Different strategies in the biochemical diagnosis of pheochromocytoma

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Chapter 4

SDH subunit mutation status in saliva:
Genetic testing in patients with pheochromocytoma

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ABSTRACT
Germline mutations occur in up to 30-40% of pheochromocytoma/paraganglioma, with mutations in the succinate dehydrogenase (SDH) subunits B (SDHB) and D (SDHD) being the most common. Blood samples are favored for obtaining high quality DNA, however leukocytes can also be obtained by collecting saliva. The aim of this study was to determine whether SDHB and SDHD gene mutations in patients with (PHEO/PGL) could be determined using a salivary sample. Paired blood and salivary samples were collected from thirty patients: nine SDHB mutation positive, thirteen with a SDHD mutation, and eight without any SDHx mutations. The Oragene DISCOVER kit was used to collect and extract DNA from saliva. Blood DNA was extracted from EDTA blood samples. The DNA purification and concentration were measured by spectrophotometry. The eight exons of SDHB and the four exons of SDHD were amplified and sequenced by PCR-based bidirectional Sanger sequencing. Total DNA yields from blood DNA were similar to those obtained from saliva DNA (mean (±SD) saliva vs. blood DNA concentration 514.6 (±580.8) ng/µL vs. 360.9 (±262.7) ng/µL (P=.2). The purity of the saliva DNA samples was lower than that of blood (mean OD$_{260}$/OD$_{280}$ ratio 1.78 (±0.13) vs. 1.87 (±0.04) (P=.001), respectively), indicating more protein contamination in the saliva-extracted DNA. This study shows that salivary DNA collected from patients with PHEO/PGL is a good alternative for extraction of genomic DNA for its high DNA concentration and acceptable purity and can be used as an alternative to blood derived DNA in screening for SDHB and SDHD mutations.
INTRODUCTION

Pheochromocytoma (PHEO)s and paraganglioma (PGL)s are rare neuroendocrine tumors derived from sympathetic and parasympathetic paraganglia in the thorax, abdomen and head and neck region. In up to 40% of patients with these tumors, a germline mutation can be detected\(^1\). Mutations in the \textit{neurofibromatosis type 1} (\textit{NF1}), \textit{Rearranged during Transfection} (\textit{RET}), \textit{von Hippel-Lindau} (\textit{vHL}), and \textit{succinate dehydrogenase} (\textit{SDH}) subunits \textit{A}, \textit{B}, \textit{C} or \textit{D} genes are the most well known causes of disease\(^2\)\(^-\)\(^4\). The number of PHEO/PGL susceptibility genes was recently increased to 21\(^5\). The pathogenesis and progression of these tumors are very strongly influenced by genetics. In particular, \textit{SDHB} mutations have been associated with more aggressive tumors, younger ages at presentation, and higher rates of metastatic disease\(^2\)\(^6\).

Genetic testing is indicated in all patients diagnosed with a PHEO/PGL below the age of 50 years. Family history, clinical presentation (location, biochemical secretion pattern, and accompanying symptoms) and the immunohistochemical characterization of the tumors, are mainly used to determine which genes will be tested\(^1\)\(^2\). Based on the most recent guideline recommendations, genetic testing for other family members should be offered if a genetic mutation is identified in the proband\(^7\).

Obtaining high quality DNA for human genetic studies is essential in the disease gene discovery process. Blood samples are favored for obtaining cells for high quality genetic testing. However, DNA can also be obtained from saliva collection, which is less expensive, noninvasive, easy to obtain (even at home), and can be readily sent to the hospital by regular mail. These advantages are very important, particularly in children, because phlebotomy is always stressful and unpleasant process for them. Therefore, we decided to investigate whether saliva could be used as an alternative method to blood for the detection of \textit{SDHB} and \textit{SDHD} mutations in patients with PHEOs/PGLs.

MATERIALS AND METHODS

Study population

Saliva samples were collected from 30 consecutive patients who were seen in the Clinical Center of the National Institutes of Health, Bethesda, US. All patients were evaluated under the clinical protocol 00-CH-0093 that was approved by the Institutional Review Board of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Nine patients had a known \textit{SDHB} mutation, 13 had a known \textit{SDHD} mutation, and 8 patients were of unknown \textit{SDHx} mutation status.

Sequencing protocol

The Oragene DISCOVER (OGR-500) (DNA Genotek Inc. Ottawa, Canada) saliva kit was used to collect and extract DNA from saliva according to the manufacturer’s protocol. Briefly, patients were asked to fast and abstain from chewing gum and smoking at least 30 minutes before saliva collection. The collected saliva samples were stored at 4° degrees Celsius before DNA extraction. DNA from EDTA blood samples was extracted as previously described\(^8\). The DNA purification and concentration were
measured by spectrophotometry. The eight exons of \textit{SDHB} and four exons of \textit{SDHD} were amplified and sequenced by PCR-based bidirectional Sanger sequencing using the saliva-extracted and blood-derived DNA. The primers used for \textit{SDHB} and \textit{SDHD} have been described elsewhere. All amplified samples were examined by agarose gel electrophoresis to confirm successful amplification of each exon. Direct sequencing of the purified fragments was then done using the Genetic Sequencer ABI3100 Applied Biosystems (Applied Biosystems Inc, Foster City, CA, USA) apparatus. Sequences were analyzed using Vector NTI 10 Software (Invitrogen, Carlsbad, CA).

