ROLE OF TUMOR SUPPRESSOR GENES IN NEUROENDOCRINE NEOPLASIAS
AND CARDIOVASCULAR DISEASE

DISSERTATION

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By

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ABSTRACT

Cancer and cardiovascular disease are the two leading causes of death in the United States. Although these two complex diseases are seemingly unrelated, our literature searches have led us to the conclusion that they are caused by many of the same biological processes, including proliferation and inflammation. For example, there exist neoplastic and cardiovascular etiologies of hypertension. We therefore wanted to address the hypothesis that tumor suppressor and other cancer-related genes are causative in the development of both cancer and cardiovascular disease. In order to address this hypothesis, we examined the underlying genetic alterations of tumor suppressor and other cancer-related genes in a neuroendocrine neoplasia, pheochromocytoma (PC) and in a specific type of cardiovascular disease, carotid artery atherosclerosis. PCs present in both sporadic and hereditary cases. The syndromic manifestations, including MEN 2, VHL, and PC/PGL syndrome, exhibit strong phenotype-genotype correlations. The causative genes for these syndromes are \textit{RET}, \textit{VHL}, and \textit{SDHB}, \textit{SDHC}, and \textit{SDHD}, respectively. Current clinical cancer genetics strives to achieve this correlation in all diseases, for this facilitates genotype-based personalized management of the patients and their families.

Our studies uncovered a number of interesting conclusions. Although it was previously thought that only 10\% of PCs were hereditary, we found in a population-based
registry of PC cases, that ~25% of apparently sporadic PC cases are caused by a germline mutation in one of the PC-associated genes. Further examination of the genetics of the remaining mutation-negative PC cases led us to the discovery of novel whole- and partial-gene deletions in \textit{SDHB} and \textit{SDHD}. When looking at cases of the Carney triad/dyad, an association of PGL, GIST and pulmonary chondroma, we found that 3/50 (6%) have germline splice-site mutations in either \textit{SDHB} or \textit{SDHC}. In addition, up to 1/3-1/2 of apparently sporadic PC cases are associated with a haplotype consisting of \textit{RET} SNPs, and this haplotype is likely in linkage disequilibrium with a low-penetrance locus in the 5’ UTR. Finally, we used three array-based platforms to look for genetic alterations in atherosclerotic carotid artery samples. Chromosomal loci 1q, 3p, 15q, and 16q were found to be altered and previous data indicates that all of these regions are altered in cancers. The data we present has significant implications, including the phenotype-genotype correlations as well as the clinical implications for patients and their physicians. It is our hope that the integration of the genetics of neuroendocrine neoplasms and carotid artery disease will represent the molecular merging of two major disease processes, and allow for common effective genetics-based personalized medical management for both cancer and cardiovascular disease.
Dedicated to my Mom and Dad
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LIST OF SYMBOLS/ABBREVIATIONS

PC: pheochromocytoma

PGL: paraganglioma

MEN 2: multiple endocrine neoplasia type 2

VHL: von Hippel-Lindau

GIST: gastro-intestinal stromal tumor

RCC: renal-cell carcinoma
CHAPTER 1

INTRODUCTION

Cancer and cardiovascular disease are the two leading causes of death in the United States. Billions of dollars each year are spent nationwide to research and address infinite hypotheses for these seemingly unrelated diseases, including those of etiology, pathogenesis, therapy and prevention. Because they appeared unrelated, even granting agencies and professional society meetings kept these topics quite separate. However, our literature searches leading to our research hypotheses lead to one exciting conclusion: cancer and cardiovascular disease are caused by many of the same biological processes. These essential processes are excess proliferation and inflammation (1). This stunning conclusion leads us to address our hypothesis that tumor-suppressor and other cancer-related genes are not only involved in the pathogenesis of carcinogenesis, but also the development of cardiovascular disease. In order to address this hypothesis, we utilized clinical cancer genetic and global genomic strategies. Because we wanted to examine the genetic alterations which could underlie both neoplasia and proliferative vascular disease, we chose as our theme, hypertension. Thus, we analyzed the genetics of a neoplastic
cause of hypertension, pheochromocytoma, and we chose to examine the genetics of intimal proliferation in carotid artery atherosclerosis.

Pheochromocytoma (PC), our model neoplasia that may cause hypertension, usually occurs sporadically and in the apparent minority of cases, is associated with one of several heritable cancer syndromes, multiple endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau disease (VHL), and heritable PC-paraganglioma syndromes (PC-PGL) (2-8). Because the majority of PC are apparently sporadic, i.e., without affected family members or syndromic associations, we wanted to determine whether unsuspected germline mutations in the relevant susceptibility genes occurred and if so, at what frequency. In addition, we also wanted to determine if germline mutations occurred in PC/PGL presentations in non-traditional syndromic forms, ie, not MEN 2, VHL or PC-PGL syndrome and in this case Carney triad/dyad.

The impeccable genotype-phenotype correlation between the causative germline mutations in the RET proto-oncogene and the clinical subtypes of MEN 2 is a paradigmatic model for which all clinical cancer genetics currently strive (9). MEN 2 is a hereditary cancer syndrome that is characterized by pheochromocytoma (PC), hyperparathyroidism (HPT), and medullary thyroid carcinoma (MTC) (3), and over 90% of all cases are due to a germline mutation in RET. Of the over 95% of MEN 2A patients with germline mutations, 84% affect a single amino acid, codon 634, and germline mutations at codon 623 are strongly associated with PC. Such a strong correlation allows physicians and clinical geneticists alike to clearly diagnose a patient, perform genetic
testing on their relatives, and appropriately screen and treat each affected individual in a personalized manner.

In addition to traditional intragenic gain-of-function mutations in \textit{RET} found in MEN 2 patients, loss-of-function mutations are associated with a small subset of Hirschsprung disease patients (HSCR [OMIM 142623]). HSCR disease is a common congenital disorder, seen in 1/10,000 live births. It is characterized by aganglionic megacolon. It has been shown that a haplotype consisting of a unique combination of \textit{RET} polymorphic loci is in linkage disequilibrium with A45A, a SNP in exon 2. In addition, it was shown that a different \textit{RET} haplotype was in linkage disequilibrium with exon 14 SNP S836S in apparently sporadic cases of MTC. Therefore, we hypothesized that a haplotype could also be associated with a subset of apparently sporadic pheochromocytomas. We therefore examined these SNPs in a subset of 104 sporadic PCs, without traditional germline mutations, from the population-based Freiburg-Warsaw-Columbus PC registry. In one of the ensuing chapters, we describe the data showing that a unique haplotype within \textit{RET} is associated with apparently sporadic PCs.

Von Hippel-Lindau disease (VHL [OMIM 193300]) is an inherited disease that affects many systems of the body, and predisposes to PC, RCC, retinal and central nervous system hemangioblastomas (10, 11). VHL is highly penetrant, and is inherited in an autosomal manner. The susceptibility gene, the tumor-suppressor gene \textit{VHL}, is found on 3p26 (7). Similar to MEN 2, VHL has various subclasses. These subclasses are distinguished by their phenotypic presentations, and also exhibit a genotype-phenotype correlation and missense mutations are particularly associated with PC (12-14).
overall frequency of PC in VHL disease is ~10-15%. A relatively new disorder, PC-PGL syndrome, has been characterized by the presence of functioning PC and/or extra-adrenal PC (PGL; can be functioning or non-functioning). Germline mutations in SDHB, SDHC, and SDHD predispose to this syndrome. We wanted to examine the genetic causes of all PCs, including PGL. Hereditary forms of PC and PGL were recently shown to be associated with the genes encoding members of the SDH complex. Succinate dehydrogenase (SDH) or mitochondrial complex II is composed of four subunits, SDHA-SDHD. Genetic linkage identified a susceptibility locus on chromosome sub-band 11q23 (PGL1), and SDHD was identified as the susceptibility gene for PGL1 families. A second locus, PGL2, was identified and mapped to chromosome 11q13, however the responsible gene and corresponding mutations remain to be identified. PGL3 results from germline mutations in SDHC (15), while SDHB was identified as PGL4 (16). Mitochondrial complex II has a dual role in energy production, with its role in both glycolysis and the electron transport chain. This complex is able to participate in both processes because of its position on the mitochondrial membrane, in both the matrix and the membrane. The essential role of this complex in energy production and oxygen sensing provides the link to the PC tumor development.

In a 2003 critical review in Nature Reviews Cancer, Eng and colleagues hypothesized that the hypoxia-inducible factor gene (HIF-1) was responsible for tying RET, VHL and the SDH genes together (17). Remarkably, this hypothesis has just been experimentally confirmed, showing a plausible interaction between RET, VHL, and the SDH genes (18, 19). This interaction is through the hypoxia inducible pathway. The
function of VHL as a key mediator of the hypoxia response has been established for many years. VHL is responsible for targeting HIF-1 for degradation under normal oxygen conditions. *SDHB* and *SDHD* mutation-positive PC/PGLs resemble carotid body growths that occur as a result of chronic hypoxia exposure in individuals living at high altitudes (20).

The Carney triad is technically not a syndrome but instead, a unique association of PGL, gastric stromal tumor (GIST) and pulmonary chondroma (21). Carney dyad comprises PGL and either GIST or pulmonary chondroma. Until now, this association of three seemingly unrelated tumors was thought to be random or coincidental. However, because of the essential PGL component in all Carney triad/dyad patients, and because the SDH genes play an essential role in many hereditary PGL, we hypothesized that these genes may play a role in the Carneys triad/ dyad as well (chapter 5).

In order to elucidate the germline contribution of the above mentioned susceptibility genes in apparently sporadic PC/PGL, we carried out our studies on a population-based PC and PGL series and those with Carney triad/dyad. The design of the studies and salient data are illustrated in figure 1.1.
Figure 1.1. Description of overall thesis project design, including all studies relating to PC/PGL.

In this thesis (Chapters 2-5), we established that germline mutations in one of RET, VHL, SDHB, SDHC, and SDHD occurs in apparently sporadic PC, and that there appears to be a genotype-phenotype correlation depending on the gene involved. We found in the Carney dyad that germline mutations in two of the SDH genes, SDHB and SDHC characterize a subset of Carney dyad patients that are hereditary. Because cardiovascular disease is characterized by two hallmark functions of carcinogenesis and also hypertension, proliferation and inflammation, we wanted to elucidate the role of
cancer-related genes in this complex disease. We did so by examining the genetics of proliferation in carotid artery disease, an additional cause of hypertension.

In order to specifically address this part of our hypothesis, we used three array-based platforms. The integration of the data from array comparative genomic hybridization (aCGH), single-nucleotide polymorphism (SNP) array, and Affymetrix U133A Expression array has allowed us to draw a number of conclusions with respect to our hypothesis. First, carotid vascular disease can indeed be caused by somatic genetic variations in a number of cancer-related genes and chromosomal regions that are highly populated with cancer-related genes. Secondly, these chromosomal regions that were particularly interesting based on the integration of the data were on 1q, 3p, 15q, and 16q. Finally, further experiments must and will be done in order to concretely elucidate the role of these genetic regions and the genes in those regions, in the development of carotid artery atherosclerosis.

In conclusion, we present novel findings showing genetic evidence of heredity by finding germline mutations and deletions in SDHB, SDHC, and SDHD for a significant percentage of apparently sporadic PC and PGL presentations as well as an association with a unique haplotype within RET. We also present the novel data of three (6%) Carney dyad patients harboring germline splice-site mutations in one of the SDH genes. Our data will impact the practice of clinical cancer genetics. Finally, we were able to draw many novel conclusions from our tri-platform genomics approach to carotid artery disease, and show the role of genetic alterations in four chromosomal regions.
CHAPTER 2

GERMLINE MUTATIONS IN NON-SYNDROMIC PHEOCHROMOCYTOMA

2.1 Abstract

The group of susceptibility genes for pheochromocytoma that included the proto-oncogene \textit{RET} (associated with multiple endocrine neoplasia type 2 [MEN-2]) and the tumor-suppressor gene \textit{VHL} (associated with von Hippel–Lindau disease) now also encompasses the newly identified genes for succinate dehydrogenase subunit D (\textit{SDHD}) and succinate dehydrogenase subunit B (\textit{SDHB}), which predispose carriers to pheochromocytomas and glomus tumors. We used molecular tools to classify a large cohort of patients with pheochromocytoma with respect to the presence or absence of mutations of one of these four genes and to investigate the relevance of genetic analyses to clinical practice.

\textit{Methods} Peripheral blood from unrelated, consenting registry patients with pheochromocytoma was tested for mutations of \textit{RET}, \textit{VHL}, \textit{SDHD}, and \textit{SDHB}. Clinical data at first presentation and follow-up were evaluated.

\textit{Results} Among 271 patients who presented with nonsyndromic pheochromocytoma and without a family history of the disease, 66 (24 percent) were found to have mutations (mean age, 25 years; 32 men and 34 women). Of these 66, 30 had mutations of \textit{VHL}, 13
of RET, 11 of SDHD, and 12 of SDHB. Younger age, multifocal tumors, and extraadrenal tumors were significantly associated with the presence of a mutation. However, among the 66 patients who were positive for mutations, only 21 had multifocal pheochromocytoma. Twenty-three (35 percent) presented after the age of 30 years, and 17 (8 percent) after the age of 40. Sixty-one (92 percent) of the patients with mutations were identified solely by molecular testing of VHL, RET, SDHD, and SDHB; these patients had no associated signs and symptoms at presentation.  

Conclusions Almost one fourth of patients with apparently sporadic pheochromocytoma may be carriers of mutations; routine analysis for mutations of RET, VHL, SDHD, and SDHB is indicated to identify pheochromocytoma-associated syndromes that would otherwise be missed.

2.2 Introduction

It is becoming increasingly apparent that tumors of a single histologic type are heterogeneous in their natural history, prognosis, and response to treatment. Tumors such as pheochromocytomas and paragangliomas may also display important molecular differences and lend themselves to genetic analysis.

It is a widespread assumption that most pheochromocytomas are sporadic and only about 10 percent are hereditary (22). When hereditary, pheochromocytoma can be a component of multiple endocrine neoplasia type 2 (MEN-2), caused by mutations of the RET gene; von Hippel–Lindau disease, caused by mutations of the VHL gene; and, rarely, neurofibromatosis type 1 (2, 23, 24). Recently, mutations of the gene for succinate dehydrogenase subunit D (SDHD) were identified for another related neuroendocrine
disease, familial paragangliomas of the neck, or glomus tumors (5). \(SDHD\) and \(SDHB\) encode mitochondrial enzymes involved in oxidative phosphorylation (25). In a study by Heutink et al., all 38 affected members of five original families tested had neck paragangliomas, but none had pheochromocytomas (25-27). However, in our pilot study of pheochromocytomas from a small series comprising 17 unrelated patients with nonfamilial disease who showed no molecular or clinical evidence of MEN 2 [MEN 2] von Hippel–Lindau disease, or neurofibromatosis type 1, we identified three unsuspected germ-line mutations of \(SDHD\) (28). In contrast, in a referral-based cohort of 19 patients with pheochromocytoma from Brazil, no mutations of \(SDHD\) were found (29). In 2001, mutations of \(SDHB\) were found in three of eight families with pheochromocytoma, paraganglioma, or both (16).

Molecular medicine makes it possible to differentiate sporadic from hereditary disease, which will affect medical management not only for the patient but also for the family. This is particularly true for inherited tumor syndromes. In the present study, we analyzed the known susceptibility genes for pheochromocytoma — \(VHL\), \(RET\), \(SDHD\), and \(SDHB\) — in a large, unselected series of registry patients who presented with this tumor in order to classify them as having either truly sporadic or hereditary disease. Those who had mutations could then be reclassified as having von Hippel–Lindau disease, MEN 2, or one of the syndromes associated with pheochromocytoma and paraganglioma. In addition, we evaluated mutation status in relation to a range of clinical features to determine which, if any, can predict hereditary disease.
2.3 Methods

2.3.1 Patients

Patients with pheochromocytomas have been consecutively registered in the population registries of Freiburg, Germany, and Warsaw, Poland, in accordance with the ethical standards of the respective countries. Two hundred ninety-eight consecutive, unrelated patients with histologically confirmed pheochromocytoma from whom blood-leukocyte DNA was available were enrolled. All patients provided written or oral informed consent. For the purposes of registration, all cases of pheochromocytoma were included except those discovered by clinical or genetic screening of persons without symptoms of illness, in order to minimize ascertainment bias toward hereditary cases. For the purposes of this study, we excluded 11 patients with neurofibromatosis type 1, since all these patients had classic cutaneous lesions and could be easily given a diagnosis of a syndromic condition without molecular genetic analyses. We further excluded 14 patients with a family history (9 with tumors related to von Hippel–Lindau disease, and 5 with tumors related to MEN-2). DNA samples were available from 70 percent of the patients in the Freiburg registry and 88 percent of the patients in the Warsaw registry. Thus, 271 eligible registrants entered our study.

Of the 271 patients, 241 presented with pheochromocytomas only, whereas 8 presented with both pheochromocytomas and functioning paragangliomas. Twenty-two presented with paragangliomas only. Paragangliomas that originate in the sympathetic nervous system are most commonly found in the retroperitoneum but can also occur in the thorax as catecholamine-secreting, "functioning" extraadrenal pheochromocytomas
Paragangliomas that originate in the parasympathetic nervous system can occur adjacent to the aortic arch, neck, and skull base as local "nonfunctioning" masses, also called glomus tumors or chemodectomas. Unless the location of the tumor is germane, we will refer to our patients as presenting with pheochromocytoma.

2.3.2 Molecular Genetic Analyses

All eight exons of SDHB, all four exons of SDHD, all three exons of VHL, and exons 10, 11, and 13 through 16 of RET were examined by analysis of single-strand conformation polymorphisms and direct sequencing, as previously described (6, 7, 16, 28, 30-32).

When a patient with a germ-line mutation was identified, his or her consenting parents were investigated for the presence of the mutation; this analysis enabled us to trace the disease and the mutation back to the previous generation. If neither parent carried the index patient's mutation, we confirmed paternity using standard microsatellite fingerprinting. Genomic DNA samples from 300 anonymous, healthy blood donors matched with the registry patients for race (white) and region were analyzed as controls.

