GENOTYPE-PHENOTYPE CORRELATIONS AMONG TWO LARGE WESTERN PENNSYLVANIA VON HIPPEL-LINDAU DISEASE (VHL) TYPE 2A KINDREDS WITH HIGH INCIDENCE OF PHEOCHROMOCYTOMA AND DIFFERENT MISSENSE MUTATIONS IN THE *VHL* GENE

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Von Hippel-Lindau disease (VHL) type 2A is a rare inherited tumor predisposition syndrome, which is primarily associated with benign tumors of the blood vessels of the central nervous system and eyes and benign tumors of the adrenal gland, though renal cell carcinoma (RCC) can also be part of this tumor spectrum**.** Two large regional VHL type 2A kindreds have been assessed over decades at the University of Pittsburgh. Both kindreds have markedly high rates of adrenal and extra-adrenal pheochromocytoma, and almost no cases of RCC. By mutational analysis, each kindred has a separate disease-causing missense mutation of the *VHL* gene: Y112H (334 T to C) in Family 1, and Y98H (292 T to C) in Family 2. Both gene changes arise from a tyrosine to histidine substitution in exon 1 of the *VHL* gene on chromosome 3p25. Phenotypic expression in the family with Y112H (Family 1) has been described in the past, however the phenotype related to Y98H (Family 2) has not heretofore been described. Although these mutations are similar and occur within the same region of the gene, I hypothesize that there are differences in disease expression between the families, particularly related to pheochromocytoma. The aim of this study is to evaluate the phenotypic expression of VHL in these genotypically different VHL type 2A kindreds.

Public Health Significance: Although VHL disease is clinically rare, the treasure trove of information provided by large VHL type 2A kindreds can help to clarify phenotype, penetrance, and survival patterns as well as further define surveillance algorithms.

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PREFACE

I would first and foremost like to acknowledge all the participants in this study for their cooperation and willingness to share their stories. Their personal perspectives on the disease have enriched this project and allowed it to be so much more important to me than just data collection. Secondly, I would like to thank my mentor Dr. Sally Carty for her guidance and support; I have learned so much from her in these two short years that will serve me well in both my professional and personal life. Darcy Thull has been invaluable to me during this process with her expansive genetics knowledge and effective teaching. Michaele Armstrong has also been a huge help to me during this project; her technological prowess and HIPAA knowledge have been invaluable. I would also like to thank Dr. Wendy Rubinstein, Dr. John Mulvihill and the late Dr. Samuel Tisherman for making this research possible and taking the time to learn more about both the disease and the family they were studying. Also, thank you to Dr. Sean Davis for his work on the Microscoft Access Database that was provided to us. Dr. Gnarra's insight into the molecular aspects of VHL has been very helpful, and his ties to the VHL Family Alliance have provided a network to share our research. Dr. Linwah Yip has also been helpful in graciously sharing her knowledge and experience. Dr. Sue Challinor, as co-Director of the Endocrine Genetics Clinic, has allowed these patients to receive the best care possible, and a forum to teach residents and fellows more about VHL. I would like to thank the Endocrine

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1.0 INTRODUCTION

Von Hippel-Lindau disease (VHL) is an autosomal dominant endocrine tumor syndrome characterized by benign and malignant tumors of various organ systems including the brain, eyes, spine, adrenal glands, para-aortic sympathetic chain, pancreas and kidney. The U.S. incidence of VHL is approximately 1 in 32,000 persons [VHL Family Alliance]. Clinical heterogeneity is a hallmark of VHL, and four phenotypes have been described based on the relative incidences of pheochromocytoma and renal cell carcinoma (RCC) (Table 1) [Chen et al, 1995; Neumann and Wiestler, 1991; Zbar et al 1996].

Pheochromocytomas are rare tumors of neural crest origin that secrete one or several catecholamines (epinephrine [adrenaline], norepinephrine, and/or dopamine), which are the hormones of the sympathetic fight-or-flight response. Pheochromocytomas have variable malignant potential (5-30%). The prevalence of pheochromocytoma in Western countries is between 1 in 6,500 to 1 in 2,500, with an annual incidence in the U.S. of 500 to 1,100 cases [Pacak et al., 2002]. Signs or symptoms include intermittent or sustained hypertension and/or "spells" of palpitations, tachycardia, headaches, episodic sweating, pallor and nausea [Lonser et al, 2003], but as many as a third of pheochromocytomas are asymptomatic at presentation. If left untreated, pheochromocytoma is fatal, primarily due to complications of hypertension that include heart attack, heart failure, stroke, kidney failure and cognitive decline [Tisherman et al, 1993]. Pheochromocytoma is a unique tumor type in that its manifestations and risks are not the

classic complications of solid tumor mass, local spread, and distant metastasis, but are much more often the complications of its' hormonal secretion.

VHL type 1 carries a low risk of pheochromocytoma (<5%), while VHL type 2 is characterized by a high rate of pheochromocytoma (>60%) [Chen et al, 1995; Zbar et al, 1996]. By 1995, approximately 77% of the families described had VHL type 1 [Chen et al, 1995]. VHL type 2 is subdivided into three categories: type 2A is characterized by pheochromocytoma, retinal angioma and hemangioblastoma, but rarely RCC; type 2B carries a high rate of RCC (up to 70%) plus pheochromocytoma, hemangioblastoma and pancreatic cysts; and type 2C carries the risk of pheochromocytoma alone [Woodward et al., 1997; Zbar et al., 1996].

Pheochromocytoma can also be sporadic, or can be a component of several other genetic conditions such as multiple endocrine neoplasia type 2A (MEN2A) (pheochromocytoma penetrance is ~50%), multiple endocrine neoplasia type 2B (MEN2B) (penetrance ~40%), or neurofibromatosis type 1 (NF1) (penetrance 0.1%-5.7%) [Neumann et al., 1993]. Recently a new form of inherited pheochromocytoma has been reported in patients with nonsyndromic familial pheochromocytoma or paraganglioma. These patients have been found to have germline mutations in the succinate dehydrogenase (SDH) genes, including SDHB, SDHC and SDHD. The majority (~97%) of mutations identified are in the SDHD gene, which is imprinted and associated with paraganglioma or pheochromocytoma susceptibility only after paternal transmission [Baysal et al., 2002, reviewed in Favier et al., 2005].

Members of 2 VHL type 2A families have been followed for decades through the University of Pittsburgh. Family 1 has over 1000 members, and has been described several times in the literature. Branches of Family 2 have been assessed briefly in the literature as part of larger studies that sought to localize the *VHL* gene, establish genotype-phenotype correlations,

and/or provide evidence of a founder effect [Brauch et al., 1995, Chen et al., 1995; Glenn et al., 1991, Hosoe et al., 1990], but Family 2 has never been described independently in the literature. The majority of both families' members reside in Western Pennsylvania, giving us a unique opportunity to study them over the years. Although both families have markedly high incidences of pheochromocytoma, and at least anecdotally may have no cases of RCC (see Results), these 2 families have different VHL-causing mutations of the same region of the *VHL* gene, Y112H in Family 1 and Y98H in Family 2. I hypothesized that the genetic differences of these families may correlate with differences in phenotypic expression, and to analyze this I systematically examined the clinical and pathologic characteristics of affected patients with documented pheochromocytoma.

1.1 BACKGROUND OF STUDY

1.1.1 Family 1

Family 1 is a famous Pittsburgh-area regional VHL type 2A kindred that was described almost five decades ago to manifest a high rate of pheochromocytoma [Tisherman, Gregg, Danowski, 1962]. This family was initially identified and laboriously tabulated by Dr. Samuel A. Tisherman, a prominent Pittsburgh internist who passed away in 2000. His work began when, as a resident trainee, he identified a 21-year-old man hospitalized for severe hypertension; familial pheochromocytoma was suspected because the family history revealed 4 other family members

with that rare tumor. In Dr. Tisherman's initial report, 7 patients with confirmed pheochromocytoma and 1 patient with suspected pheochromocytoma were identified, and in follow-up, 8 additional relatives were diagnosed with pheochromocytoma.

Working assiduously over decades, and prior to the mapping of the *VHL* gene defect, Dr. Tisherman traced the family's roots back to early 16th century Germany to the town of Leipzig in the east-central state of Saxony (Figure 1). It is believed that Family 1 arrived in America around 1670 and subsequently spread throughout the United States, with over half of the 1200 descendents of the original 11 siblings settling in western Pennsylvania [Tisherman et al., 1993]. In 1993, in a 30-year phenotype characterization, Dr. Tisherman expanded the family tree to chart 7 generations with at least 1195 known relatives; over the years, and often by the means of summer picnics, he personally met with and/or partially evaluated at least 522 Family 1 descendants [Tisherman et al., 1993]. By 1993, Family 1 had 19 known affected individuals, 16 with pheochromocytoma, 4 with retinal angioma and one with CNS hemangioblastoma [Tisherman et al., 1993], for a rate of pheochromocytoma among those clinically affected of 84.2%. Ten additional individuals were determined to have VHL type 2A by virtue of being obligate carriers. Family 1 remains the largest kindred with pheochromocytoma described in the literature to date. The present research further updates and expands this pedigree, including **20** newly described patients with VHL type 2A.

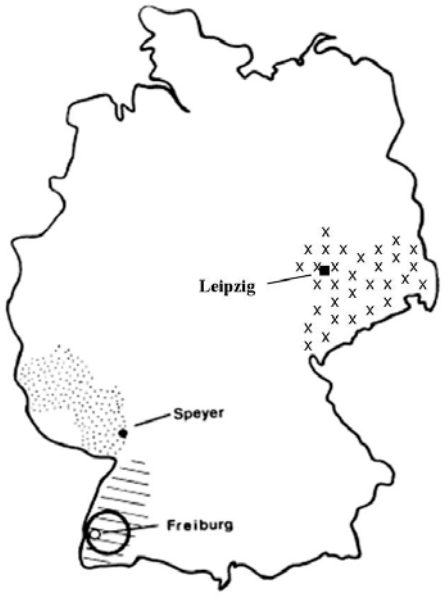

Figure 1. **Map of Germany with Regions of Origin of VHL Type 2A Families.**

The Pfalz region is *spotted* and the Black Forest is *hatched*. The *open circle* indicates the area of origin of 14 Black Forest families. The *filled circle* indicates the place of origin of the founder member of the Family 2 prior to emigration. The filled square indicates the place of origin of the ancestors of Family 1. The surrounding area marked by *x's* represents the state of Saxony. Adapted from [Brauch et al., 1995].

Dr. Tisherman's work was influential in establishing routine clinical care for patients with VHL type 2A and in clarifying the genetics of the disease. In studying multiple generations of the same family, he reported that compared to historical controls, education and surveillance could decrease mortality from pheochromocytoma [Tisherman et al., 1993]. The extensive DNA bank established by his work aided in its localization to chromosome 3p25-26 by linkage analysis [Hosoe et al., 1990] and in the isolation of the *VHL* gene by positional cloning [Latif et al., 1993]. Subsequently, a germline mutation at what was previously nucleotide 547 (now nucleotide 334) was identified in 31 Family 1 members with a diagnosis of pheochromocytoma, retinal angioma, or both [Mulvihill et al., 1997].

1.1.2 Family 2

Family 2 members with VHL type 2A have been evaluated at the University of Pittsburgh for about 2 decades. The proband was a 9 year-old boy who was found to have multifocal and bilateral pheochromocytoma as part of a work up for hypertension, which is itself unusual in children. Shortly after his pheochromocytoma surgery, his older brother was diagnosed with bilateral pheochromocytoma. Their sister was the first patient for Dr. Carty's practice; at 33 she reported pheochromocytoma symptoms, slipped on ice and suffered a pathologic T8 fracture, and was discovered to have a paraspinal mass that was metastatic pheochromocytoma. She also was found to have 13 siblings; of these, 12 carry the *VHL* gene and all 12 have since been diagnosed with pheochromocytoma. Her father and other extended family members also came to attention after their children's diagnoses.

VHL type 2A in Family 2 is likely to have originated 4 generations back, but information is only available for 3 generations back to the proband's paternal great-grandfather, who died at the age of 89 with heart failure after reported loss of sight in one eye at age 30. Some members of the Family 2 have received genetic testing and evaluation through the National Institutes of Health (NIH), and a germline mutation in the *VHL* gene at what was previously nucleotide 505 (now nucleotide 292) was identified [Brauch et al., 1995; Chen et al., 1995].

1.2 GENETICS OF VHL

VHL is an autosomal dominant disease that results from a germline mutation in the *VHL* gene located on chromosome 3p25-25 [Latif et al., 1993; Linehan, Lerman, Zbar, 1995]. The

resulting tumors can be benign or malignant, and present in endocrine glands as well as non-endocrine organs. Overall penetrance is over 90% by 65 years of age [Maher et al., 1991]. The initial manifestations can occur in childhood, adolescence or later, with an overall mean age of presentation of about 26 years [Maher et al., 1990]. Approximately 80% of individuals with VHL have an affected parent and about 20% have VHL as the result of a *de novo* gene mutation Although parental mosaicism has been described, the incidence is not known [Schimke, Collins, Stolle, 2000].

The diagnosis of VHL is established in one of 3 ways. First, in a patient with no family history of VHL, the condition is diagnosed by the presence of 2 or more CNS hemangioblastomas (including retinal angioma), or one CNS hemangioblastoma and a visceral tumor (pancreatic cysts or tumors, RCC, or pheochromocytoma). However, epididymal and renal cysts are not considered in the diagnosis since they are frequent in the general population [Melmon and Rosen, 1964; Neumann, 1987; Hosoe et al., 1990]. Second, when there is a positive family history of VHL, the diagnosis is made in a first-degree relative with a CNS hemangioblastoma (including retinal angiomas), pheochromocytoma, or clear cell renal carcinoma (diagnosed before the age of 60) [Melmon and Rosen, 1964; Neumann, 1987; Hosoe et al., 1990]. Third, molecular genetic testing is now the gold standard for establishing a diagnosis of VHL since germline *VHL* mutations have been identified in virtually all VHL families. As such, locus heterogeneity is not known to exist in VHL although allelic heterogeneity is a hallmark of the disease and is discussed further below.

The *VHL* gene consists of 3 exons that encode the *VHL* protein, including 712 nucleotides and 70 base pairs of 5' untranslated sequence in exon 1 [Maher and Kaelin, 1997]. Two mRNA isoforms differ based on the presence or absence of exon 2 [Gnarra et al., 1994].

The normal *VHL* gene product (pVHL) has many cellular roles including transcriptional regulation, post-transcriptional gene expression, protein folding, extracellular matrix formation, microtubule stability, and ubiquinylation (Hergovich et al., 2003; Kaelin, 2007; Roberts and Ohh, 2008]. Among the best characterized defects in cells with inactive pVHL is the inability to regulate the function of hypoxia-inducible factor (HIF) leading to increased activation of genes involved in angiogenesis and resulting in RCC and the other highly vascularized tumors [Kaelin, 2002].

