SDHX MUTATIONS ARE ASSOCIATED WITH THE PI3K-AKT SIGNALING PATHWAY IN VAGAL PARAGANGLIOMAS

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SUMMARY

Background: *Vagal paraganglioma (VPGL) is a very rare neuroendocrine tumor arising from the paraganglion associated with the vagus nerve. VPGL is mainly characterized by an asymptomatic course and slow growth. However, up to 19% of tumors can metastasize. Due to the rarity of this tumor, information about VPGL is limited to single cases and small sample sets; the data on molecular genetic features is extremely scarce.*

Methods: *For the first time we have analyzed the enrichment of biological pathways associated with mutations in the SDHx genes in VPGLs. Bioinformatics analysis was performed based on the results of high-throughput transcriptome sequencing on an Illumina platform for 33 tumor tissues obtained from patients with vagal paragangliomas.*

Results: *Eight pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with gene overrepresentation (top-40 mode) have been identified. Significant changes were shown for the cancer-associated PI3K-Akt signaling pathway and interconnected pathways of focal adhesion and interaction of receptors with the extracellular matrix enriched by overexpressed genes.*

Conclusion: *Our result indicates the association of SDHx mutations with changes in the PI3K-Akt signaling pathway in vagal paraganglioma. The potential mechanism of deregulation in this pathway could be linked with a state of pseudohypoxia induced by the dysfunction of succinate dehydrogenase due to mutations in the SDHx genes.*

Key words: *Head and neck paraganglioma, vagal paraganglioma, SDHx mutations, high-throughput sequencing, transcriptome, PI3K-Akt signaling pathway.*

INTRODUCTION

Paragangliomas of the head and neck (HNPGLs) are rare neuroendocrine tumors that develop from paraganglion cells of the parasympathetic nervous system localized in the head and neck region. HNPGLs are divided into several types depending on their location. The tumor presenting along the vagus nerve is called vagal paragangliomas (VPGLs) and accounts for 13% of all HNPGLs (1). VPGL can occur as single tumors on one or both sides of the neck, or together with other HNPGLs (multifocal form). Clinical manifestations of VPGL vary among patients, but in most cases, these are slow-growing and difficult to diagnose neoplasm, the resection of which is associated with a high risk of complications (2). Among all paragangliomas and pheochromocytomas (adrenal paraganglion tumor), VPGL is characterized by the highest risk of metastasis that is 16-19% (3).

HNPGLs have the highest degree of inheritance (approximately 30-40%) (4). In HNPGLs, germline and somatic mutations most often occur in the *SDHx* genes (*SDHA*, *SDHB*, *SDHC*, and *SDHD*), encoding the corresponding subunits of succinate dehydrogenase (mitochondrial complex II), among about 20 known susceptibility genes (5–6). In 2017, a large-scale study under the supervision of the Cancer Genome Atlas (TCGA) project revealed three main transcriptional clusters of paragangliomas and pheochromocytomas associated with the presence of mutations in certain genes (7). The tumors were divided into the following subtypes: 1) tumors with impaired functioning of the kinase cascade, 2) tumors characterized by pseudohypoxia, 3) tumors with impaired functioning of the Wnt signaling pathway. *SDHx*-mutated tumors were assigned to the pseudohypoxic subtype associated with the stabilization of hypoxia inducible factors (HIFs) and subsequent activation of their target genes. However, it should be noted that HNPGLs were not included in this study due to their embolization traditionally preceding surgical removal.

The present study for the first time shows associations between the presence of mutations in the *SDHx* genes and changes in biological pathways during neoplastic transformation in vagal paraganglioma. This study is important for a better understanding of mechanisms of VPGL development because the data on molecular genetics of this tumor is extremely limited in biomedical literature.

MATERIALS AND METHODS

Tissue samples

In the study, whole-transcriptome sequencing data obtained for 33 tumor tissues of VPGLs from the Vishnevsky Institute of Surgery, the Ministry of Health of the Russian Federation were used. These tumors were not embolized during the operation, which allowed using the postoperative biomaterial for transcriptome research. A written informed consent was obtained from all patients. The study was approved by the Ethics Committee of Vishnevsky Institute of Surgery and was performed according to the Declaration of Helsinki (1964). Histomorphological characterization of tumors

was carried out in the Pathology Department. The specimens contained at least 70% of tumor cells. The patient characteristics are presented in Table 1.