2.3.3 Clinical Studies

One of us performed or reviewed the clinical evaluation (personal and family history and physical examination) and medical records (detailed personal and family history, physical examination, and biochemical imaging studies). Family history was also updated at the time of blood sampling; final updates of all clinical data were performed through December 1, 2001.
Patients who were clinically or genetically identified in the present study as having a hereditary pheochromocytoma syndrome underwent clinical evaluation and surveillance for MEN 2, von Hippel–Lindau disease, and syndromes associated with pheochromocytoma and paraganglioma. The clinical screening program included measurement of serum calcitonin levels after stimulation with pentagastrin and measurement of serum parathyroid hormone levels for MEN 2; magnetic resonance imaging (MRI) of the central nervous system, MRI or computed tomography (CT) of the abdomen, and retinoscopy for von Hippel–Lindau disease (23); and MRI of the abdomen, thorax, and neck for syndromes associated with pheochromocytoma and paraganglioma. The clinical diagnosis of MEN 2 required the occurrence of pheochromocytoma and medullary thyroid carcinoma (2). In addition to pheochromocytoma at presentation, the diagnosis of von Hippel–Lindau disease required at least angioma of the retina or hemangioblastoma of the central nervous system in the index patient or a first-degree relative (33). The diagnosis of neurofibromatosis type 1 was made according to standard criteria.

Pheochromocytomas were classified according to number (solitary or multiple), location (adrenal or extraadrenal), and pathological findings (benign or malignant). Distant metastases or infiltration of surrounding tissue was required to designate a pheochromocytoma as malignant (22).
2.3.4 Statistical Analysis

For the comparison of rates from small samples, Fisher's (two-tailed) unpaired exact test was used; for larger groups, the standard two-sided chi-square test was used. P values less than 0.05 were considered to indicate statistical significance.

2.4 Results

A total of 271 patients (155 female and 116 male; age range, 4 to 81 years; mean age, 40 years) with nonsyndromic pheochromocytoma and without a family history of the disease were enrolled in the study. We identified 66 patients with deleterious germ-line mutations (24 percent); 13 had mutations of \( RET \), 30 mutations of \( VHL \), 11 mutations of \( SDHD \), and 12 mutations of \( SDHB \) (Table 2.1).
Table 2.1. Age of the Patients and Type of Tumor at Presentation According to Genetic Status.

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>SDHD Mutation-Associated MEN-2 (N=13)</th>
<th>Von Hippel-Lindau Disease (N=30)</th>
<th>SDHB Mutation-Associated Pheochromocytoma-Paraganglioma Syndrome (N=11)</th>
<th>SDHB Mutation-Associated Pheochromocytoma-Paraganglioma Syndrome (N=12)</th>
<th>Hereditary Disease (N=66)</th>
<th>Nonsyndromic Disease (N=205)</th>
<th>Total (N=271)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at presentation (yr)</td>
<td>Mean 36.4</td>
<td>18.3</td>
<td>28.7</td>
<td>25.6</td>
<td>24.9</td>
<td>43.9</td>
<td>39.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Range 21-50</td>
<td>5-49</td>
<td>5-59</td>
<td>12-48</td>
<td>5-59</td>
<td>4-81</td>
<td>4-81</td>
<td></td>
</tr>
<tr>
<td>Age at onset ≤ 18 yr (no.)</td>
<td>0</td>
<td>20</td>
<td>3</td>
<td>4</td>
<td>27</td>
<td>21</td>
<td>48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type of tumor (no.)</td>
<td>Multifocal 5</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>21</td>
<td>5</td>
<td>26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Extraadrenal 0</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>14</td>
<td>16</td>
<td>30</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*The P values are for the comparison of hereditary disease with nonsyndromic diseases.
2.4.1 Frequency Distribution and Types of Mutations

Thirteen unrelated patients (5 percent) were found to have seven germ-line mutations of RET (Table 2.1). These were missense mutations, like the majority of mutations of RET associated with MEN-2 to date (34). Haplotype analysis to exclude a founder effect was inconclusive, but relatedness seemed unlikely on reevaluation of the family histories.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation (cDNA Nucleotide)</th>
<th>Consequence (Amino Acid)</th>
<th>Independent Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET</td>
<td>T1900C</td>
<td>Codon 634, Cys to Arg</td>
<td>11</td>
</tr>
<tr>
<td>RET</td>
<td>T1900G</td>
<td>Codon 634, Cys to Gly</td>
<td>11</td>
</tr>
<tr>
<td>RET</td>
<td>G1901A</td>
<td>Codon 634, Cys to Tyr</td>
<td>11</td>
</tr>
<tr>
<td>RET</td>
<td>G1901C</td>
<td>Codon 634, Cys to Ser</td>
<td>11</td>
</tr>
<tr>
<td>RET</td>
<td>G1901T</td>
<td>Codon 634, Cys to Phe</td>
<td>11</td>
</tr>
<tr>
<td>RET</td>
<td>C1902G</td>
<td>Codon 644, Cys to Trp</td>
<td>11</td>
</tr>
<tr>
<td>RET</td>
<td>A2872T</td>
<td>Codon 791, Tyr to Phe</td>
<td>13</td>
</tr>
<tr>
<td>VHL</td>
<td>T406G</td>
<td>Codon 65, Ser to Ala</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>C416G</td>
<td>Codon 68, Ser to Trp</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>G452A</td>
<td>Codon 80, Ser to Asn</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>G490A†</td>
<td>Codon 93, Gly to Ser</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>G490T</td>
<td>Codon 93, Gly to Cys</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>G491T‡</td>
<td>Codon 93, Gly to Cys</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>G493T</td>
<td>Codon 94, Glu to Stop</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>T505C</td>
<td>Codon 98, Tyr to His</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>C5822G††</td>
<td>Codon 107, Arg to Gly</td>
<td>1</td>
</tr>
<tr>
<td>VHL</td>
<td>C570G</td>
<td>Codon 119, Phe to Leu</td>
<td>2</td>
</tr>
<tr>
<td>VHL</td>
<td>GC577, 578AT‡</td>
<td>Codon 122, Ala to Ile</td>
<td>2</td>
</tr>
<tr>
<td>VHL</td>
<td>T620G</td>
<td>Codon 136, Phe to Cys</td>
<td>2</td>
</tr>
<tr>
<td>VHL</td>
<td>T679A†</td>
<td>Codon 156, Tyr to Asn</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>A680G</td>
<td>Codon 156, Tyr to Cys</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>G695A†</td>
<td>Codon 161, Arg to Gln</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>G695C</td>
<td>Codon 161, Arg to Pro</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>C703T</td>
<td>Codon 164, Gln to Stop</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>C712T†</td>
<td>Codon 167, Arg to Trp</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>G713A</td>
<td>Codon 167, Arg to Gln</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>C775G</td>
<td>Codon 188, Leu to Val</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>C796T</td>
<td>Codon 195, Gln to Stop</td>
<td>3</td>
</tr>
<tr>
<td>VHL</td>
<td>T806A</td>
<td>Codon 198, Leu to Gln</td>
<td>3</td>
</tr>
<tr>
<td>SDHD</td>
<td>G14A</td>
<td>Codon 5, Trp to Stop</td>
<td>1</td>
</tr>
<tr>
<td>SDHD</td>
<td>C33A</td>
<td>Codon 11, Cys to Stop</td>
<td>1</td>
</tr>
<tr>
<td>SDHD</td>
<td>52del(TG)</td>
<td>Frame shift</td>
<td>1</td>
</tr>
<tr>
<td>SDHD</td>
<td>52del(TG)</td>
<td>Splice defect</td>
<td>1</td>
</tr>
<tr>
<td>SDHB</td>
<td>C112T</td>
<td>Codon 38, Arg to Stop</td>
<td>2</td>
</tr>
<tr>
<td>SDHB</td>
<td>G274T‡</td>
<td>Codon 92, Asp to Tyr</td>
<td>3</td>
</tr>
<tr>
<td>SDHB</td>
<td>C361T‡</td>
<td>Codon 121, Gln to Stop</td>
<td>4</td>
</tr>
<tr>
<td>SDHB</td>
<td>C213T‡</td>
<td>Codon 27, Arg to Stop</td>
<td>2</td>
</tr>
<tr>
<td>SDHB</td>
<td>221ins(CAG)‡</td>
<td>Codon 29, insertion of Gln</td>
<td>2</td>
</tr>
<tr>
<td>SDHB</td>
<td>C270G‡</td>
<td>Codon 46, Arg to Gly</td>
<td>2</td>
</tr>
<tr>
<td>SDHB</td>
<td>G439A‡</td>
<td>Codon 101, Cys to Tyr</td>
<td>4</td>
</tr>
<tr>
<td>SDHB</td>
<td>T708C‡</td>
<td>Codon 192, Cys to Arg</td>
<td>6</td>
</tr>
<tr>
<td>SDHB</td>
<td>G721A‡</td>
<td>Codon 196, Cys to Tyr</td>
<td>6</td>
</tr>
<tr>
<td>SDHB</td>
<td>847delTCTCT‡</td>
<td>Frame shift</td>
<td>7</td>
</tr>
</tbody>
</table>

*Some of these mutations have been described previously.
†The mutation is spontaneous.
‡The mutation is novel.
§IVS denotes intervening sequence.

Table 2.2. Germline Mutations in the Four Genes Detected in the Series of Patients with Pheochromocytoma.
The mutations of *VHL* in 30 patients (11 percent) comprised 3 nonsense and 19 missense mutations (Table 2.1). Among these 22 distinct mutations, 4 were novel. We had access to the DNA of both parents of four patients with diagnoses of pheochromocytoma (whose ages were 5, 7, 8, and 16 years). No genetic or clinical evidence of von Hippel–Lindau disease could be found in the parents, and microsatellite analysis at five different informative loci in the four families confirmed paternity. Thus, these four cases represent spontaneous germ-line mutations of *VHL*. Therefore, relationship of each carrier pair with the G490A and the G695A complementary DNA (cDNA) mutations (Table 2.2) has been excluded. The C712T cDNA mutation represents a well-known "hot spot," (13) and extensive pedigree evaluation makes it unlikely that the patients with the A680T cDNA mutation are related. It should be noted, however, that shared haplotypes usually denote ancient population-based founder effects instead of closer intermarriages that can be identified through the family history.

Eleven patients (4 percent of the 271) had seven different mutations of *SDHD*, three of which were novel (Table 2.2). Six mutations cause truncation of the putative protein, whereas one leads to a substitution of one amino acid. There were two different recurrent mutations. Haplotype analysis was inconclusive because of the small numbers, but pedigree information made relationship unlikely.

Twelve patients (4 percent) were found to have nine distinct, novel mutations of *SDHB* (Table 2.2). There were three recurrent mutations, none of which occurred in shared haplotypes; extensive pedigree evaluation also demonstrated that it was unlikely that any of the three carrier pairs were related.
None of the seven mutations of SDHD or the nine mutations of SDHB found in our patients were found in 600 control chromosomes from the 300 blood donors.

2.4.2 Clinical Presentation and Follow-up of Carriers

The age at the onset of symptoms was statistically lower in all carriers of mutations than in patients with sporadic disease, who were operationally defined as negative for mutations of any of the four susceptibility genes. Seventy percent of the patients who presented at the age of 10 or younger had germ-line mutations, and this percentage decreased steadily with increasing age to 0 percent among patients who presented after the age of 60 (Table 2.3). However, 139 registrants presented with pheochromocytoma after the age of 40, 11 of whom had mutations (8 percent). Only five of these patients had clinical findings that retrospectively suggested a hereditary pheochromocytoma. Mutations of VHL were present in 42 percent of all those who presented at age 18 or younger (20 of 48) and 74 percent of all patients with mutations who presented at age 18 or younger (20 of 27); 77 percent of those found to have mutations of VHL presented before the age of 20 (Table 2.3). In contrast, 9 of the 23 patients found to have germ-line mutations of SDHD and SDHB (39 percent) presented after the age of 30.
Table 2.3. Age at Presentation of Patients with Mutations or Sporadic Disease.

At initial presentation, 45 of 66 probands with mutations (68 percent) had only one tumor (Table 2.1). Multiple pheochromocytomas, however, were statistically more frequent among patients with mutations than among patients without mutations (21 of 66 [32 percent] vs. 5 of 205 [2 percent], P<0.001). However, multifocal tumors, as compared with solitary tumors, may be gene-specific: no patients with mutations of SDHB presented with multifocal disease, whereas 40 percent of those with mutations of VHL had multifocal disease. Twenty-eight percent of the patients with mutations of VHL, SDHD, and SDHB had extraadrenal tumors, as compared with 8 percent of patients without mutations (P=0.006).

We also identified classic syndrome-associated lesions at presentation and at final follow-up. Of 13 patients who were positive for mutations of RET, none had clinical evidence of medullary thyroid carcinoma at presentation, but medullary thyroid carcinoma developed in 12 during the follow-up period. Among 30 carriers of mutations of VHL, 5 also subsequently had other features associated with von Hippel–Lindau
disease, such as hemangioblastoma of the central nervous system or eye, pancreatic cysts, islet-cell tumors, or renal-cell carcinomas, during follow-up. In total, 10 patients were found to have associated lesions during follow-up. Of 23 carriers of \textit{SDHD} or \textit{SDHB} mutations, none had glomus tumors at presentation, but these tumors developed in 4 patients during follow-up (Figure 2.1). The majority of medullary thyroid carcinomas and glomus tumors were detected by screening.
Figure 2.1. Pheochromocytoma of the Left Adrenal Gland and Glomus Tumor of the Left Carotid Body in a Carrier of a Mutation of SDHD.
The pheochromocytoma became symptomatic five years earlier than the glomus tumor. In Panel A, transverse T2-weighted abdominal MRI shows a hyperintense left adrenal pheochromocytoma (arrow). In Panel B, contrast-enhanced transverse cervical MRI (T1-weighted images with spectral fat saturation) reveals a contrast-enhancing left cervical glomus tumor (arrows).

Because of our exclusion criteria, none of the 66 patients who were positive for mutations presented with a family history of syndrome-specific tumors. However, among patients with mutations of \textit{RET}, six had a positive family history at the final follow-up. Similarly, 12 carriers of mutations of \textit{VHL} had a positive family history at follow-up. None of the 23 patients found to have germ-line mutations of \textit{SDHD} or \textit{SDHB} had a positive family history at initial presentation. Even at follow-up, only four had family members who had been found to have clinical disease. There was a delay of up to 35 years before relatives began to have symptoms, and for 10 of 23 patients, the family history became positive only when active clinical screening was performed. Forty-two of the 66 probands with mutations (64 percent) had only one pheochromocytoma and no associated syndrome-specific lesion. Over 80 percent of patients with mutations of \textit{SDHD} (7 of 11) or \textit{SDHB} (12 of 12) presented with one pheochromocytoma, no family history, and no feature of associated syndromes. In contrast, this was true in about half of the patients with mutations of \textit{RET} (8 of 13) or \textit{VHL} (15 of 30).
2.5 Discussion

Our systematic clinical and molecular evaluation of 271 unrelated patients who presented with nonsyndromic pheochromocytoma revealed that 66 (24 percent) had a hereditary predisposition to von Hippel–Lindau disease, MEN 2, or the syndromes associated with pheochromocytoma and paraganglioma on the basis of newly discovered mutations in the *VHL*, *RET*, *SDHD*, or *SDHB* gene. Among the 66 patients with mutations, 45 percent had germ-line mutations of *VHL*, 20 percent had mutations of *RET*, and 17 and 18 percent had mutations of two newly identified genes, *SDHD* and *SDHB*, respectively. Currently, 64 percent of all probands found to have hereditary disease were identified with the use of molecular testing of *VHL*, *RET*, *SDHD*, and *SDHB* and had no family history, solitary disease, and no associated signs and symptoms at presentation.

Several previous studies, limited by small size, hinted that certain subgroups of patients with pheochromocytoma might have a higher risk of hereditary disease — those with bilateral or multifocal tumors, those who are relatively young at presentation, or both (2, 23, 35, 36). Our registry-based study addresses these issues by its complete or nearly complete identification of virtually all cases of bilateral tumors, cases of extraadrenal pheochromocytoma (paraganglioma), and cases with early onset. We can state confidently that 84 percent of all multifocal tumors (including bilateral tumors) and 59 percent of pheochromocytomas with onset at the age of 18 years or younger were found to be hereditary. Our results suggest that extraadrenal pheochromocytoma may be a striking feature of hereditary disease (P=0.006). When extraadrenal disease is found either in isolation or with adrenal pheochromocytoma, the molecular differential
diagnosis, in descending order, includes mutations of \textit{SDHB} (in 50 percent of cases), \textit{SDHD} (36 percent), and \textit{VHL} (17 percent) but not \textit{RET}.

A partial explanation for the high frequency of hereditary pheochromocytoma without a family history of disease might include spontaneous mutation in one of the susceptibility genes, decreased penetrance, and maternal imprinting. In our registry, spontaneous (ie, \textit{de novo}) mutations in \textit{VHL} accounted for 13 percent of cases of hereditary von Hippel–Lindau disease. Penetrance is known to be relatively high (approximately 70 percent by the age of 70) among patients with MEN-2 and von Hippel–Lindau disease, overall (37, 38). The penetrance of mutations of \textit{SDHD} and \textit{SDHB} is not well known because they are newly identified genes. Preliminary figures from this registry suggest a relatively high penetrance. Familial glomus tumors due to mutations of \textit{SDHD} are known to be maternally imprinted (5, 26). Overall, therefore, pheochromocytomas in patients without family histories are due to \textit{de novo} mutations, decreased penetrance, or maternal imprinting, although other causes such as gene–gene interactions and gene–environment interactions may be possible.

Genetic testing can be a powerful aid to the identification of a syndrome in such cases. For example, our study suggests that a patient with pheochromocytoma who has mutations of \textit{SDHD} or \textit{SDHB} has an approximately 20 to 30 percent likelihood of already having glomus tumors or of having glomus tumors develop. Since such tumors are difficult to treat when advanced (Figure 2.1), it is reasonable to speculate that molecular identification of a mutation in one of these two genes could lead to surveillance, early diagnosis of tumors, and more effective treatment. In our study, 68 percent of patients
found to have germ-line mutations presented with solitary tumors. It is the standard of
care for clinical cancer geneticists to offer genetic testing to patients with a minimal a
priori risk of mutation of 10 percent. This figure can be lowered if the technique of
mutation analysis is straightforward and cost effective and the alteration in medical
management saves lives, as is the case for testing for mutations of \textit{RET} (39). Our
registry-based study has demonstrated that the a priori risk of finding a mutation of \textit{VHL},
\textit{RET}, \textit{SDHD}, or \textit{SDHB} in unrelated patients who present with pheochromocytoma not
only meets but exceeds both of these criteria, even in the case of patients who are over
the age of 40 at presentation.