VHL is a tumor suppressor gene. Thus tumorigenesis occurs in accordance with Knudson's two-hit hypothesis requiring both *VHL* alleles to be inactivated for tumors to form [Knudson and Strong, 1972; Tory et al., 1989]. This is evidenced by examination of a variety of tumor types from patients with VHL that consistently show loss of the *VHL* wildtype allele, as well as the identification of somatic mutations of the *VHL* gene in patients with sporadic clear cell RCC and CNS hemangioblastomas [reviewed in Maher and Kaelin, 1997]. Over 500 germline mutations have been identified in families with VHL [Beroud et al., 1998]. In a heterogenous group of type 1 and type 2 VHL patients, these mutations include partial and complete gene deletions (15%-20% of patients) as well as missense (27%), frameshift or nonsense (27%), and splice site mutations [Beroud et al., 1998; Crossey et al., 1994; Maher and Kaelin, 1997]. Later studies using improved diagnostic techniques have allowed detection of mutations in nearly 100% of patients with confirmed VHL [Stolle et al., 1998]. The majority of mutations are private with only a handful of mutations found in 4 or more families. However, codon 167 appears to be a mutation "hot spot," conveying a high risk for both pheochromocytoma and RCC in Japanese, French, UK, and American families alike [Beroud et al., 1998; Zbar et al., 1996].

1.2.1 Genotype-Phenotype Correlations in VHL

Several VHL genotype-phenotype correlations have been described and are particularly useful in prognostic counseling regarding the risk of pheochromocytoma. The clear correlation of genotype with phenotype has already allowed for classification of 4 clinical subtypes of VHL based on risk of pheochromocytoma (above), including type 1 (RCC, pancreatic neoplasms and cysts, CNS hemangioblastoma, retinal angioma), type 2A (pheochromocytoma, CNS hemangioblastoma, retinal angioma), type 2B (pheochromocytoma, CNS hemangioblastoma, retinal angioma, RCC), and type 2C (pheochromocytoma only). In VHL type 1, about 60% of germline mutations in patients *without* pheochromocytoma are known to be microdeletions/insertions, nonsense mutations, or large deletions, whereas in VHL type 2 patients *with* pheochromocytoma, 96% of germline mutations are known to be missense, with 43% of these being at codon 238 [Chen et al., 1995] (Table 1).

Table 1. **VHL Subtypes and Genotype-Phenotype Correlations**

VHL subtype	VHL mutation	HB	RCC	Pheo
1	Deletion, Insertion, Nonsense, Missense	High risk	High risk	Low risk
2A	Missense	High risk	Low risk	High risk
2B	Missense	High risk	High risk	High risk
2C	Missense	Low risk	Low risk	High risk
HB, hemangioblastoma; RCC, renal cell carcinoma; Pheo, pheochromocytoma				

Adapted from [Bryant et al. 2003]

Before the precise location of the *VHL* locus on chromosome 3 was identified, Family 2 members were a part of a 1991 report from NIH, which first observed that different mutant alleles at the same *VHL* locus were associated with "distinct tissue specificities." Family 2 and several other smaller families were characterized by a high frequency of pheochromocytoma and a low frequency or absence of RCC and pancreatic cysts, thus distinguishing a separate VHL phenotype that was confirmed by subsequent studies and later classified as VHL type 2A. The mechanism of disease suggested by the authors was that "mutant alleles at the *VHL* locus produce different defective proteins; one mutant allele at the *VHL* locus leads to uncontrolled growth of the renal tubular epithelium while another mutant allele at this locus leads to uncontrolled growth of the cells of the adrenal medulla" [Glenn et al., 1991]. Some mutant alleles, such as a C to T change at nucleotide 712, are thought to cause uncontrolled growth at *both* tissue sites, as evidenced by VHL type 2B families with this mutation who have high frequencies of both RCC and pheochromocytoma [Green et al., 1986]. In fact, their risk of developing RCC is 32%, which is comparable to VHL type 1 [Maher et al., 1990].

The major implication from data demonstrating that missense mutations of the *VHL* gene are responsible for the pheochromocytoma-specific phenotype of the disease is that the VHL mutant protein must remain full length and be partially functional in order to produce pheochromocytoma [Chen et al., 1995]. Accordingly, mutations that result in truncation or deletion of a portion of the VHL protein do not result in pheochromocytoma, with the exception of deletions of the 3' untranslated region of the *VHL* gene that may lead to pheochromocytoma, albeit rarely [Chen et al., 1995]. A recent study by Ong et al. [2007] looked more closely at VHL-causing missense mutations, subdividing them based upon where in the protein structure the amino acid substitution was expected to occur. Two groups of missense mutations were

delineated; those that result in substitution of a surface amino acid (which includes Y98H and Y112H) and those that interfere with amino acids deeper in the core of the protein and thus disrupt structural integrity [Ong et al., 2007]. The objective of the study was to investigate a previous claim that *VHL* missense mutations associated with pheochromocytoma were more likely to involve surface residues [Stebbins et al., 1999]. Indeed, they confirmed the association between surface amino acid substitutions and pheochromocytoma predisposition, which will allow for further clarification of pheochromocyoma risk [Ong et al., 2007]. However, they found that the lifetime risks of retinal angioma, hemangioblastoma, and RCC were not significantly different between the two types of mutations [Ong et al., 2007]. Conversely, it was discovered that age at first manifestation of VHL disease was significantly earlier, and age-related risks of retinal angioma and RCC were higher, in individuals with a truncating mutation (nonsense or frameshift) compared to those with deletions or missense mutations that disrupted the structural integrity of the *VHL* gene [Ong et al., 2007].

The molecular mechanisms underlying pheochromocytoma susceptibility are not yet fully understood. As mentioned above, the best characterized role of the *VHL* gene product (pVHL) is regulating proteolytic degradation of the α subunits of the hypoxia-inducible factor-1 (HIF-1) and hypoxia- inducible factor-2 (HIF-2) transcription factors [Cockman et al., 2000; Maxwell et al., 1999]. In turn, these transcription factors are active in managing cellular responses to low oxygen and regulating transcription of a broad range of target genes implicated in angiogenesis, proliferation, and metabolism [Semenza, 2003]. pVHL inactivation stabilizes HIF-1α and HIF-2α resulting in activation of target genes that are upregulated in the highly vascularized tumors that are a trait of VHL disease. pVHL binds the HIF α subunits at a β-domain surface binding site and the elongin C protein at a second α-domain surface binding site [Kim and Kaelin, 2003;

Ong et al., 2007]. Type 1 and type 2B mutations impair pVHL binding to elongin C, which results in decreased pVHL stability and rapid degradation. On the other hand, type 2A mutations map to the HIF-binding site β-domain and do not affect the ability of pVHL to bind to elongin C [Clifford et al., 2001]. Although type 2A and type 2B surface missense mutations impair the ability of pVHL to regulate the HIF-1 and HIF-2 transcription factors, other *VHL* mutations associated with pheochromocytoma susceptibility retain the ability to regulate HIF, suggesting that HIF dysregulation is not solely responsible for pheochromocytoma development in VHL disease [Clifford et al., 2001; Hoffman et al., 2001]. Supporting this notion are studies reporting that VHL-pheochromocytoma linked mutations are associated with increased JunB expression secondary to altered protein kinase C signaling, resulting in failure of apoptosis in adrenal medullary progenitor cells in VHL disease and other causes of familial pheochromocytoma [Lee et al., 2005]. In addition, the VHL type 2A mutations Y98H and Y112H show only modest impairment in HIFα regulation but do not regulate microtubule stability, which is predicted to result in chromosome instability [Hergovich et al., 2003; Thoma et al., 2009]. In summary, dysregulation of HIF seems to play a role in the development of RCC and pheochromocytoma. However, since some *VHL* mutations that also are associated with pheochromocytoma do not interfere with HIF, there must be other mechanism(s) involved in tumor development.

These genotype-phenotype correlations have been crucial in understanding the genetic mechanisms of VHL disease and valuable in presymptomatic diagnosis. Still, penetrance and expressivity of VHL cannot be predicted solely by VHL genotype. The present study seeks to evaluate phenotypic expression of mutations within the same region of the *VHL* gene that have been identified in 2 VHL type 2A families.

1.2.2 Founder Effects and Phenotype in Families 1 and 2

Both the Y112H and Y98H mutations in Family 1 and Family 2, respectively, result in the replacement of tyrosine with histidine in exon 1 of the *VHL* gene. This nucleotide substitution leads to the loss of an *AluI* endonuclease restriction site [Mulvihill et al., 1997]. While other smaller families with pheochromocytoma have been found to have other mutations, such as G463T and C712T, the large multigenerational families studied with Y98H and Y112H mutations were the main contributing factor in the discovery of the pheochromocytoma-specific region of the *VHL* gene [Crossey et al., 1995; Mulvihill et al., 1997].

Although these 2 mutations are located within the same exon of the *VHL* gene, it is not unreasonable to believe that they may result in different phenotypic expression of the disease. Indeed, Bradley et al. (1999) demonstrated that two different missense mutations in the same codon of the *VHL* gene can cause very distinct clinical phenotypes. His group investigated a large family with a previously unreported T547A mutation in exon 1 of the *VHL* gene resulting in a Tyr112Asn missense mutation (Y112N) in the protein. The family was characterized by a high incidence of RCC and a low incidence of pheochromocytoma; specifically, out of 100 at-risk family members, there were 13 affected individuals of whom 7 had RCC; other VHL manifestations included renal cysts, retinal angioma, and CNS hemangioblastoma [Bradley et al., 1999]. Of note, there was only one case of pheochromocytoma diagnosed [Bradley et al., 1999]. Indeed, the Y98N mutation has been associated with RCC [Gnarra et al., 1994]. This is in contrast to the Y112H mutation described below which causes a Tyr to His missense mutation and is associated with VHL type 2A i.e. pheochromocytoma, but not RCC. While the Y98H and Y112H mutations also result in the replacement of tyrosine with histadine in exon 1, it will still be interesting to see if there are any phenotypic differences in these 2 families.

The Y98H (Family 2) mutation is well documented in the literature (typically referred to as the T505C mutation) and has been established through haplotype analysis as a founder mutation originating from the Black Forest region of Germany (i.e. from a different region of Germany than Family 1) [Brauch et al., 1995]. Areas of early Pennsylvania were settled to a large extent by German immigrants. The Y98H mutation has been identified in at least 16 VHL type 2A families, 2 of which live in Pennsylvania, including Family 2, and all of whom can trace their family roots to Germany. Fourteen of the Y98H families are directly from the Black Forest region of Germany and it is believed that Family 2 ancestors emigrated in the early 18th century from the Pfalz area to Pennsylvania (Figure 1) [Brauch et al., 1995]. The Pfalz region lies to the north of the Black Forest region (Figure 1). The exact region of Germany from which the other Pennsylvanian family emigrated from is unknown. Brauch et al. [1995] proposed that the Y98H mutation "originated and spread in the isolated mountain valleys of the Black Forest. Due to its economic and political status in the 1700s, the Pfalz attracted migratory workers and mercenaries." [Brauch et al.,1995]. They predicted that the mutation originated sometime before 1700, and was exported to the United States by such Pfalz Family 2 ancestors who immigrated from Germany in the 1800s [Brauch et al., 1995]. The data from this study provided crucial evidence that "among the 75 different mutations identified by Crossey et al. [1994] and Chen et al. [1995] the Y98H mutation is the most tissue-specific *VHL* mutation with nearly complete penetrance by the fourth decade of life" [Brauch et al., 1995]. However, Bender et al. [2001] demonstrated that despite high age-related penetrance, the mortality and survival rate of those with the Y98H mutation is comparable to those of the general population of Germany. As 45% of German VHL families carry the Y98H mutation, its identification has allowed for

presymptomatic genetic diagnosis and genetic counseling in this fraction of the VHL population [Brauch et al., 1995].

The Y112H mutation (Family 1) was discovered a short while after the Y98H mutation, by Zbar et al. [1996] in a study examining germline mutations in 469 VHL families from North America, Europe and Japan. Further evidence for the association of the Y112H mutation to the VHL type 2A phenotype was provided by Chen et al. [1996], when affected members of Family 1 were tested for a germline mutation of the *VHL* gene. Mutation analysis of Family 1 revealed the Y112H mutation, which had previously been identified in a much smaller family that only contained three affected individuals, all of whom had pheochromocytoma and none of whom had RCC [Chen et al., 1996]. These small numbers were insufficient to allow classification of the mutation to a specific subtype of VHL type 2. However, the identification of the mutation in Family K, along with another 22 affected patients (19 with pheochromocytoma, 0 with RCC) allowed the Y112H mutation to be clearly assigned to VHL type 2A [Chen et al., 1996].

It is interesting to note that although Family 1 can also trace their roots back to Germany, their ancestral area is far removed (approximately 200 miles) from the Black Forest region. Family 1 is believed to originate from Leipzig, in the east-central region of the country, in contrast to the southwest corner of the country where the Black Forest is located. Family 1's ancestors migrated to America in 1670 (compared to Family 2 migration in the early 1700s) and although members of the clan are scattered throughout the United States, more than 600 descendents settled in Western Pennsylvania [Tisherman et al., 1993]. The origins of the other families identified with Y112H mutation are unknown, and therefore a founder effect for this mutation cannot be established at the present time.

Although the primary risk of VHL type 2A is pheochromocytoma, the presence of other VHL-associated tumors cannot be ignored. Over a decade ago, Family 1 was known to have 4 members with retinal angioma and one member with CNS hemangioblastoma, while a larger grouping of families (NCI family 3127) that includes Family 2 had 22 members with retinal angioma and 4 members with CNS hemangioblastoma [Chen et al., 1995; Chen et al., 1996]. A later study by Bender et al. [2001] compared the frequencies of tumors in Y98H mutation carriers from Germany and the United States, which included Family 2 as well as another Pennsylvanian family. While the families clearly share common ancestors, there was marked variation in tumor frequencies. The frequency of pheochromocytoma was similar between German and American families (47% and 55%, respectively); however, the frequency of retinal angioma was higher in American families (47% vs. 36%) while CNS hemangioblastoma occurred more often in German families (41% vs. 9%). Also, while there was a complete absence of RCC in the American families, there were 4 cases in the German families (3%). The authors proposed that these differences may reflect the fact that a gene mutation alone is sufficient to cause pheochromocytoma, while both genetic and environmental factors are more influential in the development of retinal angioma and CNS hemangioblastoma; they could not determine whether the development of RCC in Y98H mutation carriers was a true consequence of the mutation or not, given the small numbers of the study and taking into account the annual incidence of RCC in the general population in Germany [Bender et al., 2001]. These data will be important in comparing VHL type 2A manifestations in 2 families from relatively similar environments, and may give us more insight into gene-environment interactions regarding VHL type 2A.

1.3 MANAGEMENT AND SURVEIILLANCE OF VHL

1.3.1 VHL Surveillance Recommendations

Studies of the natural history of VHL showed a life expectancy of 50 years before surveillance protocols were established, the main causes of death being the complications of RCC and CNS hemangioblastoma [Lamiell, Salazar, Hsia, 1989; Maher et al., 1990; Neumann et al., 1993]. Morbidity and mortality is alleged to have decreased due to syndrome delineation, earlier diagnosis, improved imaging techniques, and improvements in therapy [Lonser et al., 2003]. Surveillance is felt to be critical not only in detecting new lesions at an early stage, but also in monitoring small asymptomatic lesions for progression. Surveillance is focused primarily on hemangioblastoma (including retinal angioma), RCC and pheochromocytoma because they are the three manifestations most often resulting in severe disability or death [Chan-Smutko, Plon, Iliopoulos, 2009]. Regular clinical monitoring by a physician or medical team familiar with the spectrum of VHL disease is required in 3 groups of patients: those with known VHL, those known to have a VHL disease-causing mutation, and those at-risk by inheritance but who have not yet undergone DNA testing [Schimke, Collins, Stolle, 2000]. Screening recommendations are adapted to the individual, taking into account previously diagnosed disease, the VHL disease spectrum, and prior VHL manifestations in other family members [Chan-Smutko, Plon, Iliopoulos, 2009].

