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ANALYSIS OF *SDHX* EXPRESSION IN VAGAL PARAGANGLIOMAS

Research 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.

АНАЛИЗ ЭКСПРЕССИИ ГЕНОВ *SDHX ПРИ ВАГАЛЬНЫХ ПАРАГАНГЛИОМАХ*

Научная статья

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Аннотация

Вагальная параганглиома (ВПГ) – это очень редкая нейроэндокринная опухоль, формирующаяся из параганглия, связанного с блуждающим нервом. Молекулярно-генетические особенности ВПГ мало изучены из-за редкости этих опухолей и сложности исследования (малый размер, необходимость предоперационной эмболизации, отсутствие прилежащей нормальной ткани и др.). В результате ранее проведенных нами исследований, идентифицирован спектр мутаций в генах *SDHx*, кодирующих субъединицы сукцинатдегидрогеназы, и подтверждена их высокая частота для 33 опухолевых образцов ВПГ. В данной работе, нами выполнен анализ экспрессии генов *SDHx* в этих опухолях с использованием метода количественной ПЦР (кПЦР). Выявлено значимое повышение экспрессии генов *SDHC* и *SDHD* в исследуемых образцах ВПГ. Показано, что мутационный статус генов *SDHB* и *SDHD* не коррелирует с их экспрессией на уровне мРНК. Полученные результаты свидетельствуют о том, что идентифицированные *SDHx* мутации, вероятно, не оказывают влияния на экспрессию соответствующих генов.

Ключевые слова: параганглиома головы и шеи, вагальная параганглиома, мутации, экспрессия генов, SDHx, количественная ПЦР.

Introduction

Vagal paragangliomas (VPGs) are rare neuroendocrine neoplasms of the head and neck. VPG arises from a small paraganglion near the vagus nerve. WHO classifies paragangliomas and pheochromocytomas (PPGs) as tumours with variable potential for metastasis [1]. VPGs are described as tumours with high degree of metastatic potential; up to 19% of VPGs can metastasize [2], [3]. To date, molecular mechanisms and markers of their progression remain uncovered. Moreover, the genetics of VPGs has been poorly examined because of their rarity. Several well-known genes, comprising *SDHx*, *RET*, *VHL*, *FH*, *MAX*, etc., are linked to embryonic mutation in about 40% of PPGs [4]. Mutations in the *SDHx* genes, which encode four subunits of the succinate dehydrogenase (SDH) complex, are the major cause of hereditary VPGs [5], [6]. When *SDHx* mutations are present, oncometabolite succinate accumulates and reactive oxygen species production increases. This cause transcriptional deregulation, epigenetic abnormalities, and genomic instability contributing to tumorigenesis [7]. The spectrum of *SDHx* variants has a wide variety and covers frequent single nucleotide variants and rarer single/multiple exon deletions [8]. Despite the fact that *SDHx* variants cause severe consequences in cellular pathways, the impact of many mutations (especially, missense variants) on mRNA and protein remains clouded.

Herein, we estimated *SDHx* RNA levels and correlations between *SDHB* and *SDHD* variants and their expression in VPGs.

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 (*Proteinase 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

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Gene	Variant	Pathogenicity
SDHA *[5p15.33]*	c.C781T, p.R261C (chromosome5:231001, rs143484394)	Uncertain significance
	c.C830T. p.T277M (chromosome5:231050, rs367721665)	Uncertain significance
SDHB *[1p36.13]*	c.136C>T, p.Arg46* (chromosome1:17371320, rs74315370)	Pathogenic
	c.541-2A>G (chromosome1:17350571, rs786201161)	Pathogenic
	c.C79T, p.R27X (chromosome1:17371377, rs74315369)	Pathogenic

	c.287-2A>G (chromosome1:17355233, rs1064794270)	Pathogenic
	c.A307G, p.M103V (chromosome1:17355211, rs140178341)	Uncertain significance
SDHD [11q23.1]	c.205G>T, p.Glu69* (chromosome11:111959626)	Pathogenic
	c.335_338del, p.T112fs (chromosome11:111965547, rs587776648)	Pathogenic
	c.A305G, p.H102R (chromosome11:111959726, rs104894302)*	Likely pathogenic
	c.C242T, p.P81L (chromosome11:111959663, rs80338844)	Uncertain significance
	c.G232C, p.G78R (chromosome11:111959653, rs1592780479)	Uncertain significance
	c.G337C, p.D113H (chromosome11:111965551)	Uncertain significance
	c.170-2A>T (chromosome11: 111959589)	Likely pathogenic

*Note: * - three patients carry this variant. SDHC is not shown in the table because we did not find any mutations in this gene*

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

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

*Note: * - due to the lack or low number of mutations in SDHA, correlation was not assessed*

Our findings are important for understanding the mechanisms behind development of *SDHx*-related VPGs. These genes acts as tumor suppressors (TSs) and their inactivation lead to tumor formation [10]. Dr. Peilin Jia with colleagues showed that different genetic variants could influence TS mRNA expression in different cancer types [11]. Our findings demonstrated that *SDHB* and *SDHD* mRNA levels are likely unaffected by variants in the corresponding genes, however, the study is limited by small sample size of VPGs due to the rarity of the disease. It is possible that these mutations could affect the structure and function of the proteins rather than mRNA synthesis. For instance, it has been previously demonstrated that pathogenic mutations in the *SDHx* genes can lead to changes in the stability of the SDH complex, which can be detected by immunohistochemical SDHB staining and is highly correlated with mutations in any *SDHx* [12], [13]. We also for the first time observed significant changes in *SDHC* and *SDHD* mRNA levels. Deregulated expression of these genes may be linked to the development of VPGs. These expression changes can be caused by different mechanisms such as promoter methylation, copy number variation of chromosomes, alterations in posttranscriptional regulation and others. Thus, somatic *SDHC* promoter

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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Конфликт интересов

Не указан.

Рецензия

Все статьи проходят рецензирование. Но рецензент или автор статьи предпочли не публиковать рецензию к этой статье в открытом доступе. Рецензия может быть предоставлена компетентным органам по запросу.

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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.

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