In our opinion, although it has not yet been demonstrated by a clinical trial, the
identification of a new case of hereditary pheochromocytoma on the basis of the
identification of the disease-associated mutation should prompt genetic testing of all first-
degree relatives of the carrier to determine the presence or absence of the family-specific
mutation. Since disease is likely to develop in virtually all patients with a family-specific
mutation, it seems reasonable to offer to such patients lifelong surveillance, prophylactic
surgery, or both, depending on the precise genetic diagnosis.
CHAPTER 3

Large Deletions in $SDHB/SDHD$

3.1 Abstract

Over 30% of adrenal pheochromocytomas are hereditary. These neuroendocrine tumors are major components of three inherited cancer syndromes, multiple endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau disease (VHL), and pheochromocytoma/paraganglioma syndrome (PC-PGL). Germline mutations in $RET$, $VHL$, and $SDHB$, $SDHC$, and $SDHD$ are associated with MEN 2, VHL, and PC-PGL, respectively. The majority (>70%) of hereditary extra-adrenal PCs (catecholamine-secreting paragangliomas (PGL)) are accounted for by germline intragenic mutations in $SDHB$, $SDHC$, or $SDHD$. Therefore, a subset of hereditary PGL is not accounted for. Here we report 2 unrelated hereditary PGL families and 1 unrelated proband with large deletions in $SDHB$ or $SDHD$, of which one family harbors a germline whole-gene deletion of $SDHD$ (Fam4194) and the other two harbor a partial-gene deletion of $SDHB$. Although they were initially designated mutation negative for all the PC-associated genes after PCR-based analysis, we suspected that a large deletion or rearrangement might be present these cases. Genotyping around the PC-associated genes demonstrated that Fam4194 were consistent with linkage with $SDHD$. Using fine structure genotyping and
semi-quantitative duplex PCR analysis, we identified a ~96kb deletion spanning \textit{SDHD} in Fam4194. In addition, we were able to identify a ~1kb deletion in the 5’ region of \textit{SDHB} (including exon 1) in Fam7135 and proband 5168 using the semi-quantitative duplex PCR method. Thus, including \textit{SDHD} and \textit{SDHB} deletion analysis could increase gene testing sensitivity for PGL patients, which would aid in genetic counseling and management of the patient and family.

3.2 Introduction

Adrenal pheochromocytomas (PC) are major component neoplasias of the heritable syndromes multiple endocrine neoplasia type 2 (MEN 2) and von Hippel-Lindau disease (VHL). The causative genes for these syndromes are \textit{RET} and \textit{VHL}. In addition, pheochromocytoma/paraganglioma syndrome (PC/PGL) is characterized by adrenal PC, and/or catecholamine-secreting (often referred to as extra-adrenal PC) PGL or biochemically silent PGL. This syndrome is caused by germline mutations in \textit{SDHB}, \textit{SDHC} and \textit{SDHD} (5, 8, 11, 16, 17, 34, 40).

Germline mutations in \textit{SDHB}, \textit{SDHD}, and less commonly \textit{SDHC}, account for up to 70\% of familial head and neck PGL (41), perhaps 8\% of apparently sporadic head and neck PGL (11) and approximately 9\% of apparently sporadic PC (42). There likely exists a fourth paraganglioma locus (PGL2) at 11q13, but the gene has not yet been cloned (41). Succinate dehydrogenase, or mitochondrial complex II, consists of the aforementioned subunits, SDHB, SDHC, and SDHD, along with a fourth subunit, SDHA. This complex is encoded by nuclear genes and plays a role in the Krebs cycle and electron transport chain (43). Germline mutations in each of the components of complex II have been
shown to disrupt complex formation and subsequently decrease the enzymatic activity of the remaining complex (44). How this leads to tumorigenesis is currently unknown.

To date, more than 40 germline mutations in SDHB, SDHC, and SDHD have been reported and all are intragenic mutations with no descriptions of large deletions or rearrangements (5, 11, 40, 45). However, not all familial PC and/or PGL cases have been found to have germline intragenic mutations. Therefore, we sought to address the hypothesis that these families, as well as mutation-negative sporadic cases of PC/PGL, may carry germline large deletions or rearrangements in one of the SDH genes.

3.3 Patients
3.3.1 Family 4194

The proband, III-1, was diagnosed with bilateral carotid PGL, which were biochemically silent, at the age of 28 years (Figure 3.1). Apart from the proband, there were 5 other family members affected with biochemically silent PGL (4 with carotid and one with PGL at the base of the skull) and 8 unaffected individuals (Figure 3.1). The father (II-4) of the proband, who is an obligate carrier of the putative mutation, is currently 56 years old and has not had PGL. Three of his siblings have had PGL: II-5 (PGL at age 50; possible recurrence or second primary late fifties); II-9 (PGL at the base of the skull, diagnosed at age 50; cerebral aneurysm at age 53); and II-11 (carotid PGL at age 20; recurrence at age 43). In addition, the proband’s father has two affected cousins: II-1 (carotid PGL diagnosed in his early thirties) and II-2 (bilateral carotid PGL diagnosed at age 13; subsequently diagnosed with colon cancer at age 48).
Informed consent was obtained in accordance with and approved by the Human Subjects Protection Committee of The Ohio State University.

Figure 3.1. Family 4194 affected with PGL. *Roman numerals* denote generation number, *Arabic numerals*, individual numbers. *, Family members with DNA available for genetic testing. Numbers below the asterisks indicate affected individual’s ages at onset (year).

3.3.2 Family 7135 (Fam7135)

Proband 7135 presented with head and neck PC and was a part of the Frieburg-Warsaw-Columbus PC Registry. These PC cases have been consecutively registered in the population registries of Freiburg, Germany, Warsaw, Poland, and Columbus, Ohio, in
accordance with the ethical standards of the respective countries. Patient 7135 had a histologically confirmed pheochromocytoma from whom blood-leukocyte DNA was available, and provided written or oral informed consent. In addition, 7135 had a cousin who died from a malignant PGL, however DNA was not available from this case.

3.3.3 Proband 5168

Patient 5168 was also a part of the Freiburg-Warsaw-Columbus PC registry. This patient presented with a sporadic occurrence of multiple extra-adrenal PC and relapsing PGL of the aortic arch. In addition, this patient exhibited left neck area and bone metastasis.

3.3.4 Family BRZ01

The proband, III-3, was diagnosed with a catecholamine-secreting abdominal paraganglioma at the age of 8 years (Figure 3.2). The mother of the proband, II-2, was diagnosed with a biochemically silent carotid PGL at the age of 38 years. In addition, the proband’s maternal aunt was diagnosed with a thoracic catecholamine-secreting PGL at the age of 14 years. We were unable to obtain DNA samples from any unaffected individuals in this family.
Figure 3.2. Family BRZ01 affected with PGL. Roman numerals denote generation number, Arabic numerals, individual numbers. *, Family members with DNA available for genetic testing. Numbers below the asterisks indicate affected individual’s ages at onset (year).

3.4 Methods

3.4.1 Analysis of Sequence Variation of Pheochromocytoma-Associated Genes

Genomic DNA from 14 members of Fam4194 (6 affected and 8 unaffected), 3 affected individuals from BRZ01, 7135 and 5168 was extracted from blood leukocytes using standard techniques (Qiagen, Valencia, CA). Germline mutation analyses for all exons, exon-intron boundaries, and flanking intronic regions were performed for VHL, SDHA, SDHB, SDHC, and SDHD and for MEN 2-associated RET exons 10, 11, and 13-16. PCR was performed using Qiagen HotStarTaq kit (Qiagen, Valencia, CA) for 38
cycles with a 55 C annealing temperature for all primers for all genes. An aliquot of the PCR product was purified using Exonuclease I/ Shrimp alkaline phosphatase treatment (New England Biolabs, Beverly, MA/ USB Corporation, Cleveland, OH). The purified amplicons were directly sequenced using Big-Dye Terminators v. 20 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems).

3.4.2 Genotyping

Four microsatellite markers closely flanking each gene, VHL, SDHB, SDHC, and SDHD, and 8 SNP loci within SDHA, were chosen with a heterozygosity index >0.6 for linkage analysis (Table 3.1). In order to genotype these markers for each of the 14 family members in Fam4194, PCR using a fluorescently labeled forward primer was performed. Subsequent to PCR, the products were fractionated on an ABI 3700 DNA Analyzer (Applied Biosystems). The output provided either one or two allele size(s) for each marker and these were then analyzed using the ABI PRISM Genotyper Version 3.7 NT. We used these data to form haplotypes for the region surrounding each gene and analyzed these haplotypes for association or lack thereof with the affected family members.
<table>
<thead>
<tr>
<th>SNPs</th>
<th>Microsatellite Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA 684 T/C</td>
<td>SDHB D1S507</td>
</tr>
<tr>
<td>891 T/C</td>
<td>D1S2697</td>
</tr>
<tr>
<td>IVS6-11G/T</td>
<td>D1S199</td>
</tr>
<tr>
<td>1038 C/G</td>
<td>D1S2647</td>
</tr>
<tr>
<td>1680 G/T</td>
<td>SDHC AP0A2</td>
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<tr>
<td>1752 A/G</td>
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<td>1932 G/A</td>
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<td>D3S1620</td>
<td></td>
</tr>
<tr>
<td>D3S3630</td>
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</tbody>
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Table 3.1. SNPs in SDHA and Microsatellite markers in SDHB, SDHC, SDHD, VHL used for linkage analysis.

3.4.3 Semi-Quantitative Duplex PCR Analysis

In order to look at the gene dosage of each SDHD exon in Fam4194 as compared to a control gene GAPDH, a duplex PCR was performed individually with each exon and GAPDH. PCR was performed using Qiagen Multiplex HotStarTaq Mix (Qiagen, Valencia, CA). For all exons of our gene of interest (GOI), the optimal ratio of GAPDH:GOI genomic DNA was determined (Table 3.2). The GAPDH/GOI amplicons were quantified by spot densitometry on the Alpha Innotech Corporation ChemiImager Fluorescence and Low Light Imaging System (Alpha Innotech Corporation, San Leandro, California), with ChemiImager 4000 version 4.04 software.
<table>
<thead>
<tr>
<th>Gene of Interest (GOI)</th>
<th>Ratio GAPDH:GOI</th>
<th>Annealing</th>
<th>Cycles</th>
<th>% Agarose Gel</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1:3</td>
<td>60 deg C</td>
<td>28</td>
<td>1.4</td>
</tr>
<tr>
<td>SDHD ex2</td>
<td>1:5</td>
<td>58 deg C</td>
<td>28</td>
<td>1.4</td>
</tr>
<tr>
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<td>SDHD ex4</td>
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<td>58 deg C</td>
<td>28</td>
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</tr>
<tr>
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<td>28</td>
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</tr>
<tr>
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<td>3</td>
</tr>
<tr>
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<td>28</td>
<td>1.4</td>
</tr>
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</table>

Table 3.2. Ratio of GAPDH: GOI for each duplex PCR
3.3 SDHD deletion in Fam4194 revealed by semi-quantitative duplex PCR with spot densitometry and quantitative RT-PCR. Comparison of SDHD exons 1-4 to GAPDH control using semi quantitative duplex genomic PCR. SDHD exons are the higher molecular weight band and GAPDH the lower. All bands were quantified using spot densitometry.

3.4.4 Fine-mapping of putative SDHD deletion

Markers surrounding SDHD were selected using NCBI MapViewer (http://www.ncbi.nlm.nih.gov/mapview/) to fill in the regions between the markers used for linkage and SDHD (Figure 3.4). Whenever possible, markers with heterozygosity index of >0.6 were used (34% of markers). Published primers on NCBI UniSTS (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists) were ordered for each marker with a fluorescent tag added to the forward primer to enable use for genotyping on the ABI 3700 DNA Analyzer (Applied Biosystems).
**Figure 3.4.** Physical map showing *SDHD* and flanking genomic regions involved in the deletions. 11q23 region including *SDHD*.

We genotyped the chosen markers surrounding *SDHD* for all family members. Heterozygosity at a marker locus indicated the presence of both alleles, and we therefore concluded that that locus was outside the deleted region. On the other hand, homozygosity could indicate either true homozygosity or hemizygosity. To help differentiate the two possibilities, we compared the affected with the unaffected individuals’ genotypes. If any unaffected individuals were heterozygous, then it was possible that the affected individuals who appeared homozygous may actually be hemizygous at that marker locus, and further analysis was done. When both affected and unaffected individuals were homozygous for a particular marker, we performed semi-quantitative duplex PCR using that marker combined with GAPDH. Details are outlined above (Semi-Quantitative Duplex PCR Analysis section). Although the genetic order of the microsatellite markers is publicly available, we decided to confirm the precise order by using BLAST 2 Sequences ([http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)). This was done by aligning the sequence of the forward primer of each marker with the NCBI published chromosome 11 contig that contains *SDHD* (NT_033899) (Figure 3.4).
3.4.5 SDHD Expression analysis using quantitative RT-PCR

Quantitative RT-PCR using SYBR-green dye was used to compare the expression level of SDHB/SDHD transcript to an internal control (GAPDH) in the germline of two affected individuals (II-2, III-1) and one unaffected individual (II-3) from family 4194 (Figure 3.1). Qiagen SYBR-Green HotStarTaq Master Mix (Qiagen, Valencia, CA) was combined with a forward and reverse primer that spanned the exon/exon boundary of exon 1 and exon 2 of SDHB and SDHD. This eliminated any possibility of amplifying from (low level) genomic DNA contamination, if present at all. The PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The data were analyzed using the ABI PRISM 7700 Sequence Detection Software version 1.6 and evaluated for an increased cycle threshold. An increased GAPDH: SDHD ratio was indicative of decreased expression of our gene transcript of interest with respect to the control gene transcript (GAPDH). All samples were run in duplicate for both SDHD and GAPDH primer sets.
Figure 3.5. Expression analysis of SDHD in deletion-positive proband and her unaffected mother. The ΔΔCt is an indicator of a relative quantification of the transcript. This value was normalized against an internal control gene, GAPDH. An increase in the ΔΔCt indicates a decrease in the transcript. These data indicated a ~ 4-fold decrease in expression in the proband than compared to her unaffected mother.

3.4.6 Fine-mapping of putative SDHB deletion

Markers surrounding SDHB were selected using NCBI MapViewer (http://www.ncbi.nlm.nih.gov/mapview/) to fill in the regions between the markers used for linkage and SDHB (Figure 3.6). However, because we did not have any family members for either Fam7135 and proband 5168, we opted to first perform semi-quantitative duplex PCR for the following exons in each of the PC-associated genes: VHL
exons 1,3; SDHB exons 1,5,8; SDHC exons 1,6; SDHD exons 1,4; and SDHA exons 1,7,15. We found that of the above exons, only SDHB exon 1 was indicative of a deletion, with a SDHBex1:GAPDH ratio of <0.5 (See figure 3.6).

We then performed sequential semi-quantitative duplex PCR of the regions surrounding SDHB exon 1. We used amplicons flanking SDHB exon 1 combined with GAPDH to determine the deleted region in family BRZ01. These amplicons included one for exon 2, multiple amplicons in intron 1 (IVS1A, IVS1B), as well as amplicons in the 5’ UTR of SDHB (5’UTRa, 5’UTRb).
Figure 3.6. Partial deletion of SDHB exon (ex) 1 revealed by semi-quantitative genomic-based PCR with GAPDH as the control gene and spot densitometry of a boxed region within each band, which is consistent throughout all samples. C1-C5 denote five different normal controls. Note that exon 2 is not deleted by duplex semiquantitative PCR analysis.

Because we knew that the deletion included exon 1 but not exon 2, it was clear that the breakpoints for this deletion were in the 5’UTR and in intron 1. We therefore designed primers for amplicons in both of these regions that could be duplexed with GAPDH to show if that region was deleted or not. The primer amplicons were termed 5’a, 5’b, etc. as well as IVS1A, IVS1B, etc. See figure 3.7.
3.4.7 SDHB DNA dosage analysis using quantitative RT-PCR

Although we did not have any RNA available from 7135 or 5168 to validate the SDHB deletion using expression analysis, we wanted to confirm this deletion using another method. We opted to use quantitative RT-PCR (ICycler, BioRad, California) using genomic DNA from our patients, with GAPDH as our internal control. We first optimized the reaction by amplifying logarithmic dilutions of control (Promega) DNA with both our gene of interest primers (SDHB exon 1) and our control gene primers (GAPDH). Each reaction was run in triplicate, as well as repeated on different days to ensure accuracy. This experiment confirmed that our gene of interest and our control gene were amplified proportionally at all DNA concentrations. We therefore performed the experiment using our patient samples, Fam7135, 5168, and a number of control DNA
samples including a German normal control pool (20 German normal samples added at equal volume and equal concentration) and a Columbus normal control pool (20 Columbus normal samples added at equal volume and equal concentration). We obtained the cycle threshold (Ct) values from the experiment which gave us a relative value of DNA dosage for that sample and that gene. We then calculated the difference in the Ct value for each sample between GAPDH and SDHB to give us the $\Delta$Ct. Finally, we could divide the $\Delta$Ct from the patient samples by the $\Delta$Ct of the control samples ($\Delta\Delta$Ct of controls=1) to obtain the $\Delta\Delta$Ct (see figure 3.8). This is the most concise representation of the DNA dosage level of our patient samples as compared to the control pools.

3.5 Results

3.5.1 Germline SDHD Deletion in Fam4194

The proband of Fam4194 (Fig.3, individual III-1) was initially analyzed for sequence variations and germline mutations in the PC-susceptibility genes, RET, VHL, SDHB, SDHC, and SDHD, as well as SDHA. Mutations and sequence variants were not detected with the exception of a common single nucleotide polymorphism (SNP) in exon 7 of SDHA found in Fam4194 that is seen frequently in the general population as well.

We used four markers to genotype the regions flanking each of VHL, SDHB, SDHC, and SDHD in addition to 8 SNP loci commonly found within the SDHA exons. Haplotypes were formed from genotypes flanking each gene and inspected for association (“consistent with linkage”) or lack of association (linkage excluded) between the affected individuals and a particular haplotype. We were able to exclude linkage to VHL, SDHA, SDHB, and SDHC in Fam4194 (data not shown). However, a specific
haplotype around SDHD was found to be shared by all the affected individuals in Fam4194 but was not found in any of the unaffected individuals. Because we showed that Fam4194 had SDHD marker haplotypes consistent with linkage, we re-sequenced the four SDHD exons and confirmed the absence of coding or splice site variants. Therefore, we further explored SDHD as a potential locus for large deletion or rearrangement.