Molecular genetic testing is indicated in all individuals with a confirmed or suspected diagnosis of VHL [Rasmussen et al., 2006]. Testing is performed through sequence analysis and/or deletion analysis. Sequence analysis scans all 3 exons for point mutations and small deletions or insertions in the *VHL* gene, which account for approximately 72% of all *VHL*

mutations [Stolle et al., 1998]. Partial or complete gene deletions account for approximately 28% of all *VHL* mutations and can be detected through various methods [Banks et al., 2006; Stolle et al., 1998]. A positive result is confirmed when a mutation is identified in at least one copy of the *VHL* gene. Once a positive result is identified in a family, single-site analysis can be offered to other family members. A negative result means that neither copy of the *VHL* gene is altered; since the margin of error for this test is typically under 1-2%, this is considered a true negative result. However, somatic mosaicism for a *de novo VHL* germline mutation cannot be ruled out in individuals with VHL manifestations who do not meet strict diagnostic criteria and who do not have a detectable *VHL* germline mutation [Schimke, Collins, Stolle, 2000]. Additionally, individuals tested prior to the year 2000 using linkage analysis can consider being re-tested as improved techniques are more reliable and may reveal prior test results as false negatives. A referral for genetic counseling is critical for individuals undergoing testing in order to fully understand the implications of testing and correct interpretation of results.

Interestingly, the literature does not yet distinguish clinical surveillance recommendations by VHL subtype. The only pheochromocytoma-specific recommendation is somewhat out of date, as it antedates plasma metanephrine testing and instead recommends annual blood pressure monitoring supplemented by annual measurement of urinary catecholamine metabolites beginning at age 5 years in families with a high incidence of pheochromocytoma [Schimke, Collins, Stolle, 2000]. Overall surveillance protocols for monitoring high-risk VHL patients have been developed by several groups; what follows are the recommendations of the National Institutes of Health (NIH) group [Choyke et al., 1995] and the VHL Family Alliance. Family members who had a 50% risk of VHL prior to genetic testing, but are found not to have inherited the mutation, do not need further clinical monitoring. To summarize, more extensive screening

and monitoring for pheochromocytoma appears both sensible and necessary for VHL type 2A families.

Table 2. VHL Surveillance Recommendations

Age (y)	Recommendation
Infancy (birth-23 months)	- Annual physical examinations including retinal examination
2-10	- Annual physical and retinal examination - Urinary or plasma catecholamines every 1-2 years - Annual abdominal ultrasound from 8 years or earlier if indicated. Abdominal MRI or MIBG scan only if biochemical abnormalities found - Audiology assessment every 2-3 years, or annually if any hearing loss, tinnitus, or vertigo
11-19	- Retinal examination every 6 months -Annual physical examination and neurological assessment -24-hour urine catecholamine and meta-nephrine collection. Abdominal MRI or MIBG scan only if biochemical abnormalities found - Ultrasound of abdomen. If abnormal, MRI or CT of abdomen, except in pregnancy - Annual MRI with gadolinium of brain and spine - Audiology assessment every 1-2 years
20+	- Annual physical and retinal examination - Annual CT scan of the abdomen - 24-hour urine or plasma catecholamine and metanephrine collection. Abdominal MRI or MIBG scan if biochemical abnormalities found - Annual MRI with gadolinium of brain and spine (except during pregnancy) - Audiology assessment every 2 years - MRI of internal auditory canal (IAC) every 2 years
60	If no evidence of VHL, change MRI of brain and spine to every 3 to 5 years; CT of abdomen every other year

The classic symptom triad of pheochromocytoma is episodic headache, sweating, palpitations and/or tachycardia - symptoms which can be variously attributed to panic attack, allergy, and even (when occurring during sleep) nightmare. Blood pressure monitoring is also important in screening. Although in the general population pheochromocytoma accounts for less than 0.2% of patients with hypertension, in patients with pheochromocytoma, sustained or episodic hypertension can be the first sign of tumor, therefore such severe hypertension should always prompt diagnostic investigation for possible pheochromocytoma. It is essential to keep in mind, however, that the majority of patients with pheochromocytoma have no history of hypertension [Bravo, 1991; Manger and Gifford, 2002; Stein and Black, 1991]. In hereditary pheochromocytoma, tumors present at a younger age, are more often multiple or extraadrenal, are less likely to secrete epinephrine, and are less likely to be associated with symptoms or biochemical evidence of catecholamine production [Eisenhofer et al., 1999; Tisherman et al., 1993; Walther et al., 1999].

Biochemical screening is the standard of care in patients at risk for hereditary pheochromocytoma. Plasma metanephrine testing is currently the most sensitive test and is therefore the test of choice. Although the measurement of 24-hour urine catecholamines and metanephrines is more specific (98%), plasma metanephrines testing is more sensitive because it captures small amounts of metanephrines which are the breakdown products produced continuously by the metabolism of catecholamines, as opposed to catecholamines themselves which are only secreted intermittently during acute hypertensive episodes (Figure 2) [Guller et al., 2006; Lenders et al., 2002]. In addition, plasma metanephrine testing is simple to perform, has few causes of false positive results, and has a high predictive value in excluding pheochromocytoma except in patients with ultra-rare, strictly dopamine-secreting tumors [Sawka

et al., 2004]. As with urine screening, a drawback of plasma metanephrine testing is that it may not be reliable for the detection of microscopic recurrent or metastatic disease or very small tumors (<1cm) [Lenders et al., 2002, Young and Kaplan, 2009].

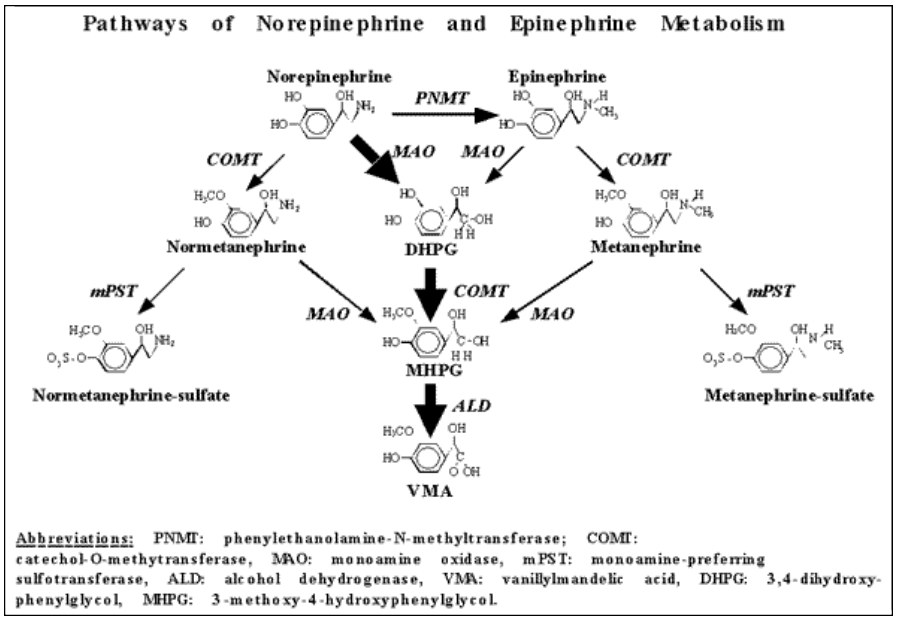

Figure 2. Catecholamine Metabolism Pathway

http://www.endotext.org/adrenal/adrenal34/adrenal34.htm

Chromogranin A and VMA (vanillylmandelic acid) are 2 other biochemical tests used in diagnosis and management of pheochromocytoma. VMA is a metabolic product of catecholamine metabolism (Figure 2) and chromogranin A is an intragranular binding protein. Following synthesis (Figure 2) catecholamines are actively accumulated by the dense-core subcellular chromaffin granules of the adrenal medulla and of pheochromocytoma tissue, by an ATP-dependent process coupled to the magnitude of the electrochemical proton gradient across the chromaffin granule membrane [Johnson, Carty & Scarpa, 1982]. Once accumulated into the chromaffin granule, catecholamines are stored in very concentrated form by binding to the

intragranular protein, chromogranin A. Blood levels of Chromogranin A are increased in 80% of patients with pheochromocytoma [Cotesta et al., 2005] allowing its use as tumor marker. Although Chromogranin A it is not specific for pheochromocytoma, it is routinely measured prior to pheochromocytoma surgery and in surveillance after surgery, to facilitate detection of metachronous disease. Because of its serious risks, which include heart attack and stroke if left untreated, all patients diagnosed to have pheochromocytoma move promptly towards resection. Radiologic tests are routinely used to localize pheochromocytoma after biochemical diagnosis. Noncontrast CT of the abdomen and pelvis is usually performed first because it is cost effective, widely available, and because the differential density of adrenal tissue on the noncontrast views has utility in distinguishing adrenal cortical tumor from other adrenal tumor types (Laursen and Damgaard-Pedersen, 1980; Young and Kaplan, 2009). Although about 10% of pheochromocytomas are extra-adrenal, about 95% of these occur within the abdomen and pelvis [Bravo, 1994]. CT has been the predominant imaging technique since 1977, with a sensitivity of 94% and a specificity of 97% [Goldstein et al., 1999]. MRI has been available since 1985 and is particularly useful in distinguishing pheochromocytoma from other adrenal masses, with a specificity of almost 100%, and a sensitivity of 83% [Goldstein et al., 1999]. Features of malignancy on CT or MR imaging include heterogeneity, central necrosis, and local or vascular invasion; size > 6 cm has also been shown to predict malignancy [Sturgeon and Kebebew, 2004]. In patients with radiologically occult disease, suspected synchronous or metastatic disease, or for whom pheochromocytoma is clinically suspected but catecholamine measurements are in the normal range, 123-I-metaiodobenzylguanidine (MIBG) scintigraphy is then indicated [Guller et al., 2006]; again, 35% of patients with VHL pheochromocytoma have no symptoms, normal blood pressure, and normal catecholamine tests [Walther et al., 1999]. MIBG is a substituted

amine resembling norepinephrine, which is taken up by adrenergic tissue. In patients with pheochromocytoma >10 cm in size, who are thus at greatly increased risk for malignancy, and in patients with extra-adrenal paraganglioma, who are thus at risk for synchronous disease [Clarke et al., 1998] MIBG scanning is recommended to survey the body for metastatic disease or multiple tumors. [Lee and Duh, 2008; Whalen, Althausen, Daniels, 1992].

1.3.2 Management of Pheochromocytoma, Hemangioblastoma, and Retinal Angioma

Two decades ago, medical students were taught that the most common presentation of pheochromocytoma was sudden death at an early age from unexplained causes. Although surgical resection of pheochromocytoma carried significant mortality in the past, it is now the standard treatment for any diagnosed pheochromocytoma. [Lonser et al., 2003; Walther et al., 1999]. Due to the severe risks of untreated pheochromocytoma including heart attack, heart failure, stroke, kidney failure and cognitive decline, surgery is generally considered urgent and with high clinical priority. Before surgery, however, careful pharmacological alpha-adrenergic blockade and intravascular volume repletion is almost always required [Lonser et al., 2003]. About 17% of patients with pheochromocytoma also have an associated cardiomyopathy diagnosed by preoperative echocardiogram, and which resolves with tumor resection. The treatment of pheochromocytoma is resection with intent to cure, which may usually be readily accomplished laparoscopically for radiologically benign-appearing tumors < 6 cm in size. In patients with VHL pheochromocytoma, cortical-sparing adrenalectomy has been shown to result in better long-term corticosteroid independence, [Baghai et al., 2002], thus in VHL the preferred initial surgical approach today is laparoscopic partial adrenalectomy [Walther et al., 2000]. Partial adrenalectomy, however, is associated with increased risk of metachronous ipsilateral

recurrence. Any suspected malignant pheochromocytoma should be entirely resected entirely using open (non-endoscopic) en bloc technique.

Hemangioma of the CNS is the most common tumor in VHL, affecting 60-80% of patients [Neumann et al., 1993; Wanebo et al., 2003]. Although these tumors are benign, their local growth can cause significant morbidity: hemangiomas can arise anywhere along the craniospinal axis and are often associated with edema, cysts, or both. Common locations are the spinal cord and cerebellum, followed by the brainstem, lumbosacral nerve roots, and supratentorial region [Lonser et al., 2003]. Symptoms related to hemangioblastoma of the brain and spinal cord vary depending on tumor location and size, and presence of associated edema or cysts, [Chan-Smutko, Plon, Iliopoulos, 2009]. Most hemangioblastomas of the craniospinal axis can be safely and completely excised [Malis, 2002; Lonser et al., 2003; Weil et al., 2003]. Because CNS hemangioma can grow at several sites simultaneously, new lesions can arise at any time, the growth pattern can be irregular and unpredictable, and the surgery itself can have morbidity, resection is generally deferred until the onset of symptoms [Lonser et al., 2003; Wanebo et al., 2003; Weil et al., 2003]. Small craniospinal hemangioblastomas (<3 cm) that are not associated with a cyst have recently been treated with stereotactic radiosurgery, but the long-term effects of this treatment have not yet been determined [Lonser et al., 2003].

Retinal angioma is a frequent occurrence in VHL, seen in as many as 60% of patients who are screened for it [Dollfus et al., 2002; Webster, Maher, Moore, 1999]. Angiomas arise in the retinal periphery, on or near the optic disc, or both [Lonser et al., 2003]. They are often multifocal and bilateral (about 50%) [Lonser et al., 2003]. Early diagnosis and treatment of retinal angioma, which now can include radiotherapy, can prevent visual loss or blindness, with most peripheral retinal tumors responding to laser photocoagulation or cryotherapy [Lonser et

al., 2003]. Tumors of the optic disc should be monitored without treatment because of the damage that some treatments including photocoagulation can cause [Lonser et al., 2003]. All patients with VHL thus require annual retinal exam, but not all patients are able to comply.

2.0 METHODS

Under a full University of Pittsburgh Institutional Review Board-approved protocol (IRB # 09010261), we retrospectively reviewed the available records of patients known to have VHL in both studied kindreds (Family 1, n=49; Family 2, n=65). We identified all patients diagnosed with VHL pheochromocytoma to date, and obtained extensive family history in patients presenting here for management of inherited pheochromocytoma management. For Family 1, we also received extensive heritage pedigree materials from Wendy S. Rubinstein MD, Samuel E. Tisherman MD, and Marlin A. Field (author of Family 1 genealogy book) [Field, 1993]. From these data we constructed de novo pedigrees for both families. We then used clinical documents, partial heritage pedigrees, and patient interview data to verify pedigree and clinical findings.

2.1 DESIGN

This is a retrospective study to investigate possible differences in the expression of VHL type 2A disease, particularly with regard to pheochromocytoma, between 2 large multigenerational kindreds who reside in Western Pennsylvania with different disease-causing missense mutations in the *VHL* gene. Many members of both families are currently followed by the Endocrine Surgery division at the University of Pittsburgh Medical Center (UPMC). One family (Family 1) was originally identified and laboriously tabulated by a late University of Pittsburgh internist

(S.E.T.). Some members of the other family (Family 2) have previously been partially described by the NIH (NCI family 3127) [Glenn et al., 1991].

