SDHx expression in vagal paragangliomas

INTRODUCTION
Vagal paraganglioma (VPG) is a rare tumor that arises from the paraganglia associated with the vagus nerve. The molecular genetics of VPGs have been poorly investigated due to their rarity and complexity (small size, embolization, lack of adjacent normal tissue). Using a collected sample set of 33 VPGs, we previously revealed a spectrum of mutations in the SDHx genes, encoding succinate dehydrogenase subunits, and confirmed their high frequency in these tumors. In the present study, we analyzed the expression of SDHx genes in VPGs using quantitative PCR. The SDHC and SDHD genes were characterized by statistically significant increased expression. We found no correlation between SDHB and SDHD mutations and their mRNA levels. Thus, the identified SDHx mutations are unlikely to affect the corresponding gene expression.

KEYWORDS: head and neck paraganglioma, vagal paraganglioma, mutation, gene expression, SDHx, quantitative PCR.

ANALYSIS OF SDHX EXPRESSION IN VAGAL PARAGANGLIOMAS

Analysis article

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Abstract
Vagal paraganglioma (VPG) is an extremely rare tumor of neuroendocrine origin arising from the paraganglia associated with the vagus nerve. The molecular genetics of VPGs have been poorly investigated due to their rarity and complexity (small size, embolization, lack of adjacent normal tissue). Using a collected sample set of 33 VPGs, we previously revealed a spectrum of mutations in the SDHx genes, encoding succinate dehydrogenase subunits, and confirmed their high frequency in these tumors. In the present study, we analyzed the expression of SDHx genes in VPGs using quantitative PCR. The SDHC and SDHD genes were characterized by statistically significant increased expression. We found no correlation between SDHB and SDHD mutations and their mRNA levels. Thus, the identified SDHx mutations are unlikely to affect the corresponding gene expression.

Keywords: head and neck paraganglioma, vagal paraganglioma, mutation, gene expression, SDHx, quantitative PCR.
Materials and methods

2.1. Tumour specimens

At the A.V. Vishnevsky Institute of Surgery, we gathered 33 formalin-fixed paraffin-embedded (FFPE) archival clinical tumour specimens from VPG patients. Postoperative material was evaluated macroscopically and fixed in 10% buffered formalin (pH 7.0-7.2). Then tumours were dehydrated in alcohols of increasing concentration (isopropanol, 50-99.7%) and poured into paraffin blocks. We also obtained several normal FFPE tissues from these patients. Prior to surgery, tumours were not embolized. There was clinical information available on the disease's progression and key clinical pathological characteristics.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from tumour FFPE specimens using the Roche High Pure FFPE RNA Isolation Kit. Four tissue sections from FFPE blocks (3-5 µm) were subjected to deparaffinization with xylene, which was subsequently removed with absolute ethanol. The deparaffinized tissue pellet was further processed for RNA isolation using the following steps:

1) tissue lysis;
2) protein digestion (Protease K);
3) RNA binding;
4) DNase treatment;
5) multiple washing steps;
6) RNA elution.

The final elution volume was 50 µL; RNA samples were stored at -80°C. The amount of RNA was measured using a Qubit2.0 fluorimeter. cDNA was synthesized using Mint Reverse Transcriptase from Evrogen. The mix, consisting of 20 μL containing 1 µg of extracted RNA, 20 μM random hexamers and 10 μL Milli-Q water, was incubated at 70°C for 2 minutes and then placed on ice. The following reagents were added to the mixture: 4 μL 5X First Strand Buffer, 2 μL dNTP mix (10 mM each), 2 μL DTT (20 mM), and 2 μL Mint Reverse Transcriptase. The reactions were incubated at 42°C for 1 hour, followed by 70°C for 15 minutes on a T100 thermal cycler. The resulting cDNA was stored at -32°C. The cDNA clean-up was performed with the QIAquick PCR Purification Kit. Quantity of cDNA was measured on the Qubit2.0 fluorimeter; 0.5 ng of cDNA from each sample was used for subsequent quantitative PCR (qPCR).