In order to test the hypothesis that a large deletion or rearrangement in or involving SDHD that is not detectable by standard PCR-based methods was responsible for Fam4194, we performed semi-quantitative duplex PCR and spot densitometry. We found that the ratio of each SDHD exon to the control GAPDH in the affected individuals was decreased when compared to the unaffected individuals (Figure 3.3).

To compare the expression level of SDHD transcript with GAPDH transcript, we obtained RNA from peripheral blood leucocytes from two affected members (III-1, II-2) and one unaffected member (II-3) of this family (Figure 3.3). Quantitative RT-PCR showed a 4-fold decrease in expression of the SDHD transcript in individual III-1 (proband) compared to her unaffected mother (II-3). Interestingly, we found that affected individual II-2 had only a 2-fold decrease in SDHD transcript when compared to the unaffected relative II-3 (data not shown).

In order to define both the 5’ and 3’ breakpoints of the SDHD deletion in Fam4194, we performed fine structure genotyping using denser markers flanking SDHD (Figure 3.4). When a marker was found to be heterozygous, that region could definitely be assigned a not deleted status. When a marker was found to be homozygous (or
hemizygous), then that region was circumstantially assigned possible deletion status for further analysis (see below). Using this strategy of sequential genotyping and status assignment, we were able to define the largest possible region of deletion spanning 178kb genomic distance, flanked by markers D11S4192 5’ of SDHD and by D11S5019 3’ of SDHD (Figure 3.4). D11S4192 is 143kb upstream of the A in the ATG of SDHD, while D11S5019 is 24kb downstream of the stop codon of SDHD (Figure 3.4).

Since the only two markers between D11S4192 and SDHD on the 5’ end, RH104183 and D11S2329E, were homozygous for both affected and unaffected family members, we did further analysis to determine if this region was hemizygous or truly homozygous in the affected individuals. Semi-quantitative duplex PCR was performed and the ratio of each marker to GAPDH was determined. The data indicated that the 63kb between and including these two markers was indeed deleted in the affected individuals, and we could therefore conclude that the 5’ breakpoint was in an 80kb region between D11S4192 and RH104183 (Figure 3.4, hatched box). We then continued fine-mapping of the 5’ breakpoint in this region, and performed semi-quantitative duplex PCR to compare the dosage of genomic DNA in that region to our control gene, GAPDH. In order to do this, we used amplicons every 10kb along this 63kb interval. We also used this strategy in the 24 kb at the 3’ end of SDHD, between the stop codon and D11S5019 in order to fine map the 3’ breakpoint of the deletion. After narrowing the region of the breakpoint sites on both the 5’ and 3’ ends of SDHD, we determined that the maximum deleted region was ~96 kb including the entire SDHD gene.
3.5.2 Germline ~1kb SDHB deletion in Fam BRZ01, Fam7135 and Proband 5168

Using semi-quantitative PCR as our deletion-screening method in these patients whose family members were unavailable for genetic testing, we excluded deletions in VHL, SDHA, SDHC, SDHD. However, we determined that SDHB exon 1 was exclusively deleted in Fam BRZ01, Fam7135 and proband 5168. In all of these patient samples, SDHB exon 2 was not deleted. By performing additional semi-quantitative duplex PCR using primers to amplify around SDHB exon 1 (Figure 3.6), we were able to further fine-map the putative deletion. We concluded in Fam BRZ01, Fam7135 and 5168 that the breakpoint of the deletion on the 5’end was between 5’a and 5’b, while the breakpoint on the 3’ end was between the end of exon 1 and IVS1A. This is approximately a 1kb deletion.

Using quantitative RT-PCR, we were able to calculate the precise DNA dosage of SDHB exon 1 compared to GAPDH (Figure 3.7) for samples 5168 and 7135. This data clearly validated our semi-quantitative duplex PCR findings to show that SDHB exon 1 had a >2-fold decrease in the patient samples when compared to the control samples.
Figure 3.8. Quantitative RT-PCR of SDHB exon 1 using ICycler. This graph shows the ΔΔCt (see text for explanation of calculation) of the two samples 7135 and 5168 compared to the controls. The ‘controls’ consists of the average of the German control pool and the Columbus control pool (which had identical ΔCt values). These data indicate that 7135 and 5168 both have >2 fold decrease in the DNA dosage of SDHB ex 1 as compared to the controls.

3.6 Discussion

Until now, germline intragenic mutations in one of the 3 SDH genes have been identified in 70% of familial PGL. We have now shown that PCR-based mutation “negative” PGL families can be attributable to large gene deletions not detectable by standard PCR-based mutation analysis. Fam4194, with at least 6 members affected with
biochemically silent PGL, was shown to carry a germline ~90 kb deletion spanning the entire \textit{SDHD} gene. Indeed, the presence of only head and neck PGL’s in this family with 5 affected relatives is consistent with \textit{SDHD} involvement (11, 42). Further, the single non-penetrant case in this family (II-4) who inherited the \textit{SDHD} deletion from his mother is consistent with maternal imprinting, a phenomenon which is well-described for \textit{SDHD}-associated PGL(5, 46). BRZ01 had a ~1 kb deletion involving the 5’ end of \textit{SDHB}. Consistent with this, extra-adrenal catecholamine secreting PGL occurred in 2 of the 3 affected family members. Whether deletion families have a different phenotype from intragenic mutation families cannot be assessed since there are, to date, only these two families with large deletions. Anecdotally, two affected members with the \textit{SDHD} deletion have such extra-paraganglial manifestations as cerebral aneurysm at 53 years and colon cancer at 48, respectively, both relatively young. This contrasts with previously published intragenic \textit{SDHD} mutation positive families with no extra-paraganglial manifestations. In our family, it is difficult to exclude or prove that the colon cancer is merely coincidental or part of the \textit{SDHD} spectrum. It may be argued that the colon cancer was coincidental given that only one family member out of 16 relatives is affected with colon cancer, about 6%, thus approximating populational frequencies. \textit{SDHB} intragenic mutations, however, have been associated with non-traditional histology renal cell carcinoma at very young ages (47).

Functional confirmation of the presence of the deletion in Fam4194 was possible by demonstration of decreased expression of \textit{SDHD} in the germline of those with the deletion. Interestingly, the proband had a 4-fold decrease in expression of the \textit{SDHD}
transcript while her affected relative II-2 had only a 2-fold decrease in \textit{SDHD} expression. This expressional difference between these two affected individuals may be explained by other factors interacting with either \textit{SDHD} and/or other subunits of complex II. We suspect that expression analysis of \textit{SDHB} in the affected individuals would similarly result in significantly decreased transcript when compared to normal controls. However, we were unable to pursue this hypothesis due to the unavailability of RNA from these patients.

We also performed \textit{in silico} scanning for known genes within the putative deletion intervals. \textit{TIMM8B} is located just upstream of \textit{SDHD} in the opposite orientation (http://www.ensembl.org/Homo_sapiens; (48)) and may be within the deletion interval. \textit{TIMM8B} a homolog of \textit{TIMM8A} and germline mutations of \textit{TIMM8A}, on Xq22, are associated with deafness-dystonia-optic atrophy syndrome (49). \textit{TIMM8B} has not been shown to be associated with disease as yet but one anecdotal report lists a family with carotid paragangliomas who also had tinnitus and deafness (48). It is equally plausible, if not more so, that the deafness and tinnitus are most likely due to the carotid body/skull base paragangliomas especially in the context of the fact that this was a Dutch family with a founder mutation known to be intragenic. All our affected individuals are not afflicted with sensorineural hearing loss or tinnitus indicating that this phenotype does not result from heterozygous deletion of this gene.

Our observations suggest that large germline deletions in the \textit{SDH} genes, at least \textit{SDHB} and \textit{SDHD}, are another genetic mechanism which are etiologic for familial PC/PGL. Therefore, deletion analysis of these genes should be offered to families or
individuals at risk for hereditary PC/PGL if standard PCR-based mutation analysis is apparently negative. If linkage-type genotyping analysis is not available clinically or not possible in the context of PCR-based mutation “negative” PC/PGL, deletion analysis could begin with SDHD in families or individuals with prominent head and neck PGL. Similarly, SDHB deletion analysis should be considered first if adrenal PC’s predominate. This type of stepped strategy, PCR-based mutation analysis followed by deletion analysis, should increase the sensitivity of gene testing and hence facilitate clinical management for PC/PGL patients as well as their families.
4.1 Abstract

Hereditary paraganglioma syndrome has recently been shown to be caused by germline heterozygous mutations in three \((SDHB, SDHC, \text{ and } SDHD)\) of the four genes that encode mitochondrial succinate dehydrogenase. Extraparaganglial component neoplasias have not been previously documented. In a population-based registry of symptomatic presentations of PC/PGL comprising 352 registrants, among whom 16 unrelated registrants were \(SDHB\) mutation positive, one family with germline \(SDHB\) mutation c.847-50delTCTC had two members with renal cell carcinoma (RCC), of solid histology, at ages 24 and 26 years. Both also had paraganglioma. A registry of early-onset RCCs revealed a family comprising a son with clear-cell RCC and his mother with a cardiac tumor, both with the germline \(SDHB\) R27X mutation. The cardiac tumor proved to be a paraganglioma. All RCCs showed loss of the remaining wild-type allele. Our observations suggest that germline \(SDHB\) mutations can predispose to early-onset kidney cancers in addition to paragangliomas and carry implications for medical surveillance.
4.2 Introduction

Hereditary neoplasia syndromes that include pheochromocytomas or extra-adrenal pheochromocytomas (paragangliomas [PGLs]) included multiple-endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau disease (VHL), and type 1 neurofibromatosis (NF1) for several decades. Recently, germline mutations in \(SDHD\), a nuclear gene encoding the D subunit of the mitochondrial enzyme succinate dehydrogenase (SDH), were described in families with head and neck PGLs (5). Subsequently, the tumor spectrum for germline mutations in \(SDHD\) was expanded to include familial and apparently sporadic pheochromocytomas (reviewed by Maher and Eng [2002]). It is interesting that germline mutations in genes encoding two other subunits of SDH (mitochondrial complex II), \(SDHB\) and \(SDHC\), were found to be associated with heritable pheochromocytoma and/or PGL (11, 17). No germline mutations in \(SDHA\) have been found in pheochromocytoma or PGL cases. Instead, homozygous \(SDHA\) mutations result in congenital severe neurodegeneration and seizures. In a population-based study, 25% of all apparently sporadic (defined as "without syndromic features and family history") clinical presentations of pheochromocytoma were found to harbor occult germline mutations in \(SDHB\), \(SDHD\), \(VHL\) (which results in VHL), and \(RET\) (which results in MEN 2) (50) [see Chapter 2]. Until now, it was believed that there were no other extraparaganglial manifestations in those carrying \(SDH\) mutations (11, 17, 41, 45, 50).

Apart from \(SDH\), germline heterozygous mutations in only one other nuclear-encoded gene that codes for a mitochondrial enzyme, \(FH\)-encoding fumarate hydratase (fumarase), has been described in a seemingly unrelated inherited cancer syndrome,
hereditary leiomyomatosis and renal cell carcinoma (HLRCC) (51). Paralleling SDH, homozygous germline mutations in FH cause severe neurodegeneration (FH deficiency) (17). Therefore, because of the mitochondrial etiologies for both SDH-related heritable PGL and HLRCC, we sought to determine the existence of PGL in a population-based registry of renal cell carcinoma (RCC) and the existence of RCC in a population-based registry of symptomatic presentations of PGL and adrenal pheochromocytomas.

4.3 Patients and Results

Family 1 was ascertained in a computerized search of the national Finnish Cancer Registry for RCC cases diagnosed at ages 15-34 years, followed by construction of pedigree and documentation through patient records, in accordance with the institutional review boards for human subjects protection of the University of Helsinki and The Ohio State University. This search revealed 244 unrelated patients with RCC. After pedigree expansion and documentation, family 1 was ascertained. The index patient received the diagnosis of RCC at age 28 years (Figure 4.1A). At the time of diagnosis, the tumor had already widely metastasized, and a palliative nephrectomy was performed. The resected kidney, encompassing the tumor, weighed 2,915 g and measured 17 × 23 cm. Histologically, the tumor was a conventional (clear cell) carcinoma that showed a mixture of clear cells and cells with granular-eosinophilic cytoplasm. The proband's mother (patient II-1) (Figure 4.1A), at age 55 years, received a diagnosis of a malignant PGL of the heart growing from the septum to the right ventricle. In her case, the tumor was considered inoperable, and it eventually led to the demise of the patient. The proband's maternal uncles (patients II-2 and II-4) had small-cell lung carcinoma,
diagnosed at age 55 years, and pancreatic carcinoma, diagnosed at age 71 years, respectively (Figure 4.1A). Both uncles’ carcinomas were unrelated to the neuroendocrine tumors found in other family members. No samples from individual II-4 were available for further analysis.
Figure 4.1. Family 1, with documented RCC, cardiac PGL, and the germline SDHB R27X mutation. A, Pedigree of family 1. Generation numbers are represented by Roman numerals. Individual numbers are in Arabic numerals. The index patient (proband) is III-1, indicated by the arrow. "Mut+" indicates mutation-positive individuals. B, Sequencing chromatogram representing part of SDHB exon 2. The sequences around codon 27 from a normal control is shown at the top ("Normal"). The germline of the proband showed a heterozygous R27X mutation with a wild-type C and a mutant T (N8168). The tumor DNA shows loss of the wild-type C allele, leaving only the mutant T (T8168). The proband's mother's germline (N8104) showed the heterozygous R27X mutation, whereas
her tumor (T8104) showed loss of the remaining wild-type allele. (Note that, although the right panel was obtained in reverse sequence, the reverse complement chromatogram, which represents a computer-generated forward sequence, is shown for ease of viewing.)

Figure 4.1. continued
Germline genomic DNA from archived normal tissue of the deceased index patient was examined for the presence of constitutional mutations in *SDHA, SDHB, SDHC*, and *SDHD* by direct semiautomated sequence analysis, as described elsewhere (50). A germline heterozygous truncating mutation in *SDHB, R27X*, was found in the proband (III-1), his mother (II-1), and his uncle (II-2). DNA was then extracted from the proband's RCC, his mother's cardiac PGL, and his uncle's lung tumor. Direct sequencing revealed somatic loss of the remaining wild-type *SDHB* allele in the proband's renal tumor and in his mother's PGL (Figure 4.1B) but not in his uncle's lung carcinoma. The uncle smoked cigarettes for >40 years, and, in the absence of loss of heterozygosity of the wild-type allele, the lung cancer is probably unrelated to the *SDHB* mutation.

Family 2 was ascertained from a population-based registry of the Freiburg-Warsaw-Columbus Phaeochromocytoma Study Group, which registers all clinical presentations of pheochromocytomas in Germany and central Poland, in accordance with the institutional review boards of The Ohio State University, the University of Freiburg, and the Institute of Cardiology, Warsaw (50) (Figure 4.2A). In this registry, there are 352 unrelated registrants, and 16 (5%) have been found to carry germline *SDHB* mutations (50); (H. Neumann and C. Eng, unpublished observations). Among those 16, two siblings (patients II-2 and II-1) were found to have RCC with solid histology diagnosed at ages 24 years and 26 years, respectively (Figure 4.2A). Both siblings also had PGL. It is interesting that patient II-1 received a diagnosis of RCC first, before his PGL diagnosis. Direct sequencing of genomic DNA, extracted from blood leukocytes, revealed a germline
heterozygous frameshift mutation in SDHB, c.847-50delTCTC. The PGL from patient II-2 and both renal tumors showed somatic loss of the remaining wild-type allele (Figure 4.2B).

Figure 4.2. Family 2, with documented PGL and RCC and germline heterozygous SDHB c.847-50delTCTC mutation. A, Pedigree of family 2. The mutation-positive individuals are indicated by "Mut+." The index patient is II-2 (5792), indicated by the arrow. B, Sequencing chromatogram in the region of nucleotides 845855 of SDHB. The wild-type sequence from a normal control is in the top panel ("Normal"). The bar below the TCTC denotes the region of deletion. The germline DNA from both the index patient

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and her brother showed the heterozygous c.847-50delTTC microdeletion mutation. Note that, because of the nature of the repeats, we cannot ascertain whether the microdeletion is c.847-50delTTC or c.848-51delCTT. When the mutant allele is present heterozygously with the wild type ("5842 germline" and "5792 germline"), the reading frame is shifted at the point of microdeletion. When the wild-type allele is lost, as in all three tumors ("5842 RCC," "5792 RCC," and "5792 PGL"), the mutant allele remains.

**Figure 4.2. continued**
To determine whether SDH also plays a role in all individuals with generic sporadic renal carcinoma, we examined SDHA, SDHB, SDHC, and SDHD for germline and somatic mutations in 60 sporadic RCCs (30 clear cell, 3 papillary, 2 granular cell, 1 mixed papillary/clear cell, 1 mixed clear cell/solid histology, 9 oncocyctic papillary, and 14 oncocytoma) diagnosed at any age. No germline or somatic mutations were found in these four genes. Because the SDHB-related RCCs were diagnosed at ages <30 years, we then examined for mutations in these four genes in 35 tumors with clear cell histology diagnosed at ages <50, but no mutations were found. Our SDHB-mutationpositive carriers with RCCs are particularly young (<30 years), and the common clear cell histology does not predominate in these (or among SDHB-related) RCCs. Thus, it would not be a surprise to find no mutations in our series of kidney carcinomas for which the predominant age at onset is approximately in the 60s and for which clear cell histology predominates.