2.2 PEDIGREE CONSTRUCTION

Pedigrees were constructed using Progeny Clinical 7.6.03 (Serial # 710390) Genetic Data Management Software (Copyright ©2007 Progeny Software, LLC, South Bend, Indiana).

2.2.1 Family 1

Many sources of data were used in constructing the pedigree for Family 1, but the majority of information was from the extensive but previously uncollated paper records of Dr. Samuel Tisherman Sr., who first described the family [Tisherman, Gregg, Danowski, 1962; Tisherman et al., 1993]. These heritage records contained extensive handwritten notes taken during colorful family reunions organized by Dr. Tisherman, in which he (and his family members) would take the blood pressure of Family 1 members to assess for hypertension. He also documented details of medical and family histories, and distributed research questionnaires regarding VHL and pheochromocytoma. Multiple sketched pedigrees were combined to create three separate branches of the family based on the descendents of the three original siblings thought to be carriers for VHL, namely individuals II-3, II-4, and II-5.

We also received extensive pedigree materials from Dr. Wendy S. Rubinstein, who worked closely with Dr. Tisherman before his death. This included a small "VHL/Pheo Study" Microsoft Access database detailing demographics, medical history, cancer history, testing, surgeries and hospitalizations of selected members of Family 1 (established by Sean Davis, MD, PhD). Additionally, our computer support personnel were able to access and display an outdated Progeny file, previously compiled by Drs. Tisherman and Rubinstein, of a pedigree for the II-5 branch of Family 1. This information was received after completion of our own Progeny database, and therefore was used very effectively to verify our own data. Another source of information used to verify relationships was a genealogy book about Family 1 [Field, 1993] which Dr. Carty purchased from the author. The book did not include any medical information but was very useful in obtaining dates of birth and death and family relationships. Based on these data and on clinical data from our own Family 1 patients, we then laboriously revised and expanded the final Family 1 pedigree (Figure 3).

2.2.2 Family 2

Based on our own clinical data, we constructed de novo a regional pedigree for Family 2. Under a full IRB-approved protocol (# 09010261) and always complying with HIPAA regulations to protect patient privacy, we accessed additional sources of information including pedigrees of family branches that were completed through their genetic counseling consultation at the UPMC Cancer Genetics Program, extensive phone conversations with identified historians of Family 2, and medical records provided to us by kindred members.

2.3 CONTACTING PATIENTS

Contacting selected members of both families was very necessary to this study. Our clinical data did not encompass all members of Families 1 and 2 known to be affected, nor did it include all medical care of affected individuals related to their VHL diagnosis; most individuals live a distance from Pittsburgh and thus receive care at other institutions, or in some cases no care. When contacting patients, a brief description of the study was given, and interested participants were emailed or mailed a medical records release and a copy of the informed consent to sign and return. They were encouraged to forward the email to other family members who might be interested in the study, but the decision to do so was their own. Returned informed consent documents were filed. Signed medical records release forms were faxed to the institutions specified by study participants at which they had received care related to their diagnosis of VHL. Some members of both Family 1 and Family 2 were also evaluated at the UPMC Endocrine Genetics Clinic program, which is co-Directed by Dr. Carty, where medical and family information was again verified.

2.4 PHENOTYPIC DATA

From all the available data, study patients were coded as affected or unaffected with VHL or its manifestations, by use of 3 categories of veracity:

A) Robust or confirmed genetic, medical and/or pathologic documentation i.e. paper records;

B) Status suspected e.g. by oral history (includes information gleaned from Cancer Genetics pedigrees); or

C) Status suspected but no other confirmatory information available.

All family members with known pheochromocytoma (pheo), hemangioblastoma (HB), retinal angioma (RA), or RCC were logged into a database. Based on all category A and B data, detailed pedigree analysis was used to identify obligate carriers as well as to construct age-related probability curves for the major manifestations of VHL type 2A.

Patients with category A data in support of VHL were considered informative and their data were used for the determination of differences between the 2 kindreds in regards to VHL type 2A pheochromocytoma phenotype. We then compared clinical parameters (age of first pheo, development of metachronous tumors, time to new occurrence of pheo, diagnosis by symptoms or screening, norepinephrine/epinephrine secretion status pre-operatively, biochemical cure, demise from disease [DOD]) and pathologic parameters (site, laterality, size, weight, multifocality, recurrence, and metastasis) for each of 100 pheos with category A data (Family 1=55, Family 2= 45) among the 2 kindreds. We also used level B pheo data for analysis of: age of diagnosis at first pheo, diagnosis by symptoms or by screening, development of metachronous tumors, and biochemical cure. This information allowed for evaluation of 30 additional pheos with respect to these 4 categories (Family 1= 10; Family 2 = 20). Mean follow-up was 13.1 y for Family 1 (range 1.75 -38.5 y) and 12.3 y for Family 2 (range 0.1-34 y).

2.5 STATISTICAL ANALYSIS

Fisher's exact test was used to compare categorical data (i.e. presence or absence of pheochromocytoma, hemangioblastoma, retinal angioma, and RCC) and student's t-test was used to compare continuous data (i.e. pheo parameters) between the 2 families. Online statistical calculators were used to determine p values (http://graphpad.com/quickcalcs/ttest1.cfm?Format=SD; http://faculty.vassar.edu/lowry/odds2x2.html). Significance was set at 0.05, and p values were 2-tailed.

3.0 RESULTS

3.1 VHL DIAGNOSIS, DEMOGRAPHICS AND PENETRANCE

Family 1 has 3 branches with VHL type 2A, consisting of 108 individuals over 7 generations; the pedigree is seen in Figure 3. In Family 1, 49/108 members (45.4%) appear to be affected with VHL to date. Additionally, based on category C evidence, another 2 Family 1 individuals (III-2, IV-2) are suspected to have the condition (47.2%).

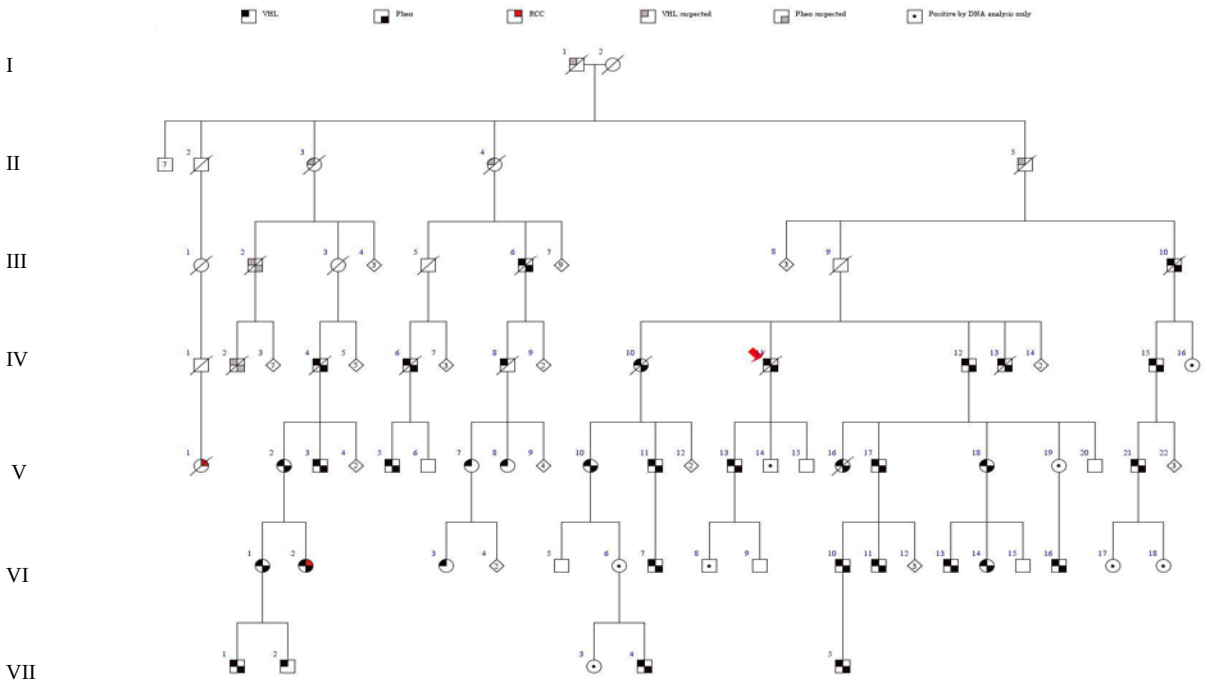

Figure 3. Family 1 Pedigree

Family 2 has 131 individuals over 6 generations as seen in the pedigree of Figure 4. Of these, 65 (49.6%) appear to be affected with VHL type 2 to date (Figure 4). An additional 6/131 Family 2 individuals (II-3, II-4, II-5, III-1, III-2, III-3) are suspected to have VHL based on category C data (54.2%).

Gender distribution of patients with VHL did not appear to differ between families: 29/49 (59.2%) of affected Family 1 members were male, and 35/65 (53.8%) of affected Family 2 members were male. Our data thus confirm an autosomal dominant inheritance pattern of VHL type 2A in both of the families in this study.

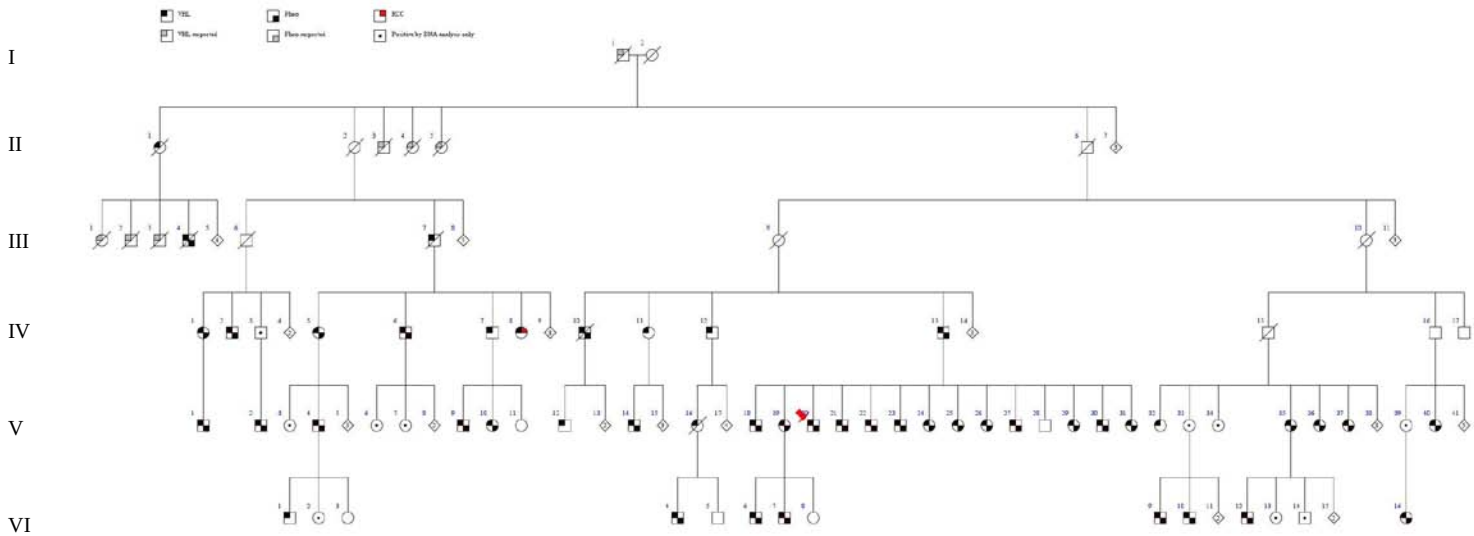

Figure 4. Family 2 Pedigree

VHL was diagnosed as described in Methods (Section 2.4) and this is illustrated schematically in Figure 5. In Family 1, the assignment of VHL diagnosis was based upon clinical and/or genetic data for 35/49 patients (71.4%), was proven by genetic analysis alone in 8/49 clinically unaffected patients (16.7%), and was determined by obligate carrier status in 6/49 kindred members (12.5%). Clinical data were assessed as category A in 31/35 (88.6%) individuals, of whom 25 had confirmatory genetic testing; clinical data was category B in 4/35 (11.4%) patients, of whom 2 reportedly had positive mutational analysis which we could not confirm for this study. Therefore in Family 1, and with the caveat that the study design did not allow for prospective genetic testing of all patients at risk, VHL has been asymptomatic to date in 14/49 identified kindred members (28.6%), and the observed penetrance of VHL was 71.4%.

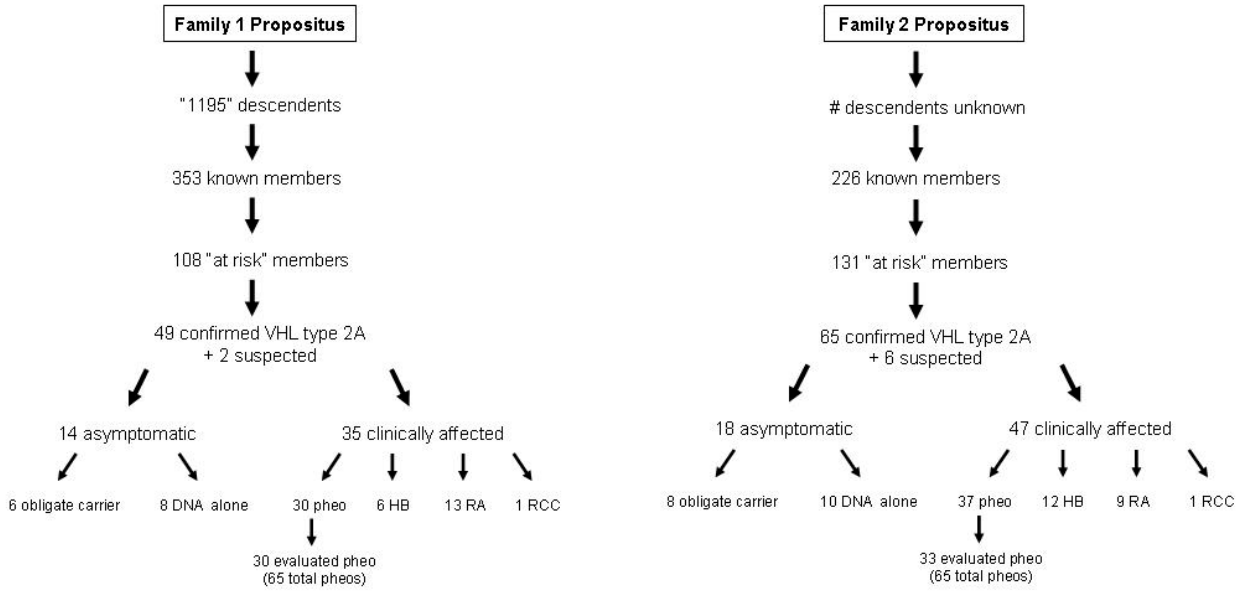

Figure 5. Schematic of VHL Type 2A Kindred Analysis

The observed diagnostic mechanisms for VHL were surprisingly similar between the 2 families. In Family 2, VHL diagnosis was based upon clinical and/or genetic data for 47/65 patients (72.3%), by genetic analysis alone in 10/65 clinically unaffected patients (15.4%), and 8/65 patients were obligate carriers (12.3%) (Figure 5). Family 2 clinical data was assessed as category A in 24/47 (51.0%) patients of whom 7 had genetic testing and as category B in 23/47 (48.9%) patients of whom 4 reportedly had genetic testing which we could not confirm. Thus in Family 2, VHL has been asymptomatic to date in 18/65 kindred members (27.7%) and the observed penetrance of VHL in Family 2 was 72.3%, which is nearly identical to that observed for Family 1 (71.4%). These data must again be viewed against the limitation that some kindred members have not received formal evaluation and many members do not have consistent follow-up. The issue of incomplete evaluation is of particular concern for Family 2, as we have anecdotally observed more fiscal/health insurance stress and greater social barriers to care among Family 2 members. This observation is reflected by a lower rate of confirmed genetic mutational analysis in Family 2 (30.8% of family members with confirmed VHL) than Family 1 (87.8% of family members with confirmed VHL), but also may reflect the fact that the majority of genetic testing that occurred in Family 1 was done under a prior research protocol and was thus free of charge to participants.