Characteristics	Number of patients
Total	32
Sex	
Male	6
Female	26
Age	
>40 years	26
≤40 years	6
Tumor localization	
Right side of the neck	11
Left side of the neck	21
Multifocal	7
Recurrence	
Yes	3
No	29
***SDHx* mutations**	
Yes	14
No	18

Table 1. Characteristics of patients with VPGLs

Transcriptome sequencing data

The total RNA was isolated from tumor FFPE tissues using the High Pure FFPET RNA Isolation Kit from Roche (Switzerland). Transcriptome libraries were prepared using the TruSeq Stranded Total RNA LT with RiboZero TM Gold kit (Illumina, USA), the principle of which is based on the removal of rRNA and subsequent preparation of libraries from the remaining material. Sequencing was performed at a single-end mode (76 bp) with double indexing on a NextSeq 500 System (Illumina). At least 30 million reads were obtained per each sample.

Bioinformatics analysis

Raw sequencing data was subjected to quality assessment using FastQC, as well as read filtering and adapter removal using Trimmomatic. The reads were then mapped to bacterial (NCBI) and rRNA databases using Bowtie2. Mapping to the reference human genome GRCh37 (hg19) was performed taking into account splice boundaries using the STAR tool. FeatureCounts (Subread package) was applied to count the number of sequences per gene. Next, the data were transferred into the R environment and analyzed using the Rtrans pipeline developed in our laboratory. The analysis of the differential expression (DE) of genes was performed using the edgeR package. Gene overrepresentation analysis (ORA) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to study the biological pathways associated with the presence of mutations in the *SDHx* genes. The top 40 DE genes with up-regulation and down-regulation were used for pathway analysis. Changes were considered significant at an adjusted P-value (FDR) ≤ 0.05 (Benjamini-Hochberg method).

RESULTS

Pathway enrichment analysis based on KEGG database was performed for two groups of tumors: VPGLs with *SDHx* mutations (14 samples) and without those (13 samples). We examined a set of 40 most variable up- and down-regulated genes between these groups (top-40) and found their over-representation in 3 and 5 biological pathways, respectively (Table 2).

KEGG ID	Pathway	Number of DE genes	Total genes in pathway	Expression direction of over-represented genes	FDR	DE genes
hsa04510	Focal adhesion	6	201	↑	0.0003	*LAMB2, ITGA11, TNXB, MYL9, COL1A2, PDGFRB*
hsa04151	PI3K-Akt signaling pathway	7	354	↑	0.0003	*LAMB2, ITGA11, NOS3, TNXB, TEK, COL1A2, PDGFRB*
hsa04512	ECM-receptor interaction	4	88	↑	0.0009	*LAMB2, ITGA11, TNXB, COL1A2*
hsa04020	Calcium signaling pathway	5	240	↓	0.0232	*SLC8A1, ADCY2, CAMK4, FGF10, PRKACB*
hsa04371	Apelin signaling pathway	4	139	↓	0.0232	*SLC8A1, ADCY2, CAMK4, PRKACB*
hsa04925	Aldosterone synthesis and secretion	3	98	↓	0.0422	*ADCY2, CAMK4, PRKACB*
hsa04024	cAMP signaling pathway	4	219	↓	0.0422	*PAK1, ADCY2, CAMK4, PRKACB*
hsa04725	Cholinergic synapse	3	113	↓	0.0489	*ADCY2, CAMK4, PRKACB*

Table 2. Significantly enriched KEGG pathways associated with *SDHx* mutations in VPGLs. ↑ - change of pathway associated with over-representation of up-regulated genes, ↓ - change of pathway associated with over-representation of down-regulated genes.