4.4 Discussion

To our knowledge, this is the first time that a gene encoding one subunit of the mitochondrial SDH complex has been implicated in renal carcinogenesis and the first time that extraparaganglial disease has been shown to be part of the PGL syndromes characterized by SDH mutations. SDH is a part of the Krebs tricarboxylic acid cycle and the mitochondrial electron transport chain, which are required for the energy metabolism of all cells (reviewed by Eng et al. [2003](17)). Anchored by SDHC and SDHD, the catalytic subunits of complex II, SDHA and SDHB, convert succinate to fumarate in an energy-dependent reaction and pass fumarate to the next enzyme in the Krebs cycle, FH.
It is interesting that germline $FH$ mutations are associated with HLRCC (51). The RCCs in HLRCC typically have type II papillary histology. Nonetheless, the renal cancers seen in our families with the $SDHB$-positive mutation are of varied histology, ranging from solid to clear cell and cells with granular-eosinophilic cytoplasm. All these carcinomas originate from epithelial cells of the proximal renal tubule. Thus, it is etiologically interesting that a defect in mitochondrial enzymes is involved in the pathogenesis of these subhistologies of RCC. The mechanism leading to neoplastic transformation after damage to the Krebs cycle and mitochondrial electron transport is still far from fully understood, but a hypothesis that these events lead to a proliferative hypoxic signal or oxidative DNA damage has been proposed (17, 52). Accumulation of reactive oxygen species could result from dysfunction in mitochondrial energy metabolism; results supporting this theory have been reported (17, 52). Another possible and not mutually exclusive mechanism by which mitochondrial dysfunction may lead to neoplasia is through the role of mitochondria in apoptosis (17). Because RCCs are referred to as "oncocytic" (i.e., replete with mitochondria), the role of SDH in RCC is plausible.

Other RCC-susceptibility genes do not encode mitochondrial enzymes. Although seemingly disparate, the majority of (or perhaps all) heritable RCC syndromes might involve the HIF-VEGF pathway. VHL, caused by germline mutations in $VHL$, is characterized by clear cell RCC, pheochromocytoma mainly of the adrenal medulla, and hemangiomas of the CNS and retina (8, 11, 53). A genotype-phenotype correlation exists in regard to the frequencies of RCC and pheochromocytoma in a particular family (54, 55). Truncating germline mutations are associated with RCC, whereas missense
mutations are associated with pheochromocytoma (54, 55). The VHL protein has several functions; for example, binding and shepherding one of the subunits of hypoxia-inducible factor (56) toward ubiquitin-mediated proteolysis in the presence of oxygen(53, 57, 58). When VHL is nonfunctional, HIF is upregulated. When mitochondrial function is impaired (e.g., as a consequence of SDHB mutations), severe energy deficits occur and oxygen free radicals are generated. When mitochondria sense the presence of oxygen free radicals (hypoxia), HIFs are activated and are translocated to the nucleus to induce gene expression (17). Thus, it may be postulated that both RCC and pheochromocytoma/PGL susceptibility resulting from SDHB deficiency could be mechanistically related to RCC and to pheochromocytoma susceptibility secondary to VHL dysfunction, via HIF (17).

Tuberous sclerosis (TSC), associated with germline mutations in TSC1 or TSC2, is characterized by renal angiomyolipomas and cysts; dermatologic lesions, such as ash leaf patches and shagreen patches; epilepsy; mental retardation; and hamartomas of the eye (59). Multifocal RCCs have been reported in several TSC cases (60, 61). Recently, loss or dysfunction of Tsc2 in the Eker rat model was shown to result in upregulation of Hif2-in RCC (62). Familial papillary RCC is a rare syndrome caused by germline mutations in the MET proto-oncogene. The ligand for MET, hepatocyte growth factor, has been shown to upregulate HIF-1 activity (63-65). Germline mutations in HRPT2 have been recently associated with hyperparathyroidism jaw tumor syndrome, and germline mutations in BHD, encoding folliculin, have been associated with Birt-Hogg-Dubé syndrome; both of these diseases have RCC as component tumors (66, 67). Although the function of folliculin and HRPT2 has yet to be elucidated, it would appear that the HIF-VEGF
pathway might be an important downstream common pathway for renal neoplasia in many heritable RCC syndromes.

The German-Polish Registry includes 16 unrelated probands with germline \textit{SDHB} mutations and a total of 31 mutation carriers (50)(H. Neumann and C. Eng, unpublished observations). Using the Registry to help estimate the frequency of RCC among \textit{SDHB} mutationpositive individuals, we approximate a 5\%-10\% prevalence. Given the age distribution of \textit{SDHB} carriers in the registry (mean age 30 years; 40\% <20 years, 15\% >40), this 5\%-10\% estimate might be slightly low. In contrast, results of the Finnish Registry search suggest a prevalence of <1\% of all early-onset RCC. If our observations can be independently confirmed, then individuals and families with germline \textit{SDHB} mutations should also undergo routine clinical surveillance for the development of early-onset renal carcinomas. Conversely, if a patient with RCC were found to harbor the germline \textit{SDHB} mutation, then annual surveillance for pheochromocytoma and PGL should be considered. On the basis of our observations, very early onset RCC with unusual histology (e.g., solid) should alert a clinician to take an extended family history. Like most heritable RCCs, (e.g., VHL-related (68)), it is possible that \textit{SDHB}-related RCC might also have a better prognosis. Of the three patients studied in this report, one is still alive after long follow-up; one has died of metastatic disease, but not until 6 years after diagnosis; and the third died of metastatic disease 1 year and 4 months after initial diagnosis. Thus, longer follow-up and study of other cases are required to investigate this aspect of the disease.
CHAPTER 5

GERMLINE SDH MUTATIONS IN THE CARNEY TRIAD/ DYAD

5.1 Abstract

Carney triad (CT) [OMIM #604287] is classified as the association of three rare component neoplasms within the same patient, namely, gastric stromal tumor (GIST), pulmonary chondroma, and paraganglioma (PGL). Carney dyad (CD) is composed of only GIST and PGL. CT appears to be sporadic, while CD shows autosomal dominant inheritance and has been shown to be familial. However, to date there is no known genetic evidence of heredity for this tumor association. Because germline mutations in SDHB, SDHC and SDHD cause both sporadic and familial PGL, we hypothesized that there would be a role of the mitochondrial complex II genes, SDHB, SDHC, and SDHD in CT and CD. In a series of 50 unrelated CT/CD probands, we show that 6% (3/50) of this series of CT/CD patients harbor novel germline mutations in either SDHB or SDHC. Of note, it is striking that all three of these germline mutations are splice-site mutations. We have shown that these mutations all have effects on the splicing of these gene transcripts and result in altered gene products. These data suggest that CT/CD patients and their famililes should be offered routine SDHx mutation analysis in order to allow for appropriate diagnosis, screening, and treatment.
5.2 Introduction

Carney triad (CT) is an extremely rare association of gastric stromal tumor (GIST), pulmonary chondroma, and extra-adrenal paragangliomas (PGL) (69). Patients usually present at an early age with multiple tumors (21, 70). The Carney dyad (CD) is a closely related but distinctly familial condition, defined by PGL and GIST but without pulmonary chondroma. PGL and GIST, independently, occur in both familial and sporadic fashions. Susceptibility loci for familial PGL have been mapped and the causative genes identified. These loci are 11q23 (PGL1-SDHD), 11q13 (PGL2-gene unknown), 1q23 (PGL3-SDHC) and 1p35 (PGL4-SDHB). Similarly, a susceptibility gene, KIT, was mapped and identified for familial GIST.

GISTs derive from the interstitial cells of Cajal (ICC) and are essential for proper gut motility. In addition, both ICC and GIST cells express CD34 and CD177, and ICC are dependent on a receptor tyrosine kinase (KIT) for proper development and maintenance. Because of this etiology, KIT was a target for mutation analysis in these patients’ GISTs. Somatic mutations in KIT have been shown to promote hyperplasia and neoplastic transformation, and are seen in 85% of GIST cases. In addition, somatic mutations in a related receptor tyrosine kinase, PDGFRα (platelet-derived growth factor receptor α), have been seen in an additional 5.25% of all GISTs (35% of those that are not associated with somatic KIT mutations) (71). However, neither KIT nor PDGFRα somatic mutations have been seen in any GISTs in the context of CT or CD (Stratakis, C, unpublished data).
Genetic mapping has identified four causative genes for familial PGL, of which three encode subunits of mitochondrial complex II, or succinate dehydrogenase complex (SDH). Interestingly, this tetrameric complex encodes a hypoxia-sensitive respiratory protein and has a key function in energy production with its role in both glycolysis and the electron-transport chain. There are four types of PGL, type 1 (associated with mutations in \( SDHD \)), type 4 (\( SDHB \)), type 3 (\( SDHC \)) and type 2 (gene unknown) (72).

The occurrence of unrelated tumors caused independently by mutations in \( KIT/\ PDGFR\alpha \) and \( SDH \) in the same patient suggests a possible convergence between their respective pathways. More specifically, this convergence is likely through the hypoxia-induced factor 1α (HIF-1α) transcription factor, which is influenced both by KIT and PDGFRα signaling pathways and the mitochondrial respiratory complexes including SDH (71). Therefore, altering either signaling pathways through receptor kinases or changes in the oxygen response system could result in the malfunctioning of HIF-1α to promote tumor development.

The Carney triad and dyad have both been seen in sporadic and familial occurrences. However, a causative gene for these familial cases of either CT or CD has yet to be identified. Germline mutations in 3 of 4 genes that form the SDH complex have been implicated in non-syndromic familial PGL. In addition, there have been 10 reports of familial GIST kindreds identified, of which 8 (80%) harbor germline \( KIT \) mutations, and 1 (10%) harbors a mutation in \( PDGFR \) (the final kindred has not been tested) (Li et al, 2005). To date, CT/CD patients tested for \( KIT \) and \( PDGFR\alpha \) are mutation-negative (Stratakis, unpublished data). We therefore hypothesized that mutations in \( SDHB, SDHC \),
and *SDHD*, the causative genes for familial PGL, were responsible and associated with CT/CD. We examine a series of 50 CT/CD patient samples for germline mutations in one of the *SDH* genes. We discovered that 3/50 (6%) harbor novel germline mutations in either *SDHB* (2/50) or *SDHC* (1/50). In addition, all three of these mutations are splice-site mutations. This paradigm-shifting finding suggests that a subset of CT/CD are hereditary. This has tremendous implications for these patients, their families, and their physicians. We suggest that all CT/CD patients are routinely tested for germline mutations in *SDHB*, *SDHC*, and *SDHD*.

5.3 Patients

Patients presenting with signs or symptoms of Carney Dyad or Triad were seen at the National Institutes of Health/ National Institute of Child Health and Human Development (NICHD). All 50 patients who were recruited for the study were seen by a single investigator and all pertinent pathology reviewed by a single pathologist, Dr. J. Aidan Carney. Informed consent was obtained in accordance with the Human Subjects’ Protection Committees of the respective institutions. Peripheral blood leukocytes were obtained from each patient and subsequently genomic DNA was extracted using standard techniques (Qiagen, Valencia, California).
5.4 Methods

5.4.1 Sequence analysis of \textit{SDHA}, \textit{SDHB}, \textit{SDHC}, and \textit{SDHD}.

Germline mutation analyses for all exons, exon-intron boundaries, and flanking intronic regions were performed for \textit{SDHA}, \textit{SDHB}, \textit{SDHC}, and \textit{SDHD}. Detailed methods can be found in chapter 3.2.4.1.

Patient samples designated mutation-positive were analyzed again for confirmation using site-specific mutation analysis. For each mutation-positive sample, we reamplified the target sequence with PCR, repeated the subsequent purification step and the sequence analysis. In addition, this sequencing was done in both the forward and reverse directions to ensure accuracy.

5.4.2 Analysis of Transcriptional Effects of Variants using RT-PCR

Using reverse-transcription PCR, we were able to elucidate the effects of each germline mutation on transcription and on splicing. For each of the three mutation-positive samples (CT8, CT35, CT2.01), RNA was extracted from fresh/frozen tumor tissue using Trizol (Invitrogen, Carlsbad, CA) followed by RNeasy Column Purification (Qiagen, Valencia, CA). Purified RNA was then synthesized into cDNA using reverse transcriptase (Gibco). The resulting cDNA was then amplified using primers flanking each variant found. The primers were specifically designed to amplify only cDNA by crossing an exon-exon boundary. After performing the PCR, the product was run on a 1.4% agarose gel and subsequently any resulting bands were cut and purified. These bands were ultimately submitted for sequencing (see found in chapter 3.2.4.1 for detailed methods) and the resulting chromatogram was analyzed for our sequence of interest.
5.5 Results

Sequence analysis of the four mitochondrial complex II genes revealed germline splice-site mutation in 3/50 (6%) of the patients tested. Two were found in *SDHB* (2/50) and one in *SDHC* (1/50).

5.5.1: CT8 *SDHC* IVS5+1 G/A Heterozygote

Our first mutation-positive proband, CT8, presented with PGL and premalignant GIST. Sequence analysis revealed a mutation at *SDHC* IVS5+1 (Figure 5.1).

**Figure 5.1.** Direct Sequencing of CT8 *SDHC* IVS5+1 G/A Heterozygote

The top panel shows the normal sequence at the boundary of exon 5 and intron 5 of *SDHC*. The yellow bar on both panels indicates the exon/intron boundary. The arrow on
the top panel is pointing to the normal G base. The arrow on the bottom panel is pointing to the variant seen in patient CT8 sample, in which the G peak is significantly decreased in height and the A peak is present.

RT-PCR analysis revealed the transcriptional effect of this splice-acceptor site variant. The result was one wild-type allele, and one mutant allele in which exon 5 was completely spliced out. This resulted in the joining of exon 4 with exon 6, and ultimately a frameshift. This frameshift caused a premature stop codon to be transcribed 36 amino acids into the 3’UTR (Figure 5.2).

**Figure 5.2.** RT-PCR results showing that SDHC exon 5 is spliced out. Exon 4 joins with exon 6 which causes a frameshift.

5.5.2: CT35 SDHB IVS1+1 G/T Heterozygote

Direct sequencing of the four SDH genes revealed a heterozygous variant at SDHB IVS1+1 in this patient sample (Figure 5.3). This patient presented with PGL and GIST.
Figure 5.3. Direct sequencing of CT35: SDHB IVS1+1 G/T heterozygote.

The top panel shows the normal sequence at the boundary of exon 1 and intron 1 of SDHB. The yellow bar on the top and bottom panels indicates the exon/intron boundary. The arrow on the top panel is pointing to the normal G base. The arrow on the bottom panel is pointing to the variant seen in patient CT35 sample, in which the G peak is significantly decreased in height and the T peak is present.

RT-PCR analysis revealed the heterozygous presence of a splice aberration, ie, the first part of intron 1 was transcribed, and then spliced out of frame to exon 2, with the frameshift resulting in a stop codon in the middle of exon 2 (Figure 5.4). This results in a significantly truncated protein, since normal SDHB has 8 exons.
Figure 5.4. RT-PCR result from CT35. Top panel shows the resulting sequence of the wildtype allele. The yellow bar indicates the SDHB exon1/intron 1 boundary. In the bottom panel, the mutant allele transcript can be seen. Notice the end of exon 1, followed by intron 1 (the first 194 bp) then joined to exon 2 resulting in a frameshift. A stop codon occurs later in the exon as a result of this frameshift.
5.5.3: CT2.01 \textit{SDHB} IVS4+1 G/C Heterozygote

Direct sequencing of the four \textit{SDH} genes indicated a variant at the boundary of exon 4 and intron 4 of \textit{SDHB} in patient CT2.01 (Figure 5.5).

\textbf{Figure 5.5.} Direct sequencing of CT02.01: SDHB IVS4+1 G/C heterozygote.

The top panel shows the wildtype sequence at the boundary of exon 4 and intron 4 of \textit{SDHB}. The yellow bar on the top and bottom panels indicates the exon/intron boundary. The arrow on the top panel is pointing to the normal G base. The arrow on the bottom panel is pointing to the variant seen in patient CT02.01 sample, in which the G peak is significantly decreased in height and the C peak is present.

RT-PCR analysis revealed this IVS4+1 G/C splice-site mutation results in the last 18 codons of exon 4 to be spliced out. Therefore, the protein is truncated but remains in frame (Figure 5.6).
Figure 5.6. RT-PCR result from CT02.01. Top panel shows the resulting sequence of the normal allele. The yellow bar indicates the SDHB exon1/intron 1 boundary. In the bottom panel, the mutant allele transcript can be seen. In this allele, 18 codons at the end of exon 4 have been spliced out, and the middle of exon 4 is spliced to the beginning of exon 5. This does not result in a frameshift.

5.6 Discussion

Carney triad/dyad is a unique and rare association of three or two tumors. The coexistence of GIST, PGL and (in triad only) pulmonary chondroma has been genetically a mystery until now. This rare tumor syndrome rarely shows familial patterns and only in CD. CT has mainly been isolated. There have been 10 reported kindreds with familial non-syndromic GIST, of which 8 (80%) harbor germline mutations in KIT, and 1 (10%) harbors a germline mutation in PGDFRα. However, these mutations have not been found when GIST is in the setting of CT or CD (71). Familial PGL has been associated with 3 of the 4 members of the succinate dehydrogenase complex, SDHD, SDHB, and
Prior to our study, it is widely believed, especially in the medical community, that CT/CD was in general not a heritable disease. Thus, our current data do represent a paradigm-shift: a subset of CT/CD (3/50 or 6%) is indeed hereditary, and are caused by mutations in either \textit{SDHB} or \textit{SDHC}. Remarkably, all 3 (100%) of the mutations are splice-site mutations, although only 3 (5%) of all published germline SDH mutations are within the splice-site (P=0.0001) (17, 73). Our observations have implications for the practice of clinical cancer genetics. If an individual presenting with PGL, without other features of known PGL syndromes, is found to have a splice-site mutation, he/she may have a higher likelihood of developing full-blown CD or CT.

Two of these novel-splice site mutations are in \textit{SDHB}. \textit{SDHB} mutation-positive pheochromocytomas are associated with aggressive, malignant disease characterized by metastases and local recurrence (see chapter 2). This has further implications for the patient’s care. The \textit{SDHB} mutation-positive CT/CD patients should receive better screening to prevent the growth of an aggressive tumor, and to be able to prevent any metastases (74).

PGL are derived from the extra-adrenal chromaffin cells. These cells are extremely sensitive to changes in oxygen tension, and it has been shown that low oxygen environment that is present at high altitudes can independently cause carotid body PGL tumors (18, 75). This is an interesting connection between the derivation of PGL and of GIST. Functionally, the convergence point of these two causative pathways is HIF-1\textsubscript{α} (hypoxia- inducible factor). Both the KIT/ PGDFR\textsubscript{α} and SDH proteins regulate the transcription factor HIF-1\textsubscript{α}, which helps cells to adapt to hypoxia (Figure 5.7).