3.2 CLINICALLY SIGNIFICANT VHL TYPE 2A

3.2.1 PATTERNS OF VHL TYPE 2A MANIFESTATION AMONG CLINICALLY AFFECTED KINDRED MEMBERS

Category A clinical features of VHL were documented in 31/35 (88.6%) symptomatic informative Family 1 members and in 24/47 (51.2%) symptomatic informative Family 2 members (Figure 5). The remaining clinical features in each family were classified as category B. Table 3 compares the type of initial manifestation of VHL type 2A by family. As may be seen, patients in Family 2 were somewhat more likely to have hemangioblastoma as the initial manifestation than did Family 1 members ($p = 0.07$), whereas Family 1 members appeared more likely to be diagnosed concurrently with pheochromocytoma and retinal angioma together as initial manifestations ($p = 0.07$), but these differences did not reach significance. Otherwise, the initial diagnosed tumor type(s) were quite similar between the 2 families.

Interestingly, one member of Family 1 (V-1) was diagnosed with RCC at age 35, but she derived from a branch of the family not known to be affected with VHL type 2A in any way. Because there was partial lack of data regarding the VHL status of her 3 prior generations, it could not be authoritatively determined whether her RCC was related to VHL type 2A or was sporadic, but our best estimate was that it was unrelated and sporadic thus she was not included in pedigree analysis. It is of course possible that her diagnosis was related to VHL type 2A. She passed away 2 years later from metastatic disease.

Table 3. Initial VHL Type 2A Manifestations in Clinically Affected Kindred Members

	Family 1 (n=35)	Family 2 (n=47)	p value
Pheo	26 (74.3%)	33 (70.2%)	0.81
HB	1 (2.9%)	8 (17.0%)	0.07*
RA	5 (14.3%)	6 (12.8%)	1.00
RCC	0	0	1.00
Pheo+HB	1 (2.9%)	3 (6.4%)	0.63
Pheo+RA	3 (8.6%)	0	0.07*
HB+RA	0	1 (2.1%)	1.00
HB+RA	0	1 (2.1%)	1.00

Pheo, pheochromocytoma; HB, hemangioblastoma; RA, retinal angioma; RCC, renal cell carcinoma
$p < 0.05$ = significant

Data on the lifelong manifestations of VHL type 2A among clinically affected kindred members are given in Table 4. The 2 families appear to be very much alike in regard to lifelong manifestations, with the main differences being related to rates of retinal angioma and hemangioblastoma. There were 13 members with known retinal angioma (37.1%) in Family 1, compared to 9 members in Family 2 (19.1%) ($p = 0.08$), and Family 1 members were more likely than Family 2 members to have been diagnosed with both retinal angioma and pheochromocytoma (14.3% vs. 2.1%) ($p = 0.08$), but these differences did not reach significance. Although Family 2 members appeared both more likely to have hemangioblastoma alone (6.4%, vs. 0), and in conjunction with other type 2A manifestations, the observed differences were not significant. Conversely, Family 1 had a somewhat higher rate of documented pheochromocytoma, with a total of 30 affected members (85.7%) compared to Family 2 with 37 total members affected (78.7%). It is interesting to note that Family 1 members appeared somewhat more likely to have multiple manifestations; 5.7% of Family 1 members had pheochromocytoma, hemangioblastoma and retinal angioma, compared to 2.1% of Family 2 members ($p = 0.57$). One member of Family 1 (VI-2) was even diagnosed with all 4 type 2A manifestations.

Table 4. Cumulative VHL type 2A Manifestations Among Clinically Affected Kindred Members

	Family 1 (n=35)	Family 2 (n=47)	p value (univariate)
Pheo+HB+RA+RCC	1 (2.9%)	0	0.43
Pheo+HB+RA	2 (5.7%)	1 (2.1%)	0.57
Pheo+HB	3 (8.6%)	7 (14.9%)	0.50
Pheo+RA	5 (14.3%)	1 (2.1%)	0.08*
Pheo only	19 (54.3%)	28 (59.6%)	0.66
Total Pheo	30 (85.7%)	37 (78.7%)	0.56
HB+RA	0	1 (2.1%)	1.00
HB only	0	3 (6.4%)	0.26
Total HB	6 (17.1%)	12 (25.5%)	0.43
RA+RCC	0	1 (2.1%)	1.00
RA only	5 (14.3%)	5 (11.0%)	0.74
Total RA	13 (37.1%)	9 (19.1%)	0.08*
Total RCC	1 (2.9%)	1 (2.1%)	1.00

Pheo, pheochromocytoma; HB, hemangioblastoma; RA, retinal angioma; RCC, renal cell carcinoma
$p < 0.05$ = significant

There were only 3 cases of RCC observed. As discussed above, in one Family 1 woman (V-1) RCC was likely sporadic. RCC was also noted in 1 Family 2 woman with VHL (IV-8) who developed it at age 44, and in one Family 1 woman with VHL (VI-2) who developed it at age 42. Both women had VHL type 2A manifestations prior to their diagnosis of RCC; RA in the woman from Family 2, and pheo and RA in the woman from Family 1. In this study, therefore, the rate of RCC observed among all 82 individuals at risk from both families was thus 2/82 = 2.4%.

Because Family 1 has been described multiple times in the literature over the years, Table 5 displays the rates of pheochromocytoma reported over the years as new diagnoses were made. Obligate carriers and DNA positive individuals were not included in the prior study data; the rates given here are solely those of clinically affected individuals. We see that in Family 1,

reported pheochromocytoma rates have been surprisingly constant, ranging from 77.4% to 88.9%, and this is in spite of the fact that not all family members were thoroughly evaluated, especially in early years when elevated blood pressure was often used as a primary means of pheochromocytoma screening.

Table 5. Reported Rates of Pheochromocytoma in Family 1: 1962-Present

	Clinically Affected	Pheo	Pheo Rate (%)
Tisherman, 1962[1]	9	8	88.9
Tisherman, 1993[2]	19	16	84.2
Mulvihill, 1997[3]	31	24	77.4
Current Study	35	30	85.7

[1] Tisherman SE, Gregg FJ, Danowski TS. *JAMA* 182: 152-156.
[2] Tisherman SE, Tisherman BG, Tisherman SA, et al. *Arch Intern Med* 153: 2550-2556.
[3] Mulvihill JJ, Ferrell RE, Carty SE, et al. *Arch Intern Med* 157: 1390-1391.

3.2.2 TUMOR-SPECIFIC PENETRANCE OF VHL TYPE 2A MANIFESTATIONS AMONG INFORMATIVE CARRIERS

Overall VHL penetrance calculations (above) were based on kindred members with VHL diagnosis by clinical manifestations, genetic analysis, and/or obligate carrier status. To determine tumor-specific penetrance, we decided to examine only those VHL type 2A carriers who were informative, and this analysis included not only category A data but also specific category B data when appropriate. This objectivity also necessarily excluded the majority of individuals in early generations in both families, mainly generations I, II and most of generation III. Some individuals in later generations were also excluded when no information was

available. Therefore, the total number of informative carriers for each manifestation of Type 2A VHL differs by tumor type, as is seen in Table 6. Table 6 also gives the cumulative (lifetime) penetrance among clinically affected family members.

Table 6. Penetrance of VHL Type 2A Manifestations Among Informative Carriers

	Penetrance by age 50			Cumulative Penetrance		
	Family 1	***Family 2***	***p value***	***Family 1***	***Family 2***	***p value***
Pheo (n=42;56)	24 (57.1%)	37 (66.1%)	0.40	30 (71.4%)	37 (66.1%)	0.66
HB (n=39;53)	5 (12.8%)	11 (20.8%)	0.41	6 (15.4%)	12 (22.6%)	0.44
RA (n=39;54)	12 (30.8%)	7 (12.9%)	0.04*	13 (33.3%)	9 (16.7%)	0.08*
RCC (n=39;53)	1 (2.6%)	1 (1.9%)	1.00	1 (2.6%)	1 (1.9%)	1.00

Pheo, pheochromocytoma; HB, hemangioblastoma; RA, retinal angioma; RCC, renal cell carcinoma
$p < 0.05$ = significant

Tumor- specific penetrance by age 50 of VHL type 2A manifestations among informative carriers was similar between the 2 studied families, with the exception of retinal angioma. By age 50, retinal angioma had been diagnosed in at least 30.8% of Family 1 members, as opposed to 12.9% of Family 2 members (p=.04), and over a lifetime, retinal angioma was known to be present in at least 33.3% of VHL carriers in Family 1, and in only 16.7% of VHL carriers in Family 2. The penetrance of hemangioblastoma appeared to be somewhat higher in Family 2 both by the age 50 (20.8% vs. 12.8%) and over a lifetime (22.6% vs. 15.4%) but without reaching statistical significance. Pheochromocytoma was the most penetrant manifestation in both families; although not statistically significant, a higher proportion of Family 2 members were diagnosed with pheochromocytoma by age 50 (66.1% vs. 57.1%), while penetrance of pheochromocytoma was higher in Family 1 over a lifetime (71.4% vs. 66.1%).

3.2.3 CUMULATIVE PROBABILTY OF VHL TYPE 2A MANIFESTATIONS BY AGE OF DIAGNOSIS

Figures 6-9 show the cumulative probabilities for pheochromocytoma, hemangioblastoma, and retinal angioma, respectively, based on age of first diagnosis for each tumor type. RCC was excluded because of the small number of cases. Ages of first diagnosis of hemangioblastoma and retinal angioma were more likely to be by self-report (category B data), as was the case for 2/6 individuals with hemangioblastoma and 4/13 individuals with retinal angioma in Family 1, and for 3/11 individuals with hemangioblastoma and 4/6 individuals with retinal angioma in Family 2. The above numbers reflect ages of first diagnosis which were unable to be verified in 1 case of hemangioblastoma and 3 cases of retinal angioma in Family 2. Age at first diagnosis of pheochromocytoma in Family 1 ranged from 6-72 years, and from 8-47 years in Family 2. As may be seen from Figure 6, Family 2 consistently had a higher probability for pheochromocytoma diagnosis at each age range up to 50 years. While all cases of pheochromocytoma were diagnosed by age 50 in Family 2, in Family 1 pheochromocytoma was diagnosed as late as 72 years of age.

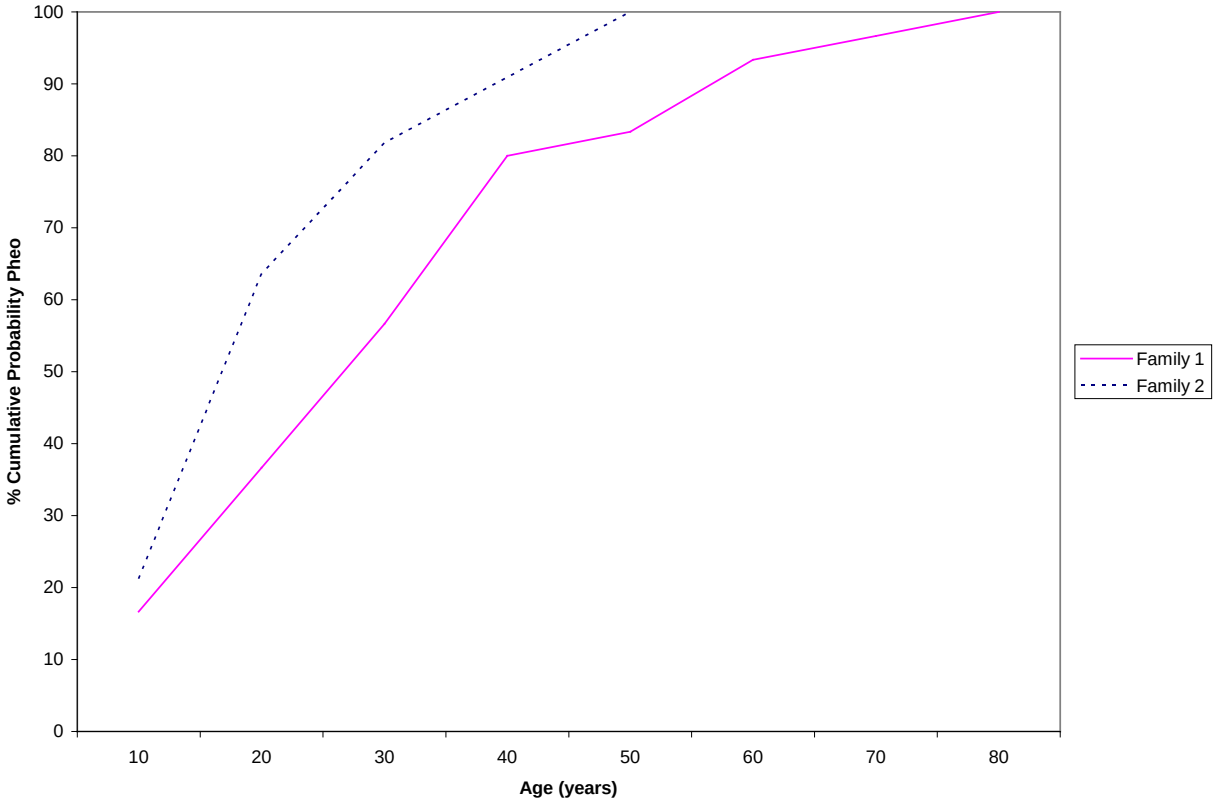

Figure 6. Cumulative Probability by Age of Diagnosis of First Pheo

Age at first diagnosis of hemangioblastoma in Family 1 ranged from 12-56 years of age, and from 9-79 years of age in Family 2. Again, Family 2 had a higher probability of being diagnosed with hemangioblastoma at each age range, as shown in Figure 7. All cases of hemangioblastoma were diagnosed by age 60 in Family 1, whereas in Family 2 hemangioblastoma was diagnosed as late as 79 years of age.

