs inactivation might be caused by an alternative genetic mechanism.

Among found genes with a high somatic mutation rate, only two genes were previously reported in association with PPGLs (\(SDHD\) and \(TP53\)). Two other SMGs, \(SLC25A14\) and \(RBM3\), are seemed to be implemented in the HNPGL pathogenesis through mitochondrial dysfunction/oxidative stress and translation regulation/response to DNA
damage, respectively. Further target study of their function may open up novel mechanisms of tumor development. Notably, carotid and vagal PGLs were characterized by a different set of SMGs. Thus, despite these tumors belonging to the same group of parasympathetic PGLs they may differ in deleterious genetic alterations. Identified mutational signatures give an understanding of the putative nature of mutation processes generating somatic mutations in HNPGLs. Moreover, we found alkylating-like signature in a case of HNPGL with germline $SDHB$ mutation. This finding assumes a novel potential mechanism contributing to tumor pathogenesis based on the disruption in the alkylation repair system. Obtained results can help to development personalized approach for patients with HNPGLs and to expand therapy options.

Despite novel and important data obtained, the results of this study are limited by the sample set size. Further investigation on a larger cohort of HNPGLs, including middle ear paragangliomas, is required for better elucidation of somatic changes underlying HNPGL pathogenesis.
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Data availability: The sequencing data presented in this study are openly available at NCBI Sequence Read Archive (SRA): BioProject PRJNA778918.

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**Figure 2.** Germline and somatic variants identified in patients with HNPGLs. A - Patients with germline mutations in PPGL susceptibility genes and candidate genes also carrying somatic variants (25 cases). B - Patients with somatic mutations in novel genes without germline variants in genes studied (7 cases) and a patient without germline but with somatic mutation in a candidate gene from the panel of genes (*PIK3CA*). The blue color of letter marks PPGL susceptibility genes and candidate genes; the bold letter additionally indicates PPGLs-related genes. Pathogenicity of variants was assessed using ACMG-AMP classification.

**Figure 3.** Loss-of-heterozygosity of chromosome 1p in a patient with a germline mutation in the *SDHB* gene. (a) VAF for each heterozygous SNP in normal tissue (blue dots) and corresponding SNP in tumor (orange dots). (b) Delta-VAF values are marked with orange dots if they are greater than 0.15 and passed Fisher’s test (p < 0.05), others are marked with green dots.

**Figure 4.** The SBS-96 numerical plot graphs for identified COSMIC mutational signatures in HNPGLs. The x-axis presents the six subtypes of substitutions each of which has an additional 16 categories to represent the combinations of bases that can prefix and postfix the mutation. The y-axis displays the percentage of each combination.
Table 1. Clinicopathologic characteristics of patients with head and neck paragangliomas.

| Characteristic                  | Number of patients, n | Carotid paragangliomas | Vagal paragangliomas |
|--------------------------------|-----------------------|-------------------------|----------------------|
| Total patients                 | 26*                   | 26                      | 15*                  |
| Sex                            |                       |                         |                      |
| Male                           | 6 (23%)               | 1 (7%)                  |                      |
| Female                         | 20 (77%)              | 14 (93%)                |                      |
| Age at diagnosis               |                       |                         |                      |
| ≥40                            | 17 (65%)              | 13 (87%)                |                      |
| <40                            | 9 (35%)               | 2 (13%)                 |                      |
| Mean                           | 49                    | 50                      |                      |
| Tumor characteristics          |                       |                         |                      |
| Single                         | 23 (88.5%)            | 13 (87%)                |                      |
| Bilateral/multiple             | 3 (11.5%)             | 2 (13%)                 |                      |
| Recurrent                      | 2 (8%)                | 1 (7%)                  |                      |
| Metastasis                     | 1 (4%)                | 0                       |                      |

* - Total numbers of patients include two patients who had both carotid and vagal PGLs and participate in the study. One of these patients carried two CPGLs and one VPGL; herein, all the tumors were analyzed.
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