\textit{SDHC}.
**Figure 5.7.** Adapted from Dahia et al, 2005, Amieux, 2004. Proposed model of the interaction of the SDH complex and KIT/PGDFRa with HIF1α. KIT and PGDFRa activate Ras and PI3K pathways, which increases levels of HIF1α. HIF1α is a key transcriptional regulator of the cellular hypoxia response. Independently, HIF1α downregulates SDHB, the key catalytic component of the SDH complex, leading to complex II dysfunction. Therefore, we provide a concrete link, through the mutations in SDH genes, which clearly shows the relationship between PGL and GIST in which the hypoxia and oxygen sensing pathways play a key role.

Recently, in cultured cell lines, it was shown that disruption of the mitochondrial complex II results in increased HIF1α activity. The increase in HIF1α is channeled through the inhibition of a hydroxylation step that is essential for VHL-dependent HIF1α degradation (76). In an independent study, transcriptional profiling of primary pheochromocytomas with SDHB, SDHD, or VHL germline mutations revealed that SDH mutations lead to upregulation of HIF1α gene targets in tumor tissue (18). Put together,
these findings suggest a reciprocal effect between HIF1α levels and mitochondrial complex II. The activity of this essential protein regulates vasculogenesis and cellular proliferation, both of which are accentuated in PGL and GIST.

Further evidence solidifies the relationship between SDH function and oxygen regulation. Increased expression of angiogenic factors were seen in cases of SDH-mutant pheochromocytomas (77). Also, families with germline mutations in VHL and SDHB have shown clinical similarities, other than pheochromocytoma, specifically renal-cell carcinoma (47). These data, in combination with the transcriptional and biochemical results mentioned above, reinforces our novel findings that functionally connect the seemingly-unrelated tumors, PGL and GIST.
CHAPTER 6

INTRONIC RET SNPs IN NON-SYNDROMIC PHEOCHROMOCYTOMA

6.1 Abstract

Pheochromocytoma is a tumor that typically arises in the adrenal medulla, of which nearly 75% are sporadic. Germline mutations in four pheochromocytoma-susceptibility genes, RET, VHL, SDHB and SDHD, have been shown to cause the 25% that are hereditary. Germline high penetrance gain-of-function RET mutations cause multiple endocrine neoplasia type 2 (MEN 2), of which medullary thyroid carcinoma (MTC) and pheochromocytoma are components, while traditional loss-of-function mutations are associated with Hirschprung disease (HSCR). We have evidence that a low penetrance founder locus, in linkage disequilibrium with a RET ancestral haplotype comprising specific alleles at three intron 1 single nuclotide polymorphisms (SNP) [so called haplotype 0] and the exon 2 A45A SNP, predisposes to the majority of isolated HSCR. Similarly, a different low penetrance locus, in linkage disequilibrium with a different intron 1 haplotype (haplotype 2) and the exon 14 S836S SNP, was found to be associated with a subset of sporadic MTC. Because we have already shown that RET likely harbors common low penetrance alleles predisposing to sporadic MTC and to sporadic HSCR, we sought to determine if RET might also act as a low penetrance
predisposing gene associated with apparently sporadic pheochromocytoma. We analyzed 104 individuals with sporadic pheochromocytoma without traditional germline mutations in \textit{RET}, \textit{VHL}, \textit{SDHD}, and \textit{SDHB} for the presence or absence of the \textit{RET} A45A and S836S SNPs and formed haplotypes and genotypes comprised of alleles at the three intron 1 SNP’s and a new upstream insertion/deletion variant. In this study, we show that pheochromocytomas are not correlated with either A45A or S836S. Despite this result, we found that sporadic pheochromocytomas are significantly associated with haplotype 0 (as in HSCR). However, unlike HSCR, this pheochromocytoma-associated haplotype 0 is not associated with the A45A SNP. Taken together with the strengthening of association with the addition of the 5’ insertion/deletion variant data, our observations may suggest the presence of a low-penetrance pheochromocytoma susceptibility locus in a region upstream of the putative loci for HSCR and apparently sporadic MTC. If identified, this locus might be helpful in the practice of genomic medicine in the future.

6.2 Introduction

Pheochromocytomas are catecholamine-producing chromaffin tumors, 90% of which arise in the adrenal medulla. Extra-adrenal pheochromocytomas, which arise in the sympathetic ganglia, are referred to as paragangliomas. Until recently, the assumption was that 10% of all pheochromocytomas are hereditary, while the remaining 90% are sporadic. In a population-based study, we found that 25% of apparently sporadic pheochromocytomas are actually hereditary (50). Despite a lack of syndromic features and family history at presentation, they were found to carry germline mutations in one of the four currently known pheochromocytoma-susceptibility genes: \textit{RET}, the susceptibility
gene for multiple endocrine neoplasia type 2 (MEN 2), VHL the gene for von Hippel-Lindau disease, SDHD, or SDHB, susceptibility genes for the pheochromocytoma-paraganglioma syndromes(50). So, 75% of this population-based series of pheochromocytomas do not have mutations in these known genes. Therefore, we postulated that these apparently sporadic pheochromocytoma cases might be accounted for by low penetrance variants in RET, for the following reasons.

The RET proto-oncogene is localized to 10q11.2, encodes a receptor tyrosine kinase expressed in neural crest and its derivatives (78, 79). Germline gain-of-function mutations cause MEN 2, which is an autosomal dominant inherited cancer syndrome that is characterized by the triad of medullary thyroid cancer (MTC), pheochromocytoma, and hyperparathyroidism (78). Loss-of-function mutations in RET are associated with a subset of Hirschsprung disease (HSCR), a common disorder also known as aganglionic megacolon (80, 81). In a population-based HSCR series, only 3% of all isolated (non-familial) cases were found to carry germline RET mutations (82). Therefore, working on the hypothesis that common low penetrance alleles in RET may be responsible for the majority of isolated HSCR, we initially performed association analyses and found that haplotypes associated with a RET exon 2 polymorphic variant (or single nucleotide polymorphism [SNP]) A45A (c.135G>A) was highly associated with the majority of isolated HSCR (83, 84). Further, this variant was found to be in linkage disequilibrium with variants at the 3’ end of RET intron (85) 1, which also was associated with the majority of isolated HSCR (86) (Figure 6.1). Statistical modeling suggests that these associated haplotypes of variants belong to an ancestral haplotype in linkage
disequilibrium with a very common but low penetrance founder mutation which accounts for susceptibility to the majority of isolated HSCR and dates back to the Stone Age (86).

Figure 6.1. Schematic representation of part of the RET protooncogene, illustrating the four IVS1 polymorphic loci analyzed and the two anchoring SNPs associated with HSCR (A45A) and MTC (S836S) [not to scale].

Interestingly, we initially found that a different RET polymorphic variant in exon 14, S836S, was associated with a subset of apparently sporadic MTC. This S836S (c.2439C>T) MTC-associated variant was in linkage disequilibrium with a haplotype comprising a different combination of variants at the 3’ end of IVS1 than that for HSCR (86). Statistical modeling suggested that similar to that for HSCR, the MTC-IVS1 haplotype was in linkage disequilibrium with a low penetrance locus within IVS1 in the vicinity of, but distinct from, that for HSCR (86).

We hypothesized that the RET proto-oncogene could also act as a low penetrance susceptibility locus that is strongly associated with apparently sporadic pheochromocytoma. In order to address our hypothesis, we decided to characterize previously established SNPs in the 3’ region of RET intron 1 and use these data to perform haplotype analysis to test whether haplotypes in this region showed association with pheochromocytoma.
6.3 Materials and Methods

6.3.1 Patients and controls

Patients with pheochromocytoma have been registered in the population-based registries in Freiburg, Germany, and Warsaw, Poland, and are described in detail previously (50). For this study, we included 104 cases of sporadic pheochromocytoma from this registry because they were identified as not having any germline mutations in RET, VHL, SDHB, or SDHD. Ninety-five race-matched normal control DNA samples were obtained from individuals from the same geographical region as the patients.

6.3.2 Analysis of RET sequence variation

Genomic DNA from pheochromocytoma patients and control samples was obtained from blood leukocytes using standard techniques. We analyzed the RET exon 2 codon 45 SNP, exon 14 codon 836 SNP and the three RET intron 1 SNPs: IVS1-126G/T, IVS1-1370C/T, and IVS1-1463T/C. PCR and genotyping of the A45A and S836S SNPs was performed as previously described (83, 87). For the IVS1 SNPs, PCR was performed using the Qiagen HotStarTaq kit (Qiagen, Valencia, CA) for 38 cycles at 55 degrees Celsius annealing. Primers available upon request. An aliquot of the PCR product was purified using Exol/Shrimp alkaline phosphatase (88) treatment. The purified amplicons were directly sequenced using Big-Dye Terminators v. 20 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA).
In a search for SNPs in \textit{RET} intron 1, a 16 base pair insertion/deletion variation was found in the location IVS1+8406 to +8421del16 within the 22kb spanning this intron, but 13.2 kb upstream of IVS1-1463T/C (Boru, G. and Eng, C., unpublished data). To establish the status at this locus in the pheochromocytoma samples, the 16-bp insertion/deletion polymorphism was detected using fluorescent genotyping. Primers were designed flanking the deletion and the forward primer was labeled with a fluorescent tag (F primer: 5’ HEX CGG CTG AGA GGA GCT TAC AC 3’; R primer: 5’ GTT TCT TCA AGC TGA CAA TCC TGA TGC 3’). PCR was performed using the Qiagen HotStarTaq kit (Qiagen, Valencia, CA) for 38 cycles at 55 degrees Celsius annealing temperature. The PCR products were analyzed on the ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) using the ROX-400HD size standard in order to determine the precise size of the two alleles.

6.3.3 Formation of haplotypes and genotypes and statistical analyses

Allele frequencies at the three intronic SNP loci as well as the 16bp insertion/deletion locus were determined and subsequently haplotypes consisting of the different combinations of the alleles for just the three SNPs as well as at all four loci were constructed (Table 6.1). The haplotypes were based on those previously established, designated 0-4 which represent unique combinations of the three \textit{RET} intronic SNPS: IVS1-126G/T, -1370C/T, -1463T/C (10) [Table 6.1 and Figure 6.1]. The extended haplotypes, which include the insertion/deletion locus (0a-4b) were based on the original haplotypes but given the suffix ‘a’ or ‘b’ depending on their status at the IVS1+8406 to +8421del16 locus (a=deletion absent; b=deletion present). The frequencies of both the
haplotypes and the genotypes (the combination of two haplotypes) were resolved and then compared between controls and individuals with pheochromocytomas.

Comparisons were performed using a standard Chi-square calculation with Yates’ correction as described previously (8-10). Nominal statistical significance was considered when P<0.05. In addition, allele frequencies were compared and significance was established using the same criteria. Comparison of distributions of haplotypes or genotypes against clinical features were performed using standard Chi-square analysis with Yates’ correction. Proportion of individuals diagnosed at a certain age per haplotype was compared using odds ratios (± 95%) confidence intervals.

6.4 Results

6.4.1 Association analysis between RET S836S and A45A and individuals with pheochromocytoma compared to controls

Since the S836S (c.2439C>T) SNP was found to be associated with apparently sporadic MTC (11) and in linkage disequilibrium with intron 1 haplotype 2 (10), our initial hypothesis was that the RET haplotype in linkage disequilibrium with S836S was a general low penetrance gain-of-function locus which would also predispose to isolated pheochromocytoma. In order to test this hypothesis, we determined the allele frequencies at this SNP in cases with pheochromocytoma and controls. However, the frequency of the variant c.2439T allele in individuals with pheochromocytoma was no different from that of normal controls (3.8% vs. 3.7%, P>0.05, data not shown). In addition, it was found that the A45A variant was not associated with sporadic pheochromocytoma (P>0.05, data not shown).
6.4.2 Association analyses of RET intron 1 variations in individuals with pheochromocytoma and controls

We have analyzed 104 apparently sporadic pheochromocytoma cases and 100 normal controls at each of four loci in intron 1 of the RET proto-oncogene: three SNP’s towards the 3’ end of the intron and one 16 base-pair insertion/deletion polymorphism 5’ of the SNPs. Of these four loci, allele frequencies at three showed significant differences between individuals with pheochromocytoma and normal controls (Table 6.2). Among a total of 208 pheochromocytoma chromosomes, there were 82 (39.4%) with the T variant at the IVS1-1370C/T locus, and 126 (60.6%) with the wild-type C allele. The T variant was statistically significantly under-represented when compared to normal controls (chi-square 4.50, P = 0.034). Similarly, the IVS1-1463 polymorphic C allele was under-represented in cases compared to controls (chi-square 8.28, P=0.004). Conversely, the IVS1+8406 to +8421del16 deletion was over-represented in individuals with pheochromocytomas compared to the controls (P = 0.022). The allele frequencies at IVS1-126 did not show a significant difference between the two groups (P=0.70; Table 6.2).
Table 6.1. Haplotypes based on the Combination of Allelic Variants at Four Polymorphic Loci Located within RET intron 1

6.4.3 Haplotype analysis of RET intron 1 polymorphic loci in cases and controls

Haplotypes were initially constructed which comprised only the three 3’ intron 1 SNPs and previously designated as haplotypes 0-4 (10). The distribution of pheochromocytoma cases and normal controls cross these haplotypes were significantly different (Chi-square 8.81, P = 0.032; Table 6.3). Of note, haplotype 0 was over-represented among cases compared to controls (Table 6.3).
Table 6.2. Comparative Studies between Pheochromocytoma Cases and Controls of Allelic Frequencies at Four RET Intron 1 Polymorphic Loci Using Chi-Squared Analysis with Yate’s Correction

<table>
<thead>
<tr>
<th>Locus</th>
<th>Cases Alleles</th>
<th>Controls Alleles</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS1-126 G → T</td>
<td>63 (0.333)</td>
<td>61 (0.321)</td>
<td>P = 0.76</td>
</tr>
<tr>
<td>No. of wild-type G alleles (allele freq)</td>
<td>146 (0.697)</td>
<td>129 (0.678)</td>
<td></td>
</tr>
<tr>
<td>χ² with Yate’s correction (P value)</td>
<td>4.15</td>
<td>6.15</td>
<td></td>
</tr>
<tr>
<td>IVS1-126 C → T</td>
<td>82 (0.404)</td>
<td>95 (0.45)</td>
<td></td>
</tr>
<tr>
<td>No. of wild-type C alleles (allele freq)</td>
<td>124 (0.609)</td>
<td>95 (0.5)</td>
<td></td>
</tr>
<tr>
<td>χ² with Yate’s correction (P value)</td>
<td>4.59</td>
<td>6.09</td>
<td></td>
</tr>
<tr>
<td>IVS1-1465 T → C</td>
<td>146 (0.697)</td>
<td>153 (0.821)</td>
<td></td>
</tr>
<tr>
<td>No. of wild-type C alleles (allele freq)</td>
<td>63 (0.303)</td>
<td>34 (0.179)</td>
<td></td>
</tr>
<tr>
<td>χ² with Yate’s correction (P value)</td>
<td>8.28</td>
<td>5.03</td>
<td></td>
</tr>
<tr>
<td>Deletion 319 → 303</td>
<td>131 (0.63)</td>
<td>98 (0.515)</td>
<td></td>
</tr>
<tr>
<td>No. of wild-type 303 alleles (allele freq)</td>
<td>77 (0.37)</td>
<td>92 (0.484)</td>
<td></td>
</tr>
<tr>
<td>χ² with Yate’s correction (P value)</td>
<td>5.28</td>
<td>0.022</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3. Distribution of haplotypes comprising alleles at three intron 1 SNP loci in pheochromocytoma cases and controls

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62</td>
<td>34</td>
</tr>
<tr>
<td>1</td>
<td>81</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>60</td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

χ² = 8.81; df = 3; P = 0.032.
Using the information for each locus, we subsequently constructed haplotypes consisting of various combinations and permutations of variants at all four loci, the three 3’ IVS1 SNPs and the insertion/deletion polymorphism 5’ of these SNPs, adding the designation a or b depending on the absence or presence, respectively, of the polymorphic 16 nucleotides (Table 6.1). The frequencies of each haplotype were compared between individuals with pheochromocytoma and normal controls. The haplotype distribution between the cases with pheochromocytoma and that of the normal control group was found to be statistically significant (chi-square 25.27, P = 0.0014; Figure 6.2).

**Figure 6.2.** Frequencies of haplotypes comprising specific alleles at four intron 1 polymorphic loci in pheochromocytoma cases and controls.
Figure 6.3. Frequencies of RET genotype in pheochromocytoma cases and controls.

Each genotype is comprised of a pair of unique haplotypes.

Haplotype 2b was the most common haplotype in the pheochromocytoma samples. This haplotype comprises the IVS1-126, and IVS1-1463 polymorphic alleles in the presence of IVS1+8406del16. Haplotype 2b represents 30.5% of all pheochromocytoma haplotypes, and haplotype 1a the second most common (27.6%) [Table 6.5 and Figure 6.2]. In the controls, haplotype 1a was the most common (35%) and 2b, second most common, while 3b was the least frequent. Haplotype 0b was over-represented amongst cases compared to controls while haplotype 2a under-represented in cases versus controls (Table 6.5 and Figure 6.2).
We first analyzed the genotype composition (pair of haplotypes) with the haplotypes established previously, 0-4 (86). The distribution between sporadic pheochromocytoma cases and controls was significantly different (P=0.037; Table 6.4).

We then analyzed the four-locus genotype composition of each sample and compared the two groups. Genotypes 1b/0a and 1a/2b predominate in individuals with pheochromocytomas (42%) while genotypes 1a/1a and 1a/2b were in the most common amongst controls (42%). The overall distribution of the genotypes in the two groups was compared and found to be statistically significant (chi-square 23.50, P = 0.0014).