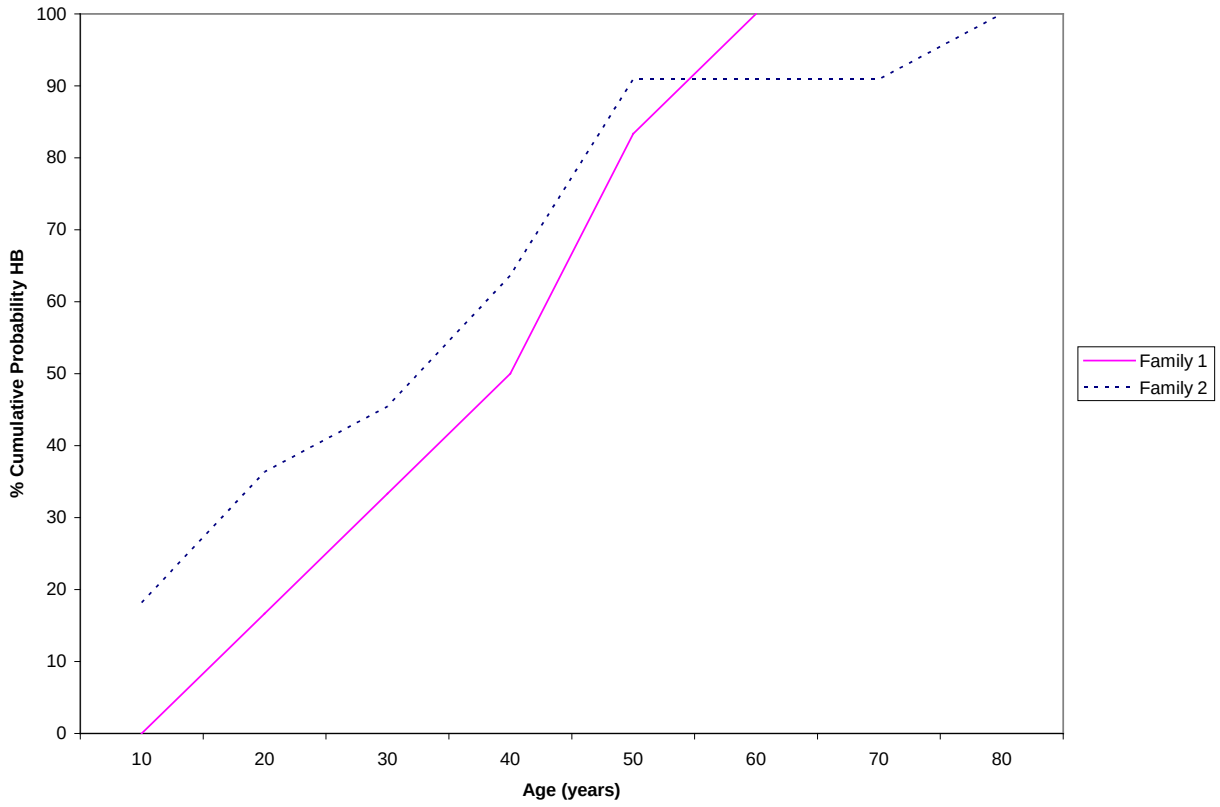

Figure 7. Cumulative Probability by Age of Diagnosis of First HB

The range of age at diagnosis of first retinal angioma was 10-55 years of age in Family 1, and 23-55 years of age in Family 2. Shown in Figure 8, Family 1 consistently had a higher probability of being diagnosed with retinal angioma at each age range. All cases of retinal angioma were diagnosed by age 60.

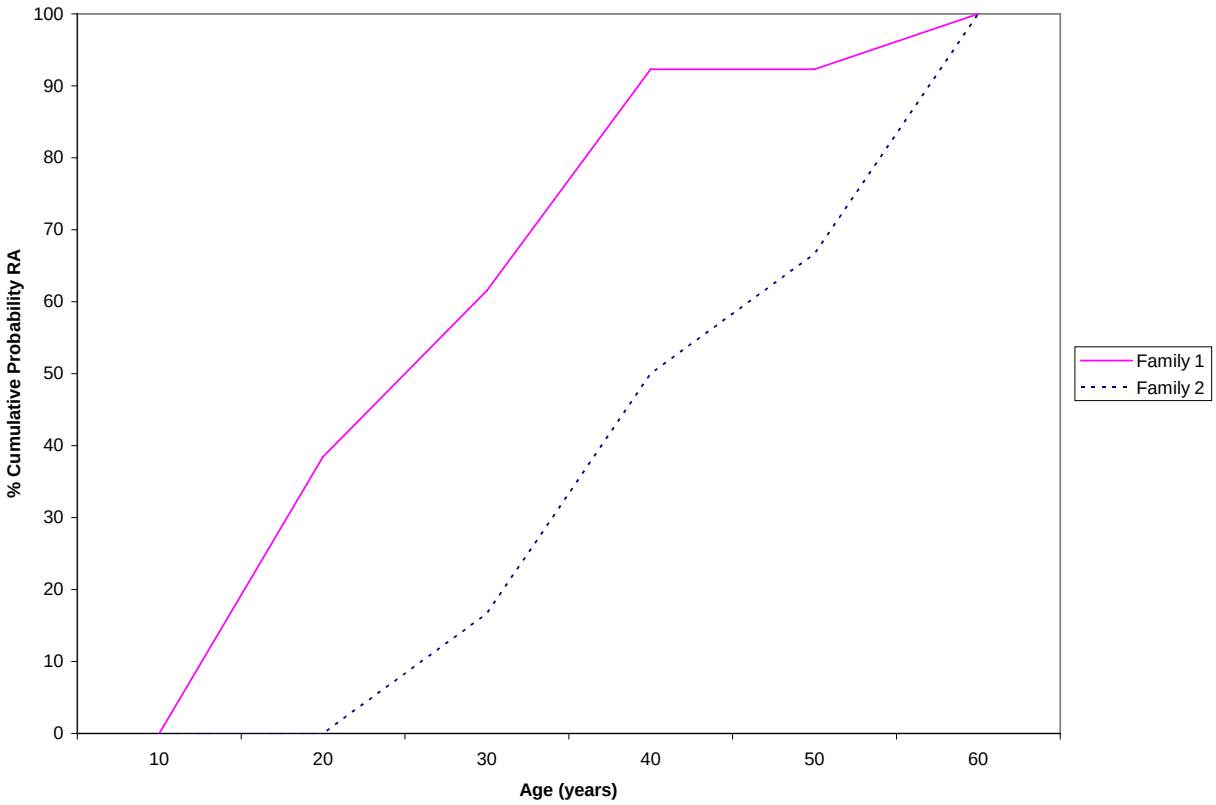

Figure 8. Cumulative Probability by Age of Diagnosis of First RA

3.3 EXPRESSION PATTERNS OF PHEOCHROMOCYTOMA

We were able to include a total of 30 individuals from Family 1 and 33 individuals from Family 2 in the analysis of expression patterns of pheochromocytoma, representing a total of 130 pheochromocytomas (which were interestingly distributed as 65 pheochromocytomas from each family) (See Appendix B for full data set). Of these, 30 data sets included category B data i.e. based on patient report (Family 1, n=10; Family 2, n=20). The clinical parameters examined

included: age at first pheochromocytoma diagnosis, development of metachronous tumors, time to recurrence, diagnosis by symptoms or screening, and hormonal secretion status pre-op. Pathological parameters included: size, weight, laterality, extraadrenal location(s), and multifocality (for synchronous tumors). Pheochromocytoma data were also examined by measures of morbidity and mortality, which included recurrence, metastasis, biochemical cure with resection and individuals who died of disease (DOD). Not all pheochromocytomas were informative for each parameter measured, thus the specific n is denoted under each category in Table 7.

Table 7. Comparison of Pheo Parameters Between VHL Type 2A Families

	Family 1	Family 2	p value
Clinical Parameters			
Age First Pheo Dx (n=30;33)	28.8	19.7	0.02*
Dx by Symptoms (n=52;49)	42 (80.8%)	33 (67.3%)	0.17
Tumor Secreted Only Norepinephrine (n=27;33)	19 (70.4%)	16 (48.5%)	0.12
Metachronous Pheo (n=65;65)	21 (32.3%)	13 (20.0%)	0.16
Time to Recurrence (y) (n=21;12)	7.8	12.8	0.08*
Pathologic Parameters			
Size (cm) (n=41;33)	3.60	3.20	0.33
Weight (g) (n=29;18)	33.3	30.1	0.81
R Laterality (n=63;54)	20 (31.7%)	19 (35.2%)	0.84
L Laterality (n=63;54)	22 (34.9%)	17 (31.5%)	1.00
Extra-adrenal (n=63;54)	9 (14.3%)	15 (27.8%)	0.11
Abdominal (n=9;15)	7 (77.8%)	11 (73.3%)	1.00
Zuckerkandl (n=7;11)	2 (28.6%)	7 (64.0%)	0.33
Chest (n=9;15)	0	3 (20.0%)	0.27
Neck (n=9;15)	1 (11.1%)	0	0.37
Synchronous (Multifocality) (n=65;55)	26 (40.0%)	33 (60%)	0.04*

Pheo, pheochromocytoma; Dx, diagnosis; y, years
p < 0.05 = significant

The category of most interest was the age at first diagnosis of pheochromocytoma. We observed (Table 7) that pheochromocytoma was diagnosed at a significantly younger mean age in Family 2 (mean 19.7 y, range 8-47 y, p =0.02), compared to a mean age of 28.8 y in Family 1 (range 6-72 y). Additionally, Family 2 pheochromocytomas were more likely to be multifocal (p =0.04), since 60% of Family 2 tumors were synchronous compared to 40% of tumors in Family 1. Furthermore, a larger proportion of pheochromocytoma in Family 2 was extra-adrenal, with 27.8% classified as paraganglioma, compared to 14.3% in Family 1, although the difference did not reach significance.

The most common location of paraganglioma in both families was the abdomen (77.8% and 73.3% in Family 1 and 2, respectively). Although Family 2 abdominal paragangliomas were much more often at the Organ of Zuckerkandl (located at the bifurcation of the aorta) (64.0% vs. 28.6%) again the numbers were small and the difference did not reach significance. Chest paragangliomas were more common in Family 2 (20.0% vs. 0). There was one neck paraganglioma in Family 1 (11.1%) and none in Family 2. Although not statistically significant, pheochromocytoma in Family 1 also appeared to be more likely to be diagnosed by symptoms rather than routine screening. Pheochromocytomas in Family 1 were also more likely secrete norepinephrine only preoperatively, which was the case for 70.4% of pheochromocytoma in Family 1, compared to 48.5% of pheochromocytoma in Family 2. Size, weight and laterality (R vs. L adrenal gland) were shown to be the same between the 2 families.

We defined metachronous pheochromocytoma to be a new tumor arising at a site different from that of a previously resected pheochromocytoma. Local recurrence at the site of prior resection, and distant metastasis, were also tracked. Metachronous pheochromocytoma was frequent in both families (33.2% and 20% in Family 1 and 2, respectively), occurring at a mean

of 7.8 y (range 1-25 y) after the first diagnosis in Family 1 compared to a mean of 12.8 y, (range 1-31 y) in Family 2 (p=0.08). Pheochromocytoma in Family 1 was shown to be significantly more morbid across almost all measures, except recurrence (Table 8). Accordingly, 100% of informative pheochromocytoma in Family 2 was biochemically cured post-operatively, whereas only 76% of pheochromocytoma in Family 1 was shown to be biochemically cured ($p < 0.05$). Additionally, 20% of tumors in Family 1 were metastatic ($p < 0.05$), compared to 4.8% of tumors in Family 2. Note that multiple metastases at the time of death i.e. autopsy findings were not counted individually, and that malignant pheochromocytoma is not determined by histology, only long-term follow-up. Rates of pheochromocytoma classified as recurrent were the same between the 2 families. The details of the 5 individuals diagnosed with pheochromocytoma in Family 1 who died from complications of the tumor (dead of disease [DOD]) are given in Table 9. In Family 2, there is no evidence that anyone has died yet due to complications of pheochromocytoma. However, one member did die of brain hemangioblastoma, another complication of VHL type 2A often leading to morbidity and mortality (Table 7).

Table 8. Measures of Morbidity and Mortality of Pheochromocytoma

	Family 1	Family 2	p value
Biochemical cure (n=50;49**)**	38 (76.0%)	49 (100%)	0.0002*
Recurrence (n=63;62)	1 (1.6%)	3 (4.8%)	0.36
Metastasis (n=64;62)	13 (20.0%)	3 (4.8%)	0.01*
DOD (n=30;33)	5 (16.7%)	0	0.02*
DOD, dead of disease			

Table 9. Individuals Dead of Disease (DOD)

	Individual	Age of Death	Cause	Details
Family 1	V-16	35	Metastatic pheo, MI	Organ of Zuckerkandl pheo, 6
				Metastatic pheo (liver), 11
				Metastatic pheo (liver, bowel, lung, periaortic LN), 35
	IV-4	56	Infarction of cerebellum	Cerebellar HB, R periadrenal pheo dx at autopsy
	IV-6	48	MI	L pheo, 37
				R pheo, 48
	IV-11	36	Metastatic pheo, MI	L pheo, 21
				R pheo, 27
				L pheo, 27
				Metastatic pheo, 34
	IV-13	24	Hrt and renal failure; pheo dx by autopsy	Retrospective dx by radiographic adrenal mass and clinical course
Family 2	IV-10	38	Brain HB	Brain HB, 18+35

Pheo, pheochromocytoma; MI, myocardial infarction (heart attack); HB, hemangioblastoma

3.4 SUMMARY OF PHENOTYPIC FINDINGS

In conclusion, we observed autosomal dominant transmission of VHL type 2A in both of the studied kindreds. We documented that pheochromocytoma was the most common tumor type in both families, with a penetrance of 71.4% and 66.1% in Family 1 and 2 respectively. Penetrance was estimated using both clinically affected individuals and carriers of a *VHL* mutation and was the same between the families. Furthermore, we observed phenotypic differences based on

genotype. Malignant pheochromocytoma occurred in Family 1 in 20% of patients and in Family 2 in only 4.8% of patients. Pheochromocytoma in Family 1 (Y112H) was significantly more likely to be lethal, to present at a later age, and to be unifocal, whereas pheochromocytoma in Family 2 (Y98H) was significantly more likely to present at a younger age, to be multifocal, and to benign in behavior. In addition, although not statistically significant, we observed trends in Family 1 towards earlier metachronous pheochromocytoma, diagnosis by symptoms rather than routine screening, and more secretion of norepinephrine alone, while Family 2 pheochromocytoma was more likely to be extra-adrenal, and to be biochemically cured with resection.

4.0 DISCUSSION

This study is unique in its clinical and phenotypic characterization of two very large multigenerational VHL type 2A kindreds. Both have been described previously in the literature, but one family (Family 2) has never been described as a whole in detail, and characterization of the other family (Family 1) has not been updated for over 12 years. With 24 members affected with pheochromocytoma at the time of last report in 1997, Family 1 is currently the largest kindred with pheochromocytoma described in the literature [Mulvihill et al. 1997], yet my thesis work considerably expands the family by adding 20 new cases of VHL, including 6 new diagnoses of pheochromocytoma. Furthermore, this work describes for the first time the phenotypic expression of an even larger kindred with inherited pheochromocytoma (Family 2) in which we have diagnosed at least 37 members to date with pheochromocytoma. This is the also first study of its kind to examine the expression patterns of pheochromocytoma, the most common manifestation of VHL type 2A, between 2 families with the 2 most frequent VHL type 2A mutations, Y112H and Y98H (Family 1 and Family 2, respectively).