**Statistics**

Data are presented as mean ± standard deviation (SD). Differences in mean OD\textsubscript{260}/OD\textsubscript{280} ratios between DNA extracted from blood or saliva were calculated with the paired T-test. A two-sided \(P<0.05\) was considered statistically significant. Analysis was preformed with SPSS statistics (version 22.0; IBM/SPSS, Armonk, New York).

**RESULTS**

We were able to confirm 9 out of 9 \textit{SDHB} mutations and 11 out of 13 \textit{SDHD} mutations in these patients both in saliva and blood-extracted DNA. Eight patients were screened negative for any \textit{SDHx} mutations (Table 1). The chromatograms of sequencing analysis obtained both from the saliva and blood of a patient with a known \textit{SDHB} mutation at exon 4 can be seen in Figure 1. In two (6.7%)

![Figure 1. Example of chromatogram of forward and reverse DNA sequence from saliva (A) and blood (B) results of a patient with a mutation in exon 4 of the \textit{SDHB} gene. (see color image on page 185)](image)
Table 1. Sequence variants of patients with SDHB and SDHD mutations tested in saliva

<table>
<thead>
<tr>
<th>Genes</th>
<th>Number of patients with sequence variance</th>
<th>Sequence variant</th>
<th>Saliva</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DNA quantity (ng/µL)</td>
<td>OD\textsubscript{260}/OD\textsubscript{280} ratios</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>Variant: SDHB intron 1, c.73-8A&gt;G</td>
<td>103.0</td>
<td>1.65</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 1, c.72+1G&gt;T</td>
<td>1028.0</td>
<td>1.54</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 2, c.79C&gt;T p.R27</td>
<td>750.0</td>
<td>1.78</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 2, c.136C&gt;T, p.Arg46X</td>
<td>62.0</td>
<td>1.92</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 3, c.271A&gt;T, p.Arg91X</td>
<td>199.3</td>
<td>1.85</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 3, c.203G&gt;A, p.C66Y</td>
<td>250.3</td>
<td>1.80</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 4, 380 T&gt;G p.1127s</td>
<td>352.2</td>
<td>1.78</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 5, c.526G&gt;T p.Glu17x</td>
<td>520.6</td>
<td>1.92</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>SDHB exon 7, c.683_684 del AG, p.Glu228GlyfsX27</td>
<td>200.0</td>
<td>1.67</td>
</tr>
<tr>
<td>SDHD</td>
<td>1</td>
<td>SDHD exon 4, c.341A&gt;G p.Tyr114Cys</td>
<td>203.1</td>
<td>1.75</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>SDHD exon 2, c.57delG p.Leu20CysfsX66</td>
<td>367.0 (±172.6)</td>
<td>1.68 (±0.06)</td>
</tr>
<tr>
<td>SDHD</td>
<td>9</td>
<td>SDHD exon 3, c.242C&gt;T, p.Pro81Leu</td>
<td>721.4 (±953.0)</td>
<td>1.82 (±0.11)</td>
</tr>
</tbody>
</table>

Values are expressed as a number or mean (±SD). SDHB, succinate dehydrogenase subunit B; SDHD, succinate dehydrogenase subunit D.
patients with \textit{SDHD} mutations at exon 2 (c.57delG p.Leu20CysfsX66), the salivary DNA samples were not successfully genotyped. Total DNA yields from saliva were similar to the ones obtained from blood DNA (mean (±SD) saliva vs. blood DNA concentration 514.6 (±580.8) ng/µL vs. 360.9 (±262.7) ng/µL) \((P=.2)\). The purity of the saliva DNA samples was lower than blood (mean \(\text{OD}_{260}/\text{OD}_{280}\) ratio 1.78 (±0.13) vs. 1.87 (±0.04) respectively; \(P=.001\)).

**DISCUSSION**

This is the first study that describes the use of saliva for the screening of \textit{SDHB} and \textit{SDHD} mutations in patients with PHEOs/PGLs. We have shown that saliva can be used as an alternative method for the genetic testing of patients with PHEOs/PGLs and their family members and that the rate of mutation detection is similar to blood-extracted DNA. In contrast to blood collection, which can be a major obstacle for obtaining DNA, this noninvasive method can be very helpful for children, in particular. In addition, the sample can be collected at home and sent to the hospital by regular mail, which would increase the patient's comfort and compliance significantly. This is particularly useful in screening large pedigrees of patients with a known mutation.

Our analysis shows that the total DNA yields are comparable between blood and saliva. However, the purity of saliva was lower, indicating more protein contamination in the saliva extracted DNA; to date more than 2000 proteins and peptides have been detected in human saliva\(^9\).

We were not able to amplify and sequence the DNA extracted from the saliva of two patients with an \textit{SDHD} mutation at exon 2 (c.57delG p.Leu20CysfsX66). Although we tried to optimize the PCR conditions, we were not able to visualize the PCR products in 1.5% agarose gel. However, we were able to amplify, sequence, and identify the mutation in the blood of these patients. It is probable that an unknown factor in the saliva of these patients impeded DNA denaturation or primer annealing.

In conclusion, salivary DNA is a good alternative for extraction of genomic DNA. DNA extracted from saliva is of acceptable quality and quantity and therefore, can be used as an alternative to blood-derived DNA for the genetic screening of \textit{SDHB} and \textit{SDHD} mutations. Saliva collection is an easy-to-use, noninvasive method, in contrast to blood collection, which can be a major drawback for both children and adults. This method would potentially improve compliance among patients and, especially, related family members of patients with a known mutation during the genetic screening process.

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