### Table 6.4. Polymorphic Allelic Frequencies in Pheochromocytoma Cases and Controls

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0a</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>0b</td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>1a</td>
<td>56</td>
<td>68</td>
</tr>
<tr>
<td>1b</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>2a</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>2b</td>
<td>62</td>
<td>52</td>
</tr>
<tr>
<td>3b</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4b</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 18.86; \text{df} = 8; P = 0.016. \]

### Table 6.5. Distribution of Haplotypes Comprising Alleles at Three Intron 1 SNP Loci in Pheochromocytoma Cases and Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td>20</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>00</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>03</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 14.94; \text{df} = 7; P = 0.037. \]
Genotypes 0b/0b and 1b/0a were particularly over-represented in cases compared to controls (Table 6.6 and Figure 6.3).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b/0a</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>2b/0b</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>1a/1b</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>1a/1a</td>
<td>1.4</td>
<td>19</td>
</tr>
<tr>
<td>2b/2b</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>1a/2b</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>0b/0b</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

$\chi^2 = 23.50; \text{df} = 7; P = 0.0014.$

**Table 6.6.** Distribution of Genotypes (pairs of three-SNP haplotypes) in Pheochromocytoma Cases and Controls
6.4.5 *Analysis of RET haplotypes and genotypes and clinical features*

Clinical data for each of the pheochromocytoma samples with respect to the tumor location, patient gender, and age at diagnosis was compared against *RET* intron 1 haplotype and genotype. The patients’ ages at diagnoses were divided into three groups: under 40 years, 40-59 years, and >= 60 years of age (Table 6.7). The overall distribution of the genotypes for individuals with pheochromocytoma across each of the three age groups was statistically significant (chi-square= 31.09, P= 0.028; Table 6.7). In contrast, the overall distribution of haplotypes across each of the age groups was not significantly different. However, there tended to be more individuals carrying the haplotype 0b diagnosed under the age of 40 years (8 of 21 or 0.38, 95% CI 0.21, 0.59) compared to those with haplotype 1b under the age of 40 (3 of 39 or 0.07, 95% CI 0.02, 0.21). The location of the pheochromocytomas (adrenal or extra-adrenal) and the gender distribution of the patients were not found to be statistically significantly different among haplotypes or genotypes (data not shown).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>&lt;40</th>
<th>40–59</th>
<th>&gt;=60</th>
</tr>
</thead>
<tbody>
<tr>
<td>0b/0b</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>1a/1a</td>
<td>0</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>1a/1b</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1a/2b</td>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>1b/0a</td>
<td>2</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>2a/1a</td>
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<td>2b/0a</td>
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<td>0</td>
</tr>
<tr>
<td>2b/0b</td>
<td>0</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>2b/2b</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>2b/2b</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

χ² = 31.09; df = 18; P = 0.028.

**Table 6.7.** Distribution of Four Variant Haplotypes Comprising Alleles at Four Intron 1 Loci in Pheochromocytoma Cases and Controls
6.5 Discussion

In this study, we have found that a haplotype consisting of a unique combination of alleles at four RET intron 1 polymorphic loci is strongly associated with and over-represented in individuals with sporadic pheochromocytoma compared to controls. This haplotype, which we denote 0b, comprises the wildtype allele at IVS1-126 and IVS1-1463, with a 16 basepair intron 1 deletion 5’ of these SNPs. Consistent with this observation, the genotype 0b/0b was found to be over-represented in individuals with pheochromocytoma compared to race-matched normal controls.
Our observations were initially surprising since we have previously shown that in Hirschprung disease patients, haplotype 0 is also the most common and the most over-represented haplotype in HSCR compared to controls (86). However, in HSCR, haplotype 0 is in absolute linkage disequilibrium with the presence of the exon 1 A45A polymorphism in contrast to our pheochromocytoma series where the variant is not over-represented and not in linkage disequilibrium with haplotype 0. Further, the pheochromocytoma-haplotype 0 association was strengthened when the status at the upstream 16-bp insertion/deletion locus was taken into account. These data suggest that a low penetrance pheochromocytoma susceptibility locus might be in linkage disequilibrium with haplotype 0b and would likely lie 5’ of the 16-bp insertion/deletion locus. Our observation that haplotype 0 is not in linkage disequilibrium with A45A in pheochromocytoma cases additionally suggests that the two putative ancestral haplotype/founder loci, the HSCR-specific haplotype 0-A45A and the pheochromocytoma-related haplotype 0, which is not associated with A45A, diverged during the Stone Age (86).

The association with pheochromocytoma is strengthened when genotypes comprising pairs of haplotypes are examined, e.g. 0b/0b occurs in 12 cases and 1 control. Thus, we may postulate that this putative low penetrance locus acts in an additive or autosomal recessive manner. Our observations have an alternative explanation, i.e., that the actual intron variants per se are lending low penetrance susceptibility to pheochromocytoma. This is a less likely explanation. Although there are several putative transcription factor binding motifs within intron 1, where one may postulate
differential binding strengths dependent on the presence or absence of variation, the fact that the identical intron 1 SNP haplotype, haplotype 0, has been found to be over-represented in HSCR as well, argues against this alternative. To better define the putative low penetrance pheochromocytoma locus, the identification and analysis of further intron 1 and further upstream SNP’s should be performed.

Interestingly, we found a significant association between the patients’ age of diagnoses and genotype. This is especially noteworthy because there is not a significant correlation with the individual haplotype. There are two explanations for these seemingly contradictory observations. First, it is possible that samples sizes are relatively small, and so, the effect of individual haplotype was not obvious. Second, this observation supports our above postulate that the additive effect of haplotypes, i.e. the genotype, dictates the outcome, and in this case, age of onset. Analogously, four-fifths of German HSCR cases homozygous for the A45A polymorphism had the short-segment phenotype (89), although this particular association was not found amongst Spanish HSCR cases (83, 84).

In summary, we have genetic evidence that a low penetrance founder pheochromocytoma susceptibility locus may exist in a region 5’ of the insertion/deletion polymorphism within intron 1 of the RET proto-oncogene which might account for a subset of apparently sporadic pheochromocytoma. Further, either the combination of intron 1 variants associated with haplotype 0b or the putative founding locus itself can modulate age-of-onset.
CHAPTER 7

ROLE OF TUMOR-SUPPRESSOR GENES
IN ATHEROSCLEROSIS

7.1 Abstract

Atherosclerosis is a disease of the vasculature, characterized by the plaque formation in the inner lining of the artery. This plaque can alter the blood flow and result in heart attack or stroke. There are many causes of atherosclerosis, including the interactions of the cells in the artery wall with each other and with the blood passing through, as well as inflammation and proliferation. Because carcinogenesis is also involves inflammation and excess proliferation, we wanted to address the hypothesis that atherosclerosis is a proliferation genetically reminiscent of carcinogenesis. In order to address this hypothesis, we used three array-based platforms including array comparative genomic hybridization (aCGH), the 10K single nucleotide polymorphism (SNP) array, and finally the U133A Affymetrix Expression array. The integration of the data resulting from these three experiments allowed us to draw a number of significant and novel conclusions. First, there is a possible gain of 15q and 3p in disease samples. Secondly, significant loss of heterozygosity (LOH) is seen at 1q, 3p, 16q, and LOH seen at 15q in a small number of samples (this does not reach statistical significance). The regions of 1q,
3p, 15q, and 16q have high numbers of genes that could be significant for future studies in the genetics of carotid artery atherosclerosis.

7.2 Introduction

Atherosclerosis is a slow, complex disease. Of Greek origin, the word atherosclerosis- ‘athero’ meaning gruel or paste, and ‘sclerosis’ or hardness, is indicative of the phenotypic characteristics that this disease causes. This disease occurs when a plaque forms in the inner lining of the arteries. This plaque is composed of fatty substances, cholesterol, calcium and exhibits increased inflammation compared to surrounding regions of the artery. The result of the plaque formation can cause decreased or altered blood flow, which can ultimately lead to a heart attack or stroke (American Heart Association, 2005).

Atherosclerosis is a complicated interaction of risk factors, including the cells of the artery wall, blood, and environmental influences. Inflammation plays a major role in all stages of atherosclerosis (90). When the endothelium of the artery encounters certain environmental cues, such as excess adipose tissue or hypertension, these cells augment the expression of adhesion molecules that promote the sticking of blood leukocytes to the inner lining of the arterial wall. These leukocytes, once in the artery wall, communicate with endothelial cells and smooth muscle cells (SMCs). These two cell types are the endogenous cells of the arterial wall. Much of this communication is dependent upon inflammatory mediators (91).

The next stage in the development of atherosclerosis is the migration of the SMCs to the innermost layer of the arterial wall, the intima. This is a result of the inflammation
in the early stage atheroma. Subsequently, the SMCs, endothelial cells and monocytes secrete matrix metalloproteinases (MMPs). MMPs have been shown to play a significant role in the biological and genetic development of atherosclerosis. MMPs modulate numerous functions of vascular cells, including proliferation, migration, angiogenesis, and apoptosis (92). The SMCs begin to proliferate, and the cellular environment then undergoes a series of modifications, which ultimately propagate the inflammatory response. The atherosclerotic lesion progresses and the calcification and cholesterol deposition occur, forming the stable plaque.

The genetic mechanisms that influence the formation of a carotid plaque are unknown. Gene expression profiling studies have indicated increased inflammation in the plaque tissue compared to the normal artery, confirming the above observations (93). However, further genetic studies have not been performed on carotid artery disease.

Since carcinogenesis is a process which involves uncontrolled cellular proliferation and inflammation, we hypothesized that atherosclerosis is a proliferation reminiscent of carcinogenesis. We addressed this hypothesis by identifying the role of tumor-suppressor genes in carotid artery disease, using a number of array-based platforms. These included array comparative genomic hybridization (aCGH), microarray expression analysis, and the 10K Affymetrix SNP array. Using these methods, we show that carotid artery disease shows significant genomic variations in numerous of chromosomal regions. These alterations include LOH at 1q, 3p, and 16q, a possible gain of 15q, and a possible loss at 3p. We also show that there are at least two sub-groups within our patient population, and that this may be due to treatment that those patients
were receiving during the time of tissue extraction (surgery). Our observations suggest that this type of cardiovascular disease shows numerous novel and previously established genetic alterations that may be able to distinguish an individual who is genetically likely to developing a plaque from one who will not develop a plaque.

7.3 Methods

7.3.1 Patients

A total of 215 tissue samples were obtained from patients undergoing carotid endartectomy to have an atherosclerotic plaque removed. The anonymized carotid plaque specimens were received from the Ohio State University vascular surgical section, under the direction of Dr. William Smead. Each carotid plaque specimen was flash frozen in liquid nitrogen. All plaque samples were then stored at -80°C until genetic material was extracted.

Among these 215, 17 had matching peripheral blood specimens (30cc, ACD-anticoagulated), from which germline DNA was extracted using standard techniques.

7.3.2 DNA and RNA Extraction and Purification

The first step in DNA and RNA extraction from the fresh-frozen carotid plaque specimens was to pulverize the tissue into tiny powder-like fragments. This step was crucial in obtaining a high quality and quantity of genetic material. This was done by keeping the tissue (in a small bag) in liquid nitrogen under sterile conditions and pounding it with a blunt end rubber mallet. The pounding was done on a 10lb metal round disc that was kept at -80°C until just before use, in order to keep the specimen as cold as possible. After each hit with the hammer, the sample was again put in liquid
nitrogen. When the tissue was a powder like substance, 1ml Trizol per 25-50mg of tissue (for RNA extraction) and 1ml of DNAzol per 25-50mg of tissue (for DNA extraction) was added. The tissue/ Trizol or DNAzol mixture was then homogenized using an electric tissue homogenizer to ensure a homogeneous solution. Subsequent to the second homogenization step, we extracted the DNA or RNA using standard methods (Invitrogen, Carlsbad, CA).

After the RNA extraction step using Trizol, the RNA was quantified using a nucleic acid quantification station. We then used <1ug of extracted RNA in a purification step using RNeasy RNA Purification Kit (Qiagen, Valencia, CA). The resulting DNA was then quantified and used for the genetic analyses.

7.3.3 Array Comparative Genomic Hybridization (aCGH) Experiment

The Spectral Chip 2600 BAC array containing 2632 BACs at approximately 1Mb interval was used (Spectral Genomics, Houston, TX) for each sample for analysis of genomic alterations. Approximately 250ng of genomic DNA from 7 pairs of patient tissue (disease) /patient blood (germline) was sent to the research laboratories at Spectral Genomics. The experiments were performed according to the manufacturer’s protocol at the Spectral Genomics research laboratory. DNA from both the reference sample and the test sample was labeled with Cy5 and Cy3, respectively, by random priming. A dye swap experiment was performed to avoid dye bias.
The experimental data were returned in the form of a plot. Each plot represented one chromosome for one sample, in which both a blue (Cy 5) and red (Cy3) line connected a number of dots. Each dot represents one BAC location. A deviation of both the blue and red lines from the middle line represented a significant gain or loss at that chromosomal location (Figures 7.1, 7.2).

7.3.4 aCGH Validation using Semi-Quantitative Duplex PCR

In order to validate our aCGH, we performed duplex PCR to determine the ratio of the BAC of interest to our control gene, GAPDH. Because each BAC was so large, we divided each into 5 equal regions and designed PCR primers in each of the 5 regions. Each PCR primer pair must amplify a product that is ~200bp in order to amplify clearly with our 118bp GAPDH primer pair. After designing the primers within the BAC, the ratio of BAC: GAPDH was determined for the samples used in the initial aCGH experiment as well as an additional 20 for a validation set. A cutoff ratio of >1.5 was used to represent gain, while a cutoff ratio of <0.5 was used to represent a loss (Table 7.1)
Table 7.1. aCGH Validation of BAC 114M1 on 3p. BAC 114M1 was divided into five regions, a-e. The ratio of BAC region: GAPDH is shown. Those highlighted in green represent gain.

7.3.5 SNP Array Experiment

XbaI mapping SNP array 130 (Affymetrix, Santa Clara, CA) was used. This array covers 10,043 SNP loci distributed on all of the human chromosomes except Y chromosome, resulting in a resolution around 300kb. The analyses were performed according to previously described methods and the manufacturer’s protocol. Overall, the
procedure was as follows. 250μg of genomic DNA was digested with XbaI restriction enzyme, ligated with an adaptor, and amplified by PCR. The resulting amplicons are fragmented, labeled with biotinylated ddATP using terminal deoxynucleotidyl transferase, and hybridized to the array. The array is scanned for fluorescence. We analyzed the SNP array data using dChipSNP (94).

7.3.6 SNP Array Data Loss-of-Heterozygosity (LOH) Analysis

dChipSNP2004 was used to analyze the SNP array data. First, the 10K SNP cel and txt files were imported into the program using the ‘open group’ command. These files contain the call for each SNP for each sample. In order to perform LOH analysis, we combined the SNP call data for pairs of normal and tumor samples to make the LOH calls. The order of the tumor/normal pairs was established using ‘array list file’ and then analyzed for LOH. The resulting data can be clearly viewed to determine regions of LOH (Figure 7.3).
7.3.7 Affymetrix Expression Array Experiment

The Affymetrix U133A 2.0 Genechip platform was used on 20 diseased tissue samples and 5 normal artery samples. The methods used for sample preparation and hybridization are based on the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA). The U133A chip is a single array representing 14,500 well-characterized human genes. Sequences used in the design of the array were selected from GenBank, dbEST, and RefSeq. The sequence clusters were created from the UniGene database and then were refined by analysis and comparison with a number of other publicly available databases. Further details about this GeneChip can be obtained from the Affymetrix website (www.affymetrix.com). In order to analyze the microarray expression data, we used dChip (Li and Wong).
7.3.8 Expression Array Validation using Quantitative RT-PCR

In order to validate the data we obtained from our expression array, we used the widely accepted method of quantitative rt-PCR using the Qiagen SybrGreen master mix (Qiagen, Valencia, CA). The RNA samples used for validation were those included in the expression array experiment and addition 20 samples used for validation purposes. We chose 10 genes of interest (GOI), of which 5 were significantly over-expressed and 5 were significantly under-expressed on the array. We ran the pcr using this master mix and the primers for each GOI and control gene (GAPDH) concurrently and in triplicate on the Bio-rad Icycler (Bio-rad, Hercules, CA). The resulting data gave a threshold cycle value (Ct) for each sample for each gene. We then calculated the \( \Delta \text{Ct} \) (Ct gene of interest-Ct GAPDH). This difference is directly proportional to the expression value. We then compared this data to our original expression array data and found that all genes tested could be validated.

7.4 Results

In order to examine the genomic profile of the carotid artery atherosclerosis samples, we took a three-pronged approach. This approach included aCGH to look for large genomic alterations, SNP array to examine the samples for smaller genomic changes/ LOH, and finally expression array analysis. We used a tripartite approach for genomic and functional genomic characterization of atherogenesis by integrating the data from all three platforms, in which we were able to look for genes in the chromosomal regions identified in either aCGH or SNP array and determine if the expression pattern of these genes correlates with the gain or loss seen. From our aCGH data, we found a
possible gain of 15q and a possible loss of 3q. Our SNP array data showed LOH at 1q, 3p, 16q. Our expression array data was examined for genes over- or under-expressed in any of these genomic regions.

7.4.1 aCGH

We used aCGH to scan all chromosomes for large genomic deletions or alterations in 5 pairs of atheroma/germline DNA pairs. We found that in one sample, VNPxx, there was a possible gain of chromosome 15q (Figure 7.1). These data were validated using duplex semi-quantitative PCR.

Figure 7.1. aCGH Plot for Chromosome 15.
In addition, we found that 2/5 disease samples showed a possible gain of 3p (Figure 7.2). This gain was validated using semi-quantitative duplex PCR (Table 7.1).

**Figure 7.2.** aCGH plot for chromosome 3. Two disease samples showed possible gain at this BAC location (see arrow).

7.4.2 SNP Array Results

After our aCGH scan for larger genomic alterations, we then analyzed 12 pairs of normal blood/atheromas for LOH across the genome. We analyzed the data for LOH using two methods, the dChipSNP software and our validation method in which we closely examined regions of the genome in which >2 pairs have LOH at a particular SNP locus. Both types of analyses determined three regions of LOH: 1q, 3p, 16q (Figure 7.3).
Figure 7.3. SNP array data in physical distance by chromosome. Top panel shows chromosomes 1-7, bottom panel shows chromosomes 8-X.
The label across the top of each panel indicates the normal blood/disease artery pair. There are 12 pairs. Along the lefthand column is the chromosome number. Each small block indicates the LOH call at a particular SNP for that normal/disease pair. The colors indicate the following: blue=LOH; yellow=retention of heterozygosity (ROH); red=conflict; gray=non-informative; white=no call. The vertical blue line along the right hand side indicates significance of LOH. The red line is the threshold of significance.