4.1 VHL TYPE 2A EXPRESSION AMONG KINDRED MEMBERS

Family 1 consists of at least 49 members with confirmed VHL type 2A, of whom 35 are clinically affected and 14 are asymptomatic. Family 2 has at least 65 members with confirmed

VHL type 2A, of whom 47 are clinically affected and 18 remain asymptomatic. All of the most common manifestations of VHL type 2A (pheochromocytoma, hemangioblastoma, retinal angioma, and RCC) are represented in each family, with pheochromocytoma being the most common manifestation in both families, and RCC being the least common tumor type. This phenotypic profile is consistent with the definition of VHL type 2A. Following pheochromocytoma, retinal angioma was the most frequent manifestation in Family 1, whereas hemangioblastoma was the most frequent manifestation in Family 2. It is interesting that there were 3 total cases of RCC diagnosed between the 2 families as this tumor type is such an uncommon feature of VHL type 2A. Although it could not be determined incontrovertibly that one case of "sporadic" RCC in Family 1 was not part of VHL type 2A, if this case is counted, our observed rate of RCC among VHL carriers in Family 1 would be about 4% which is a marked difference from many studies of type 2A kindreds that did not find any members affected with RCC. Indeed, no previous publications involving either kindred have mentioned any of the 3 cases of RCC that we discovered and report here.

We observed no significant differences in the expression patterns of VHL type 2A manifestations among the 2 families. Pheochromocytoma was found to be highly penetrant among VHL carriers in both families (71.4% and 66.1% in Family 1 and 2, respectively), and was the most frequent manifestation in clinically affected kindred members (85.7% and 78.7% in Family 1 and 2, respectively). However, the mean age at which pheochromocytoma was diagnosed in Family 2 was significantly younger (19.7 y) than in Family 1 (28.8 y) (discussed further below), and had a higher probability of being diagnosed by age range up to 50 years. All cases of pheochromocytoma in Family 2 were diagnosed by age 50, whereas in Family 1 there were 6 cases diagnosed after the age of 50. This is an important phenotypic difference between

VHL Type 2A families. It is one that should be used to better design screening algorithms (Section 4.4). We show here that phenotype affects age at first biochemical evaluation, and even well-being and longevity.

As far as non-pheochromocytoma manifestations of VHL type 2A, the rates of retinal angioma differed the most between the 2 families, with Family 1 being more likely to be diagnosed with retinal angioma alone and in combination with other manifestations. In total, about 37% of Family 1 members had retinal angioma, compared to approximately 19% in Family 2 ($p = 0.08$). Family 1 also consistently had a higher probability of being diagnosed with retinal angioma at each age range up to 60 years old. Although this may be a real difference between the phenotype of VHL type 2A in these families, we argue that the differing retinal angioma rates may actually reflect differences in screening compliance and/or delivery of medical care between the families. As mentioned previously, in general, Family 2 presents for medical care much less often than Family 1 and thus is less likely to have routine screening, especially for retinal angioma, a manifestation that unless symptomatic would be unlikely to be diagnosed if there are financial constraints. Similarly the higher rate of hemangioblastoma in Family 2 could represent a true phenotypic difference between the families, or may be a coincidental finding as the rates did not near statistical significance.

The observed lower rates of HA and RA as compared to pheochromocytoma in both families support the theory that the development of retinal angioma and hemangioblastoma are more dependent on a combination of genetic and environmental factors, whereas a gene mutation alone may be sufficient to cause pheochromocytoma. In other words, by Knudsen's well-known "two-hit hypothesis" for carcinogenesis, in individuals with a germline *VHL* mutation, the "first hit" is likely to be responsible for the failure of apoptosis in adrenal medulla precursor cells that

ultimately leads to pheochromocytoma; as such, loss of heterozygosity (LOH) has not yet been observed in this tumor type. Whereas certain unknown environmental influences may cause a "second hit" to occur that inactivates the remaining functional copy of the *VHL* gene and could be responsible for the development of retinal angioma or hemangioblastoma; indeed, these tumor types have been shown to display LOH (loss of heterozygosity). Therefore, we feel the differences in the rates of retinal angioma and hemangioblastoma between the 2 families most likely reflect discrepancies in compliance and follow-up, as well as exposure to different environmental factors.

Although not described in the Results, there were some interesting observations in Family 1 among the 3 branches of the family known to be affected with VHL (II-3, II-4, II-5) (Figure 3). Out of 35 total clinically affected individuals in Family 1, 21 were in branch II-5 and 7 each were in branches II-3 and II-4. In regard to pheochromocytoma, 81% of II-5 branch members had pheochromocytoma as their only manifestation of the disease, as opposed to 29% of II-4 branch members and 0% of II-3 branch members ($p<0.05$). Conversely, 86% of II-3 branch members had pheochromocytoma in addition to another type 2A manifestation, as compared to 19% and 14% in branches II-5 and II-4, respectively ($p<0.05$). Another interesting finding relates to retinal angioma penetrance. As many as 57% of branch II-4 members had retinal angioma as their only manifestation of the disease, compared to 14% in branch II-3 and 0% in branch II-5. No one in any of the branches had hemangioblastoma as their only manifestation of the disease, and branch II-4 had no cases or hemangioblastoma at all. Taken together, these data suggest that branch II-5 members were more likely to be affected with pheochromocytoma, while branch II-3 members often had pheochromocytoma along with another type 2A manifestation, and branch II-4 members often had retinal angioma as their only

manifestation. Indeed, during conversations with members of branch II-4, they expressed that they had inherited the “eye part of the disease” and were seemingly unconcerned or unaware of their pheochromocytoma or hemangioblastoma risks as most relatives affected with these tumor types were more distantly related to them. Some of these phenotypic differences between the branches of Family 1 are certainty significant and are consistent with the variability often found in families with autosomal dominant conditions. It is possible that the variations within Family 1 may be explained by distinct environmental and genetic factors.

4.2 PHEOCHROMOCYTOMA EXPRESSION AMONG KINDRED MEMBERS

A total of 65 pheochromocytomas from each family were evaluated, representing 30 individuals from Family 1 and 33 individuals from Family 2 with pheochromocytoma. Clinical and pathological documentation was available for approximately 77% of pheochromocytoma examined. Significant findings in Family 1 included a shorter time to new occurrence of pheochromocytoma, a higher rate of metastasis, a lower probability of being biochemically cured postoperatively, and a higher proportion of individuals who died from complications of pheochromocytoma. Significant findings in Family 2 included an earlier age at diagnosis of first pheochromocytoma, a higher rate of multifocal tumors, and a higher proportion of patients with paraganglioma (especially those located at the Organ of Zuckerkandl).

The mean age of diagnosis of pheochromocytoma between the 2 families may be the most interesting finding of this study. Pheochromocytoma in Family 2 was diagnosed at a mean age of 19.7 years (range 8-47 y), compared to a mean age of 28.8 (range 6-72 y) in Family 1 ($p<0.05$). The average age of pheochrmocytoma diagnosis in Family 1 is consistent with reports

in the literature suggesting that in overall VHL disease pheochromocytoma presents at an average age of 30 years. However, it is rather interesting that pheochromocytoma in Family 2 presented almost 10 years earlier than the mean age of diagnosis, with the majority of cases (63.6%) being diagnosed before the age of 20 years. Alternatively, in Family 1 only 36.6% of pheochromocytoma was diagnosed by the age of 20, though the majority (56.6%) was diagnosed by the age of 30. If it is indeed true that having a germline *VHL* mutation is sufficient to cause pheochromocytoma, then the earlier age of diagnosis of pheochromocytoma in Family 2 could suggest that the specific mutation (Y98H) is less able to promote apoptosis of adrenal medulla precursor cells than the Y112H mutation. Of course, this finding of young age of pheochromocytoma diagnosis may be unique to Family 2 and not hold true for all individuals with Y98H mutations.

Individuals in Family 2 were also more likely to be diagnosed with synchronous disease (50% vs. 27.7%) and a greater proportion of total pheochromocytoma in Family 2 was multifocal (60% vs. 40%) ($p<0.05$). It has been reported that up to 58% of VHL pheochromocytoma is multifocal [Walther et al. 1999], so rather than it being unusual that Family 2 has a high rate of synchronous disease, the more interesting finding may be that Family 1 has a lower-than-expected rate of synchronous pheochromocytoma. Although not quite statistically significant ($p=0.11$), Family 2 was also found to have a higher rate of paraganglioma (27.8% vs. 14.3%). This higher rate of paraganglioma in Family 2 may help explain the higher rate of synchronous disease, and is in keeping with the findings of Clarke et al. [1998] that describe a higher risk for synchronous disease in patients with paraganglioma. In VHL overall, about 12% of pheochromocytoma is predicted to be extra-adrenal and interestingly, paraganglioma has only been seen in families with missense mutations, most frequently those with Y98H mutations

[Walther et al., 1999]. Therefore, Family 2 had a rate of paraganglioma over 2 times higher than expected. The majority of paraganglioma in both families was located in the abdomen (77.8% and 73.3%), with the Organ of Zuckerkandl being the most frequent location in Family 2 (64%). The more rare locations of paraganglioma are also worth mentioning; there were 3 cases of chest paraganglioma in Family 2, and 1 case of neck paraganglioma in Family 1. Of the 15 cases of paraganglioma diagnosed in Family 2, 8 were the initial manifestation of VHL type 2A in that individual (53.3%). In Family 1, of the 9 cases of paraganglioma, 4 were the initial manifestation of VHL type 2A in that individual (44.4%) ($p<0.05$). This is a notable finding because at the present time, if a patient were to present with a paraganglioma, testing algorithms would invariably first lead to *SDHB/SDHD* gene testing rather than to *VHL*. Granted, the individuals in this study have a family history of VHL, but as will be discussed later, they are not all fully aware of this diagnosis or its implications, therefore they may not be able to assert this during an evaluation. Lastly, compared to pheochromocytoma which is malignant in about 5-10% of cases, paraganglioma has been reported to be malignant 14-50% of the time, but despite Family 2 having a higher rate of paraganglioma, Family 1 had a higher rate of metastatic disease (discussed further below).

The rate of metachronous pheochromocytoma was higher (32.3% vs. 20%) and occurred sooner in Family 1, at a mean of 7.8 years after first diagnosis, compared to 12.8 years in Family 2 ($p=0.08$). It is important to keep in mind that time to re-operation as a means to determine occurrence rate of metachronous disease is an inaccurate measure of the true rate, because we do not know exactly *when* the tumor occurred, only when surgery was performed. This is a limiting factor for both families, however the data may be more accurate for Family 1 who generally had stricter biochemical follow-up. Additionally, Family 1 pheochromocytoma was also

significantly more morbid than that of Family 2 across measures of metastasis, biochemical cure, and number of individuals dead of disease. Approximately 5-10% of pheochromocytoma is malignant, with metastatic and recurrent disease being the best predictors of malignancy. Therefore, the 20% rate of metastatic disease observed in Family 1 (compared to 4.8% in Family 2), is a very significant finding ($p<0.05$). Interestingly, the rates of recurrent disease were higher in Family 2 (1.6% vs. 4.8% in Family 1 and 2, respectively), although sometimes it was difficult to determine recurrent disease based on operative reports, or in 1 case in Family 2 where no operative report was available. Also, while all informative pheochromocytomas in Family 2 were shown to be biochemically cured post-operatively, 24% of pheochromocytoma in Family 1 continued to secrete high levels of catecholamines post-operatively ($p<0.05$), which could be suggestive of subclinical synchronous or metastatic disease. Lastly, we know of 5 individuals in Family 1 who have died from complications of pheochromocytoma, usually as the direct result of a heart attack. To the best of our knowledge, no one in Family 2 has died from pheochromocytoma, although some individuals had symptoms suggestive of pheochromocytoma at the time of their death. Taken together, this data suggest that pheochromocytoma in Family 1 is more morbid and lethal than pheochromocytoma in Family 2 which tended to be more indolent in nature.

4.3 BIOCHEMICAL PHENOTYPE OF VHL TYPE 2A PHEOCHROMOCYTOMA

The biochemical profile of pheochromocytoma associated with VHL disease has been extensively studied, usually in comparison to MEN2-associated pheochromocytoma to highlight their distinct biochemical and clinical phenotypes. It is well established that VHL-associated

pheochromocytoma tends to secrete norepinephrine/normetanephrine, while MEN-2-associated pheochromocytoma is characterized by epinephrine/metanephrine secretion [Eisenhofer et al., 2001; Walther et al., 1999]. This finding was confirmed by our study, as the majority of pheochromocytoma in both families (70.4% and 48.5%, respectively) was shown to secrete norepinephrine only. The underlying mechanism of this specific secretion profile is related to the expression of 2 enzymes in the catecholamine metabolism pathway: tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine synthesis, and phenylethanolamine*N*-methyltransferase (PNMT) is responsible for converting norepinephrine to epinephrine [Eisenhofer et al., 2001] (Figure 2). Pheochromocytoma in MEN2 is characterized by an adrenergic biochemical phenotype as it expresses both PNMT and TH, whereas pheochromocytoma in VHL expresses negligible PNMT and greatly reduced amounts of TH, thus displaying a nonadrenergic biochemical profile [Eisenhofer et al., 2001].

It is these differences in enzyme expression that contribute to the distinct clinical phenotype of pheochromocytoma in each disease; MEN2 patients have a higher frequency of hypertension (usually paroxysmal in nature) and symptoms than VHL patients due to higher rates of catecholamine synthesis and the fact that epinephrine has more potent vascular and metabolic effects than norepinephrine [Eisenhofer et al., 2001; Hjemdahl, Belfrage, Daleskog, 1979]. In other studies, only 8-30% of patients with VHL had symptoms ("spells"), and about 18% of patients had hypertension (usually sustained) [Eisenhofer et al., 2001; Walther et al., 1999]. In our study, a large proportion of pheochromocytoma in both families was diagnosed by symptoms (which included spells and/or hypertension); 80.8% of pheochromocytoma in Family 1 was diagnosed by symptoms, compared to 67.3% in Family 2 (p=0.17). As several members in each family had multiple pheochromocytomas, some that presented with symptoms and others

that were diagnosed through screening, we were however unable to say how many individuals in general had signs and symptoms of pheochromocytoma. Another notable finding from our study was that 2 adrenal pheochochromocytomas in Family 1 that were both diagnosed as a result of symptoms were found to be non-functional. Although it is not uncommon for paragangliomas to be non-functional, this is a rare finding for pheochromocytoma that by definition is located in the adrenal gland. Although symptoms were present in both individuals, their severity varied; one individual (IV-15) presented with hypertension, headaches, sweating, palpitations, nervousness/anxiety, and nocturia while the other individual (V-2) had hypertension alone. We did not identify any individuals with non-functioning pheochromocytoma in Family 2.

As MEN2 and VHL are caused by mutations in separate genes (*RET* and *VHL*, respectively), it is believed that the differences in clinical and biochemical expression of associated pheochromocytomas are due to the expression of different genes that control catecholamine synthesis [Eisenhofer et al., 2001]. It would be interesting to see if there were any differences in biochemical expression of VHL-associated pheochromocytoma based on mutation type. Although most likely not attributable to mutation type, it is still a notable finding that the majority of the pheochromocytoma in our study was diagnosed by symptoms even though they secreted primarily norepinephrine.

4.4 VHL TYPE 2A SCREENING RECOMMENDATIONS

As mentioned in section 3.2.1, the rates of pheochromocytoma reported in the literature in Family 1 since 1962 have remained consistently high. Certainly, we have come a long way since Dr. Tisherman's initial investigation into the underlying cause of a familial aggregation of

pheochromocytoma. Detection methods for pheochromocytoma have greatly improved and localization of the *VHL* gene has allowed for presymptomatic diagnosis in at-risk family members and confirmatory diagnosis in affected individuals. Pheochromocytoma is a very frequently occurring tumor in VHL type 2A and a very serious complication of the disease, as death can result from severe hypertension or from metastatic spread. It is crucial to screen carefully and consistently for pheochromocytoma, and it has become very clear that relying on infrequent measures of blood pressure as the sole means of screening is dangerous. In Dr. Carty's practice in endocrine surgery, patients with known VHL Type 2A (any route of diagnosis) or patients at risk who are unable to comply with recommended mutational analysis, are asked to undergo examination that includes yearly plasma metanephrine testing, radiologic imaging as indicated by prior or current disease, yearly retinal exam, and spine MRI every other year.