2.3. Quantitative PCR

The qPCR was done on an AB 7500 RT-PCR System. Primers and probes from TaqMan Assays for target genes, SDHA_Hs00188166_m1, SDHB_Hs00268117_m1, SDHC_Hs01698067_s1, SDHD_Hs0198144_g1, and reference gene, GAPDH_Hs00266705_g1 were used in amplifications. Reactions were performed in 25 μL reaction solutions, including following reagents: GenLab Polymerase (1U), 1X PCR Buffer GenLab (25 mM, MgCl2), ROX (0.001X, Evrogen), dNTP (0.2 mM), TagMan Mix (250 nM), Milli-Q water, and cDNA. PCR conditions were 95°C – 15 minutes followed by 40 cycles of 95°C – 15 seconds and 60°C – 60 seconds. All reactions were carried out in triplicates for each sample and gene. The AB 7500-generated data were proceeded using our own ATG software based on the ΔΔCt approach with calculation of the reaction efficiencies (E). As a result, the relative mRNA expression levels were obtained. We used nonparametric Wilcoxon test for analyzing qPCR data to compare mRNA level differences between tumour and normal tissues. P-values<0.05 were considered statistically significant. Correlations were estimated for SDHx mutational status and mRNA expression levels based on the Spearman correlation coefficient using STATISTICA 10.

Results and discussion

Exome sequencing and analysis had previously determined the SDHx mutation status in the set of VPGs under study [5],[6]. The ACMG-AMP criteria were used to interpret the pathogenicity of the identified variants [9]. We found five patients with variants in the SDHB gene (Table - 1). The most frequently mutated gene was SDHD; nine patients were characterized by mutations in this gene. SDHA variants were present in two patients, but the SDHC gene was no mutated in VPGs.

Table 1 - The list of SDHx variants in VPGs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>c.C781T, p.R261C (chromosome5:231001, rs143484394)</td>
<td>Uncertain significance</td>
</tr>
<tr>
<td></td>
<td>c.C830T, p.T277M (chromosome1:17350571, rs786201161)</td>
<td>Pathogenic</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.136C&gt;T, p.Arg46* (chromosome1:17371320, rs74315370)</td>
<td>Pathogenic</td>
</tr>
<tr>
<td></td>
<td>c.541-2A&gt;G (chromosome1:17350571, rs786201161)</td>
<td>Pathogenic</td>
</tr>
<tr>
<td></td>
<td>c.C79T, p.R27X (chromosome1:17371377, rs74315369)</td>
<td>Pathogenic</td>
</tr>
</tbody>
</table>

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QPCR was used to estimate the SDHx mRNA levels (Table 2). The expression levels of the SDHA and SDHB genes did not change statistically significantly (p=0.08 and p=0.5, respectively). The relative SDHC and SDHD expression levels were significantly increased by more than 10-fold in 60% and 48% of VPGs, respectively (p = 0.43 for each gene). However, Spearman correlation analysis showed no statistically significant association between the presence of SDHB and SDHD mutations and changes in their expression (Table 2).

Table 2 - Relative SDHx expression levels in VPGs and correlations with mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Decrease, %</th>
<th>Increase, %</th>
<th>Max increase, times</th>
<th>Max decrease, times</th>
<th>Average change, time</th>
<th>Spearman correlation coefficient</th>
<th>P value (Spearman test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA*</td>
<td>54.5</td>
<td>15</td>
<td>4</td>
<td>6</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SDHB</td>
<td>61</td>
<td>12</td>
<td>4.5</td>
<td>6</td>
<td>1.4</td>
<td>-0.21</td>
<td>0.20</td>
</tr>
<tr>
<td>SDHC*</td>
<td>23</td>
<td>60</td>
<td>31.8</td>
<td>171</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SDHD</td>
<td>20</td>
<td>48</td>
<td>26.5</td>
<td>34</td>
<td>0.7</td>
<td>-0.10</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Note: * - due to the lack or low number of mutations in SDHA, correlation was not assessed
methylation, which lead to lacking of the protein expression, was previously reported in parasympathetic paragangliomas and [14].

**Conclusion**

SDHx mutations and their mRNA levels were not found to be related in the set of VPGs studied, and the expression of the corresponding genes may not be directly impacted by these mutations. However, further studies with an expanded sample set of VPGs may improve the statistical power. Significantly deregulated expression was found in the SDHC and SDHD genes, which may be associated with the development of VPG.

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**Conflict of Interest**

None declared.

**Review**

All articles are peer-reviewed. But the reviewer or the author of the article chose not to publish a review of this article in the public domain. The review can be provided to the competent authorities upon request.

**References**