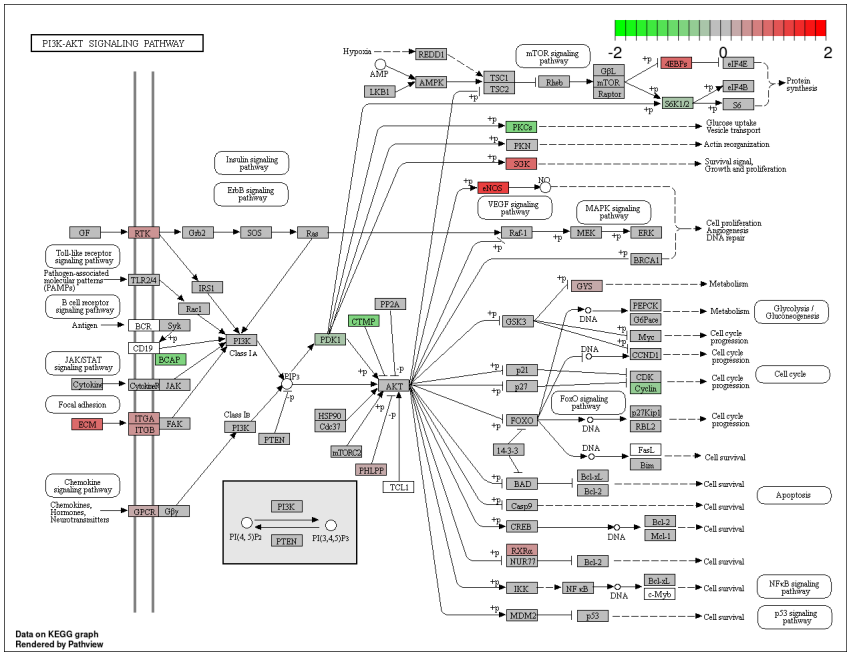

Figure 1. Visualization of the PI3K-Akt signaling pathway (KEGG). The components of the pathway are marked in color depending on the value of the logarithm of fold change (Log2FC) of DE genes.

The most significant changes associated with mutations in the SDHx genes were found in the pathways enriched by up-regulated genes: 'focal adhesion' (hsa04510), 'extracellular matrix (ECM)-receptor interaction' (hsa04151), and 'PI3K-Akt signaling pathway' (hsa04512) (FDR ≤ 0.001). Notably, the statistical significance of the changes in these pathways was increasing with the increase in the number of DE genes included in the analysis (from top-40 to top-1000, data can be provided upon request).

Importantly, the molecular pathways 'focal adhesion' and 'ECM-receptor interaction' represent a single complex system that ensures the vital activity and functionality of cells in the extracellular environment. These pathways are also involved in intracellular signaling processes. An example is the PI3K-Akt signaling pathway through membrane protein integrins (ITGA and ITGB) and focal adhesion kinase (FAK) (Figure 1). Figure 1 shows the color-coded DE genes, encoding for the components of the PI3K-Akt signaling pathway, associated with SDHx-mutated VPGLs (KEGG visualization).

DISCUSSION

Paragangliomas and pheochromocytomas with mutations in the *SDHx* genes are traditionally classified as a pseudohypoxic subtype of tumor associated with impaired functioning of enzymes of the tricarboxylic acid cycle (8). Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate and is involved in the respiratory electron transport chain. Pathogenic mutations in the *SDHx* genes lead to the accumulation of succinate that can have an oncogenic effect, resulting in alterations in cell signaling and chromatin structure maintenance (9–10).

In the study, we found a significant enrichment by over-expressed genes of a well-known cancer-associated PI3K-Akt signaling pathway in *SDHx*-mutated VPGLs. In most cases, the activation of the PI3K-Akt signaling pathway occurs as a result of mutations in the *PIK3CA* or *PTEN* gene and is often a driver event in carcinogenesis (11). The work of Joel T. Adler and colleagues showed the expression of the phosphorylated Akt (pAkt) in tumor tissues of pheochromocytomas (12). In vitro activation of the PI3K-Akt pathway was also confirmed in PC12 rat pheochromocytoma cells. The activation of this pathway has also been reported in other neuroendocrine tumors, but its association with *SDHx* mutations remains unexplored (13).

We hypothesize that the PI3K-Akt signaling pathway might be associated with a pseudohypoxic state modulated by mutations in the *SDHx* genes. As is known, the activation of the PI3K-Akt-mTOR signaling cascade leads to an increase in the mRNA transcription and translation of the HIF1A protein (14). In addition, the PI3K-Akt signaling pathway can also lead to HIF1A stabilization through the inhibition of the GSK3B kinase, which phosphorylates HIF1A, leading to its degradation through a VHL-independent pathway (14). Probably, the PI3K-Akt pathway is partially used by tumor cells to maintain the state of pseudohypoxia that was initially induced by the dysfunction of succinate dehydrogenase. However, it should be noted that the majority of genes over-represented in the PI3K-Akt signaling pathway intersect with the pathways' 'focal adhesion' and 'ECM-receptor interaction' (*LAMB2, ITGA11, TNXB, COL1A2,* and *PDGFRB*), which indicates their close linkage to SDHx-mutated VPGLs. A similar relationship was shown in glioma stem cells that demonstrated the activation of the PI3K-Akt-SOX2 signaling cascade and tumor progression in the presence of collagen/fibronectin in the extracellular matrix (3D model) (15).

CONCLUSION

Our study for the first time reports biological pathways that could be involved in the pathogenesis of VPGLs carrying mutations in the *SDHx* genes. The most statistically significant changes have shown for three pathways 'focal adhesion', 'ECM-receptor interaction', and 'PI3K-Akt signaling pathway' that have common over-represented components in *SDHx*-mutated VPGLs. In addition, the deregulation of the PI3K-Akt signaling pathway' might be associated with the pseudohypoxic state caused by succinate dehydrogenase deficiency.

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Declaration of Interests

Authors declare no conflicts of interest.

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