In order to confirm and validate the LOH calls from dChipSNP, we calculated %LOH at each SNP locus using our validation approach. We examined each SNP locus for which >2 pairs had LOH for interesting genes (Table 7.2).
<table>
<thead>
<tr>
<th>CHR</th>
<th>SNP ID</th>
<th>% LOH</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1511423</td>
<td>0.67</td>
<td>CAPON</td>
</tr>
<tr>
<td></td>
<td>1513573</td>
<td>0.5</td>
<td>PPAPA2-pappalysin2</td>
</tr>
<tr>
<td></td>
<td>1510124</td>
<td>0.29</td>
<td>ESRRG-estrogen rel. rec. 2</td>
</tr>
<tr>
<td></td>
<td>1510869</td>
<td>0.4</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1517583</td>
<td>0.5</td>
<td>DISC- disrupted in schizophrenia</td>
</tr>
<tr>
<td></td>
<td>1510130</td>
<td>0.5</td>
<td>SIPA1L2-signal ind.prolif-assoc 1</td>
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<tr>
<td></td>
<td>1512587</td>
<td>0.5</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>2</td>
<td>1508000</td>
<td>0.29</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1507817</td>
<td>0.4</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1512596</td>
<td>0.4</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1511169</td>
<td>0.33</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>1513627</td>
<td>0.5</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>1511956</td>
<td>0.5</td>
<td>GRM7-glutamate rec gene fam</td>
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<tr>
<td></td>
<td>1509695</td>
<td>1.0</td>
<td>SYN2-synapsin 2</td>
</tr>
<tr>
<td></td>
<td>1508204</td>
<td>0.6</td>
<td>PD2RN3-pdz domain cont</td>
</tr>
<tr>
<td></td>
<td>1516412</td>
<td>0.29</td>
<td>TRPC1</td>
</tr>
</tbody>
</table>

Table 7.2. SNP array validation, showing chromosomes 1-3 as examples. Table shows the chromosome number, followed by SNP ID’s for which >2 pairs (out of 12) showed LOH. The %LOH and genes in that region follow the SNP ID.

7.4.3 Affymetrix U133A Expression Array Results

A total of 25 samples were analyzed using the U133 Affymetrix Expression array. Of these samples, 20 were diseased carotid artery plaque tissue specimens, and the remaining 5 were normal arteries. The normal arteries were from normal individuals and banked at a transplant center; the arteries were of a quality that would be appropriate to act as vascular donation for transplantation and grafting.
7.4.3.1 Unsupervised clustering of the expression data

Using dChip2004 software, we analyzed our expression data for gene expression levels. The data was normalized and processed using methods described by Li and Wong (95). We first performed unsupervised clustering of the 25 samples to determine if the samples clustered based on their atherosclerosis disease status. We found that of the 20 disease samples, there was only one sample (VNP4) that clustered with the normal samples. In addition, we found that there were two subgroups of the disease samples (Figures 7.4). There are a number of possibilities for the two subgroups of disease samples, including different types of atherosclerosis, potential for rupture, and manner of treatment at the time of or even prior to surgery. Since our samples were anonymized at the time of surgery, we are unfortunately unable to correlate clinical data with genetic data.
Figure 7.4. Unsupervised clustering of the 25 samples used for expression array analysis.

The group in the red box includes the five normal and one diseased sample that does not group with the rest of the diseased samples (VNP4). There are two clear groups of disease samples, one to the right and one to the left of the group of normal samples in the red box.

7.4.3.2 Supervised Clustering Analysis

The scientific literature is immersed with discoveries regarding the role of hundreds genes in various cancers. Cancer is a disease of excess proliferation and inflammation, both characteristics of atherosclerosis. For this reason, we searched the
literature and established a list of 16 genes known for their significant roles in cancer. We wanted to determine if the expression levels of these genes would cluster our samples based on their atherosclerotic disease status. This adds another variable to determine if these cancer genes are consistently over- or under-expressed in samples that have atherosclerosis or not. Our results were astonishing, we found a marked degree of clustering within the sample set based on their atherosclerotic disease status (Figure 7.5). The samples were clearly divided into the 5 normal samples, and two clear groups of diseased samples. The disease sample that previously clustered with the normal samples clustered with its disease counterparts.
**Figure 7.5.** Supervised clustering of 25 samples used for Affymetrix expression analysis. The samples in the red box are 5 normal samples. The second original branch of the cluster tree then divides into two more subgroups, which correspond to those seen in the unsupervised clustering.

7.4.3.3 Ingenuity Pathways Analysis of Expression Array Data

A web-based software program, Ingenuity Pathways Analysis, was used to analyze the expression data for common functions and pathways. This software allows the user to input their array data and determine common pathways in this biological state. This software makes use of the world's largest curated database consisting of millions of individually modeled relationships between proteins, genes, complexes, cells, tissues,
drugs, and diseases. We found that the most common function/pathway involving the genes from our data was cancer (Figure 7.6). This included cell death, apoptosis, and proliferation.

**Figure 7.6.** Ingenuity software data analysis.

Importantly metastasis and invasion, the functions of cancer which are not recognized properties of atherosclerosis, were towards the bottom of the list of data. The data from this software program further supports our hypothesis that atherosclerosis is functionally similar to carcinogenesis.
7.5 Discussion

Atherosclerosis is a complex, slow growing process in which excess proliferation and inflammation play a major role. Because of the involvement of these processes, atherosclerosis is reminiscent of carcinogenesis. We therefore examined the role of tumor-suppressor and other cancer-related genes in atherosclerosis, specifically carotid artery disease, using a three-pronged approach. This approach included the latest technologies available to examine the genome, including array comparative genomic hybridization (aCGH), SNP array, and Affymetrix U133A Expression array. The combination of these three platforms enables thorough comparison of the genome in normal arteries, diseased arteries, and in the individual’s germline.

The integration of the data we obtained from our three-pronged approach revealed a number of interesting genomic regions of interest. We found a possible gain in chromosome 15q in the aCGH data. Also, when examining the SNP array data, we saw that there were a couple of SNPs for which >2 samples had LOH at 15q. In addition, chromosome 3p is a hot-spot for cancer-related genes. We found a possible gain of 3p as well as LOH at 3p in our atherosclerosis samples. Finally, we found significant LOH at 1q and 16q in our SNP-array data. Interestingly, there are a number of genes at many of these chromosomal locations that could play a significant role in atherosclerosis in this region.

Of the genes in our regions of interest, including 1q, 3p, 15q, and 16q, we have uncovered a few that are worth pursuing in future studies. Many of these genes add to and support our hypothesis that atherosclerosis is reminiscent of carcinogenesis. The
long arm of chromosome 1, 1q, has been implicated in a number of human cancers, including bladder cancer (96), gastric cancer (97), and Wilms’ tumor (98). The small region of LOH seen in our atherosclerotic plaque samples is located within a gene, DISC1, or disrupted in schizophrenia 1.

The long arm of chromosome 3, 3p, is a hotspot for cancer-related genes. It is significant that this genomic region showed LOH from our SNP array as well as a possible gain in a number of samples from our aCGH experiment. This chromosomal region includes FHIT, RASSF1, VHL, and many other cancer-related genes. Cancers associated with alterations in this region include renal cell carcinoma (99), lung carcinoma (100), and colorectal cancer (101) to name just a few. It is worthwhile to examine this region further, when our region of LOH is fine-mapped to a more precise region.

We found on chromosome 15 a significant gain in one atherosclerotic plaque sample. Because our sample size is small we will pursue this result further using a larger validation sample set. However, this seems to be an interesting result. The possible gain seen in our aCGH data in 1 plaque sample is located 3’ of the Prader-willi gene location. This location includes a number of cancer-related genes, including GABRB, TP53BP1, and RAD51. We also see a small region of LOH on chromosome 15q from our SNP array data; however this region does not overlap with the possible gain found in our aCGH data.

Finally, we found LOH at chromosome arm 16q. Significantly, the SNP at which the LOH was seen, rs1157118, is located within the gene CDH13, or cadherin 13.
Cadherins are a family of membrane receptors that play a key role in the regulation of organ development during embryogenesis. In addition, they are responsible for formation of stable cell-cell junctions and maintenance of normal tissue structure (102). They often function as tumor-suppressor genes (103). Because the excess proliferation and inflammation seen in atherosclerosis that is reminiscent of carcinogenesis involves the key interactions of the endothelial cells and smooth muscle cells, CDH13 is the first target gene that would be tested for mutations and further genetic alterations in future studies. This gene may play an intricate role in the development of atherosclerotic plaques.

Interestingly, of the 4 regions we have found to be genetically altered in atherosclerosis, 2 of these, 3p and 16q, contain fragile sites and the genes causing the genomic instability are well-characterized, FHIT and WWOX, respectively. Common chromosome fragile sites show susceptibility to DNA damage, leading to alterations that contribute to cancer development. The cloning and characterization of fragile sites have demonstrated that fragile sites are associated with genes that relate to tumorigenesis. Identification of the basis of instability at fragile sites and the related genes provides an entree to understanding of important aspects of chromosomal instability, a prominent feature of neoplastic genomes. FHIT/FRA3B and WWOX/FRA16D, the most sensitive common fragile genes in the human genome, function as tumor suppressor genes. (104). This result is significant and beyond coincidental that 2 of our 4 regions are also the most common fragile sites. This result must be looked into further to establish the role, if any, of these genes in atherosclerosis.
There are a number of drawbacks to our experiment that must be addressed. First and foremost, our genetic data on these samples would be the most useful if we could ultimately correlate the genetic findings with the clinical characteristics of each patient. For example, we found that when performing both supervised and unsupervised clustering using our expression array data, our atheroma samples clustered into two distinct groups (figures 26, 27). There are many possible reasons for this, perhaps that one group had unstable plaques and the other did not. However, because the samples in our study were anonymized as soon as they were removed from the patients at the time of surgery, we are unable to determine any genotype-phenotype correlations.

Another limitation of this experiment is uncertainty of the cell type presenting each of the genetic characteristics. Since our only option to extract DNA and RNA from a carotid artery plaque specimen is the method of pulverizing the entire sample into one heterogeneous powder mixture, we must accept the fact that there are many different cell types within that population of cells from which we extract the genetic material. We plan to address this issue in the future by using the following method. We plan to take 4 disease samples that clearly showed LOH at 3p, fix them in formalin and then in paraffin blocks. We will have these blocks cut into slides and stained for trichrome, factor X, and smooth muscle actin. This will allow us to identify the types of cells in each region of the sample. We will use laser-capture microscopy to pick up each particular cell type and extract DNA from that cell type alone. Finally, we will test each cellular compartment DNA for LOH at the original markers in 3p. This will tell us specifically which cell types have the genetic make-up that we are proposing.
Our studies provide the foundation for future experiments which will reflect our understanding of the genomic basis of atherosclerosis suggested by our investigation. In addition to the above next steps, we have additional future plans because this project is a work in progress. Primarily, we plan to examine specific genes chosen from the integration of our three array platform experiments in more detail. We will then determine if any germline or somatic mutations may be a causative factor in this atherosclerotic plaque. If such mutations exist, this is a direct target for familial screening, diagnostics, and treatment. Even in the case of mutation-negative patients, the target genes such as \textit{CDH13} could also be used the focus of drug discovery and development for atherosclerosis.

In conclusion, we have explored the genetics of atherosclerotic plaques using three array-based platforms. Using the data from these platforms, we have concluded that there are four region of genomic instability seen in these carotid plaques, 1q, 3p, 15q, and 16q. Because of the genes found in each of these regions, and the roles of these regions in numerous cancers, we can conclude that atherosclerosis is a proliferative and inflammatory disease that is reminiscent of carcinogenesis.
Hypertension is a symptom of many complex diseases, including cancer and cardiovascular disease. These complex diseases are not only connected through hypertension, but also through the key processes of proliferation and inflammation. These processes are the core of the existence of cancer and cardiovascular disease. In this thesis, we have examined the hypothesis that cancer-related genes have a significant role in both of these causes of hypertension, neoplasms and vascular disease. In order to address this hypothesis, we studied a neoplastic cause of hypertension, the neuroendocrine neoplasias PC and PGL, and a cardiovascular cause of hypertension, atherosclerosis of the carotid arteries. We examined genetic alterations in these diseases using a clinical cancer genetics and global genomics approach.

In the preceding chapters, we covered data which further elucidated genetic causes of apparently sporadic pheochromocytoma. This tumor is indeed no longer the ‘10% tumor’. We analyzed the Freiburg-Warsaw-Columbus population-based registry (now called the European-American Pheochromocytoma Registry) for mutations in the PC-associated genes and have shown in chapter 2 the results indicating that 25% of apparently sporadic PC/PGL presentations are hereditary and that molecular genetic
analysis is a more accurate and appropriate method of screening for these tumors. This is significantly higher than the previously thought 10%. Our observations suggest that all presentations of PC/PGL irregardless of syndromic feature association or family history should be offered germline genetic testing. Further, our data also point to an association of *SDHB* with malignant disease and intra-abdominal, extra-adrenal occurrences, while *SDHD*-associated PGL favours head and neck sites. Thus our data have changed the practice of clinical cancer genetics.

We subsequently wanted to determine if there still remained heritable causes of the remaining 75% of apparently sporadic PC/PGL cases. In order to genetically examine these patient samples, we looked for evidence of hereditary disease, eg, a family history (chapter 3). When found, we performed deletion testing on these patients in the PC-associated genes. By using both quantitative real-time PCR and semi-quantitative duplex PCR, we found 1 family in this registry that harbored a whole-gene deletion of *SDHD*. In addition, we found 2 families and 1 unrelated individual proband harboring a deletion of *SDHB* exon 1.

The discovery of germline deletions in the *SDH* genes has significant impact on clinical cancer genetics. To date, most hereditary cancer syndromes and disease are identified and diagnosed by a simple test for intra-exonic germline mutations. This test will also detect any variants within the ends of the introns, or splice-site mutations. However, any PCR-based mutation detection approach will not detect a large deletion or rearrangement. Oftentimes, such a deletion will be mistaken for a normal sequence on both alleles. Only recently, many research and a few clinical labs have also begun testing
for large deletions in their screening of a particular disease-related gene
(www.genome.utah.edu/DMD/clinical_test.shtml, genes.uchicago.edu/clinic/CustomTest.html).

Deletion tests are currently being used for $BRCA1$ or $BRCA2$ mutations in hereditary breast and ovarian cancer syndromes, as well as for $PTEN$ in Cowden syndrome and Bannayan-Riley Ruvalcaba syndrome (BRRS) (105). Our data indicate that all patients that are mutation-negative for germline exonic and splice-site mutations in the PC/PGL-associated genes should be offered testing for a deletion or rearrangement in the disease-associated gene.

In chapter 4, we discuss finding the first extra-paraganglial component of $SDHB$-associated heritable PGL. Early-onset renal cell carcinoma was seen in two unrelated registrants who harbored different $SDHB$ germline mutations. This was the first study in which such clinical and genetic data were incorporated using a population-based registry. This indication of the role of a mitochondrial enzyme causing RCC is plausible considering the high number of mitochondria within RCCs. In addition, RCC is a major component of VHL disease, in which the $VHL$ gene plays a significant role. $VHL$ is involved in the hypoxia-inducible pathway as are the $SDH$ genes (18). This is an important observation for cancer genetic counseling. Before our study, we managed patients by surveillance of only PC/PGL. Our data suggest that those with $SDHB$ mutations should be screened for early onset RCC as well.

Carney triad/dyad was previously thought to be a unique and coincidental combination of seemingly unrelated tumors. However, we show novel findings which describe three cases of Carney dyad as hereditary. Three (6%) unrelated probands
harbored unique germline mutations in SDHB and SDHC. Even more striking, is that all three mutations are splice-site mutations. Because mutations in SDHB are associated with malignancy and metastases (106), the Carney dyad patients harboring SDHB mutations should be meticulously screened in order to prevent metastatic disease.

In our final study designed to establish the role of genetics in apparently sporadic PC cases, we examined 104 patient samples from the PC/PGL registry for a number of SNPs in the RET proto-oncogene. We found that a specific combination of the SNP loci in intron 1, haplotype 0, is significantly associated with the apparently sporadic PC cases compared to controls. In addition, we believe that this haplotype is in linkage disequilibrium with a novel, undiscovered founder locus that we propose is in the 5’ portion of the gene. Given our data, this founder locus could account for 1/3-1/2 of our apparently sporadic PC in a low penetrance fashion. Clinically, this would allow for physicians to give patients harboring these particular haplotypes a low risk for developing a tumor and screen them appropriately.

A few patterns arise when we incorporate all of the data we have obtained in the past few years together. First, SDHB- and SDHD- mutation-positive individuals exhibit specific phenotype-genotype correlations. PC patients that harbor SDHB mutations are more likely to have aggressive, malignant disease with metastases. In addition, these patients often present with extraparaganglial neoplasias, including RCC and thyroid carcinomas (106) On the other hand, SDHD mutation carriers are more likely to have multifocal PGL and tumors located in the head and neck region.
Our final chapter examines the genetics of the cardiovascular causes of hypertension. We examine the role of cancer-related genes in atherosclerosis of the carotid arteries. Our results were striking- we found that by integrating the three platforms of array-based genome analysis, there were only four regions of genomic variation. These genomic regions included 1q, 3p, 15q, 16q. In each of these chromosomal regions, there are specific genes and trends of gene functions that corroborate our suggestion that cancer-related genes play a significant role in the development of this complex disease. By further examining the genes in these regions, we hope to elucidate precisely what genes could play a role in the development of atherosclerosis.

Although we were unable to correlate our genetic findings with clinical characteristics of these patients because of IRB-related (not scientific) issues, our data suggest that we had two discrete groups within our population of carotid artery disease patient samples. It is possible that these groups represent any number of characteristics, such as treatment, type of plaque, and whether the plaque was or would become symptomatic. It has been shown that individuals with symptomatic compared to non-symptomatic plaques portray extremely different expression patterns. In addition, symptomatic plaques are molecularly and biochemically more active than the asymptomatic plaques, or active plaque growth precipitates stroke symptoms (107).

We have shown that the genetic of neoplastic and cardiovascular disease causes of hypertension involve genomic alterations in cancer-related genes and chromosomal regions. Our persistence in addressing the genetics of PC and PGL has led us to the
conclusion that medical geneticists must be aware of many genes that are responsible for syndrome-associated lesions when they are in a “sporadic” setting. The genotype-phenotype correlations that we have shown will shed light on the pathogenesis and tissue specificity of these mutations. Our data will aid in the counseling of patients with either PC or PGL, whether it is presented alone or in a syndromic context. Our goal is that our studies will allow for the development of targeted molecular-based treatments for patients with PC/PGL. We also provide the groundwork for future experiments that will allow for novel molecular-genetic based therapies to become available for atherosclerosis. Given the genetic link between neoplasm and vascular disease that our data describes, the treatments for both of these causes of hypertension could molecularly overlap.


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