4.5 PSYCHOSOCIAL ISSUES AMONG KINDREDS

These families were very interesting to study for many reasons aside from their type 2A VHL manifestations. What is initially striking about the families is their sheer size and fecundity. The number of individuals in generation V of Family 2 is particularly staggering. Typically with heritable genetic diseases, families are carefully planned, particularly with autosomal dominant conditions in which each child born is at 50% risk of being affected with the disorder. Of course, in early generations little was known about VHL and its inheritance patterns. Even though each individual I spoke with was unique, I did observe a general pattern of thinking and behavior between the 2 families. Members of Family 1 were generally less knowledgeable about their

VHL disease and their family history, while members of Family 2 were more educated about VHL disease and their family history but at the same time were less likely to seek consistent medical care. It is unclear whether the inconsistent follow-up observed in Family 2 is due to financial concerns, bravery or denial. In both families, there was a clear persisting misconception that if you are not experiencing any symptoms, then you do not have pheochromocytoma. Most family members live a distance from Pittsburgh, and were opposed to traveling for evaluation thus it may also be that their local doctors are not as knowledgeable about the management of VHL and pheochromocytoma.

It was surprising that both families downplayed the seriousness of the disease, with denial being more outwardly evident in Family 1. One particular case in Family 1 illustrates the implications in some members of this family: a 21 year-old man was first diagnosed with pheochromocytoma at the age of 8 and had yearly follow-up in his community by 24-hour urine testing until age 16. At this point, he did not return for any further evaluation, although he continues to live with his father who has been dying from metastatic pheochromocytoma for several years. At 21 this young man had testicular discomfort, on abdominal CT had another pheochromocytoma, and only then consulted one of the physicians in Dr. Carty's group, but when his disease was discussed he replied, "and what is VHL?". He also did not realize that he could pass the disease on to his future children. He was referred to Endocrine Genetics Clinic, where he had the opportunity to meet with a genetic counselor for education and to have his questions answered. He elected to pursue genetic testing for the benefit of his future children and was found to carry the Y112H mutation, which was previously identified in other members of his family. He also did not seem to comprehend how serious his father's disease is, saying he was "doing fine" when he is clearly one of the worst cases in the family. The patient is getting

married soon was somewhat overwhelmed and stressed by the information that was provided. Although certain members of Family 1 were well-informed about VHL, this case illustrates that there is limited information sharing among some members (of any kindred).

Family 2 members seemed to be more concerned about the disease, yet did not seem to understand the importance of close follow-up. Almost every member of Family 2 that I spoke with commented on the 14 siblings in generation V of whom 13 have pheochromocytoma. Many wondered if the mother of these children also had VHL and if that might be why 13 of the children were affected. Although not shown in the Family 2 pedigree, there were at least 3 reported cases of consanguinity, though the known pairs were somewhat distantly related and as far as we know, only one parent was affected with VHL in each instance. We have no evidence that the parents of the 14 siblings in generation V were close relatives, although it cannot be ruled out of course. Consanguinity was also noted in Family 1, though most of the cases occurred in early generations.

4.6 LIMITATIONS OF STUDY

Careful consideration has gone into the acquisition and processing of this data, but there are still a number of limitations to this study. First, although these families make up a large proportion of individuals identified to date with VHL type 2A, since the study only includes 2 families the findings cannot be generalized to all type 2A VHL, nor to all individuals with either the Y98H or Y112H mutation. The families are very large, but the sample size is still relatively small.

Although many steps were taken to ensure that all family members with VHL were identified, surely there are some members that we missed and possibly some members were

misidentified. We were careful to verify data through a variety of sources if possible, but sometimes second-hand information was the only kind we were able to obtain and verification could not be obtained through clinical or pathological documentation. There were multiple members in each family who were lost to follow-up and who could not be contacted to discuss the study, their personal history of VHL, or to obtain their medical records. Also, I was unable to obtain some family members' medical records when the surgery occurred so long ago that the records had since been destroyed.

Many members of each family did not receive consistent follow-up, which could potentially lead to underestimation of rates of occurrence or re-occurrence of manifestations if a thorough evaluation did not take place. Additionally, we identified about 18 individuals in 3 smaller kindreds with VHL/pheochromocytoma who either had the Y98H mutation (Family 2) or were a part of Dr. Tisherman's files but had not undergone genetic testing to the best of our knowledge. We used many sources to attempt to link these members to one of the 2 studied kindreds, but were unable to do so. So although our phenotypic characterization of the families was as thorough as possible, it is by no means complete.

This is a study of genotype-phenotype correlations, and we did indeed find some significant differences between the 2 families in the expression of type 2A VHL disease, particularly related to the clinical and pathological phenotype of pheochromocytoma. Both families are large and the majority of the members live in Western Pennsylvania. However, as discussed previously there are certainly differences between the families with regard to compliance and delivery of medical care, and we can assume different lifestyles and exposures to environmental factors. As we are unable to take these variables into account in our analysis, we cannot determine if the specific *VHL* gene mutations truly account for the observed phenotypic

differences between the 2 families. Furthermore, we have insufficient knowledge regarding the mechanism of action of pheochromocytoma development with each mutation to hypothesize why we observed the differences in mean age at diagnosis, time to metachronous disease, and rate of synchronous and metastatic disease.

4.7 FUTURE DIRECTIONS

The establishment of our Inherited Endocrine Tumor Database allows for the continued tracking of these VHL type 2A families, as well as the opportunity to add newly identified VHL kindreds. It allows for the review of medical record information in order to perform retrospective research on VHL disease, as well as other inherited endocrine tumor syndromes, such as MEN1 and SDH where there are also large regional kindreds that are followed. We hope that through the Inherited Endocrine Tumor Database we have the chance to continue follow Family 1 and Family 2 since they provide a unique opportunity to learn about the natural history of VHL type 2A.

Continued follow up could provide information about changes in patterns of behavior and screening over time in the 2 families. In regard to Family 1 in particular, which has been described independently several times in the literature, our study serves as starting point to further update the characterization of this most interesting regional family. We suspect that since Family 1 was first identified in 1962, a 50-year history of the family would be appropriate, paying particular attention to changing mortality rates and causes over time. We would hypothesize that survival of individuals with VHL has improved over the 50 years that the family has been followed. Other factors to assess, that would be expected to have improved over time,

would include age of death (adjusted for birth year), adverse events associated with a high risk of mortality (stroke/MI/cardiomyopathy/malignant pheochromocytoma etc), and operative mortality from pheochromocytoma (hard to fully attribute to genetics since operative improvements have occurred independently). This future study would contribute greatly to the cancer genetics literature, helping to prove that knowing one's genetic status does indeed make a difference in morbidity and mortality through presymptomatic genetic diagnosis and early surgical intervention.

5.0 CONCLUSION

VHL type 2A is an autosomal dominant inherited endocrine tumor syndrome characterized by a high risk for pheochromocytoma, retinal angioma, and hemangioblastoma, and a low risk for renal cell carcinoma. Our study of 2 large Western Pennsylvania VHL type 2A kindreds, each with a different mutation in exon 1 of the *VHL* gene (Y98H and Y112H), confirms this phenotypic expression profile.

The 2 families displayed similar expression of VHL type 2A manifestations, including high penetrance and rates of pheochromocytoma and low penetrance of renal cell carcinoma. The rates of retinal angioma and hemangioblastoma differed most between the 2 families, but we hypothesize that these differences are more likely due to discrepancies in follow-up/compliance and exposure to environmental factors, rather than to mutational differences between the families. Interestingly, there was a distinct difference in phenotypic expression of pheochromocytoma between the 2 families, as evidenced by measurement of a variety of clinical and pathological parameters. It appears that pheochromocytoma in Family 1 (Y112H) is significantly more morbid and lethal than pheochromocytoma in Family 2 (Y98H) which seems manifest pheochromocytomas that are more indolent in nature. However, pheochromocytoma in Family 2 was diagnosed on average a decade earlier than in Family 1 (19 vs. 29 years of age), which coincidentally is also a decade earlier than the average age of pheochromocytoma diagnosis in overall VHL. There were multiple members in each family with metachronous

disease, but metachronous pheochromocytoma happened sooner after the first diagnosis in Family 1, although the difference of 5 years (8 vs. 13 years) between the families was not quite statistically significant. Family 2 was more likely to have multifocal disease and to be diagnosed with extra-adrenal paraganglioma (both overall and as the first manifestation of the disease).

These unique pheochromocytoma expression profiles may be attributable to the mutational differences of the families, particularly as germline mutations alone have been hypothesized to be responsible for the development of pheochromocytoma. However, the molecular mechanism of action that causes pheochromocytoma has yet to be elucidated, but as alluded to before may involve protein kinase C signaling and microtubule stabilization. As both the Y98H and Y112H mutations are missense mutations located in exon 1 of the *VHL* gene that replace tyrosine with histitine, it will be difficult to tease out differences between them that could result in unique phenotypic expression of pheochromocytoma. Aside from the genotype-phenotype information that can be gleaned from these families, they are an invaluable source of information regarding expression of VHL type 2A. They are extremely fascinating human beings both clinically and psychosocially and it will be interesting to continue to follow them in the years to come.

APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL LETTER

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: Sally Carty, MD
From: Christopher Ryan, PhD, Vice Chair
Date: 4/24/2009
IRB#: PRO09010261
Subject: Inherited Endocrine Tumor Registry for Research and Surveillance

The University of Pittsburgh Institutional Review Board reviewed and approved the above referenced study by the expedited review procedure authorized under 45 CFR 46.110. Your research study was approved under 45 CFR 46.110 (5).

The IRB has determined the risk to be minimal risk. Please note that the waiver for the requirement to obtain informed consent to use protected health information to identify potential research subjects has been approved.

Approval Date: 4/22/2009
Expiration Date: 4/21/2010

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

APPENDIX B

DOCUMENTED PHEOCHROMOCYTOMA DATA

Individual	Pheo #	Age at Dx	Location, Laterality	Paragang	Synch.	Recurr.	Mets	Dx Scrn vs. Sx	Secretory pre-op	Size (cm)	Weight (g)	Biochem cure	Time to Recurrence (y)	F/U
Family 1														
III-6	1	54	L adrenal					Sx		7.5	146.0	?	1	
III-6	2	55	R retroperitoneum	Y				Sx		1.4		?		No f/u-deceased
III-10	1	51	L adrenal							7.0		?		No f/u-deceased
IV-4	1	56	R adrenal					Autopsy finding		3.0		No		DOD
IV-6	1	37	L adrenal					Sx		8.5		Implied	11	
IV-6	2	48	R adrenal							5.0		?		No f/u-deceased
IV-10	1	69	R adrenal					Sx	Norepi	3.0	22.5	Y		7y2mo-deceased
IV-11	1	21	R adrenal		Y			Sx		3.0		Implied	6	
IV-11	1B	21	Preaortic (abd mets)		Y		Y	Sx		8.0	88.0		6	
IV-11	2	27	L adrenal		Y			Scrn					7	
IV-11	2B	27	Periadrenal (abd mets)		Y		Y	Scrn					7	
IV-11	3	34	Distant mets				Y		Epi, norepi			No		DOD
IV-12	1	72	R adrenal					Scrn	Norepi	0.6	5.1	Y		4y5mo
IV-13	1	24	L adrenal					Sx				No		DOD
IV-15	1	21	R adrenal					Sx	Nonfunctional	2.2	4.2	Implied	12	
IV-15	2	33	L adrenal					Scrn		6.0		Y		38y4mo
V-2	1	40	R adrenal					Sx	Nonfunctional	1.7				27y0mo
V-3	1	38	L adrenal					Sx	Norepi	2.8	27.1	Y		13y4mo

V-5	1	16	L adrenal					Sx		5.0		Implied	7	
V-5	2	23	Retroperitoneal, abd LN				Y						25	
V-5	3	48	Glomus vagale	Y										No f/u
V-10	1	38	L adrenal					Sx		2.5		Y		26y0mo
V-11	1	42	L adrenal					Sx	Norepi	6.8	152.0	Implied	10	
V-11	2	52	L adrenal bed		Y	Y	Y	Sx	Normet, norepi	1.2		N	1	
V-11	2A	52	L adrenal LN		Y							N	1	
V-11	3	53	Thoracic LN				Y-regional	Sx	Met, normet			N	1	
V-11	4	54	Mediastinal LN				Y	Sx	Normet, norepi	5.0		N	1	
V-11	5	55	Retroperitoneal LN		Y		Y	Scrn				N	2	
V-11	5B	55	L adrenal bed LN		Y		Y	Scrn				N	2	
V-11	5C	55	R abd mass	Y	Y			Scrn				N	2	
V-11	6	57	Cervical region		Y		Y-distant	Sx				N		
V-11	6B	57	Lung + multiple mets (chest, abd, pelvis, lung)		Y		Y-distant	Sx				N		2y8mo
V-13	1	31	Zuckerkandl	Y				Scrn	Normet	5.0	35.0	Y		3y8mo
V-16	1	6	Zuckerkandl	Y				Sx					5	
V-16	2	11	Liver				Y						25	
V-16	3	36	Liver, bowel, lung, peri-aortic LN				Y	Autopsy finding	VMA, norepi					DOD
V-17	1	25	R adrenal		Y			Sx		4.0		Y		31y11mo
V-17	1B	25	L adrenal		Y			Sx				Y		31y11mo
V-18	1	40	R adrenal					Sx	Norepi	2.5	4.0	Y		1y9mo
V-21	1	35	L adrenal		Y			Sx	Norepi	4.0	19.4	Y		3y0mo-deceased
V-21	1B	35	R adrenal		Y			Sx	Norepi	3.5	12.0	Y		3y0mo-deceased
V-21	1C	35	R Porta Cava	Y	Y			Sx	?	3.0	6.0	Y		3y0mo-deceased
VI-1	1	22	L adrenal					Scrn				Implied	8	
VI-1	2	30	R adrenal					Sx				Y		16y2mo
VI-2	1	24	R adrenal		Y			Sx		7.0		Y		13y0mo
VI-2	1B	24	R adrenal		Y			Sx		2.3		Y		13y0mo
VI-2	1C	24	R adrenal		Y			Sx		1.5		Y		13y0mo
VI-7	1	8	R adrenal					Sx	Norepi	2.3	6.0	Y	13	13y1mo
VI-7	2	21	L adrenal					Sx	Norepi, normet	4.8 (per CT)		awaiting rsxn		

VI-10	1	13	L adrenal					Scrn	Norepi	2.4		Y		17y3mo
VI-11	1	10	L periaortic	Y				Sx	Norepi	1.6		Implied	4	
VI-11	2	14	R adrenal					Sx	VMA	1.6		Y		18y4mo
VI-13	1	10	R adrenal					Sx	VMA	2.5		Implied	2	
VI-13	2	12	L adrenal						VMA			Y		20y3mo
VI-14	1	12	R adrenal					Sx	VMA	5.5	45	Implied	15	
VI-14	2	27	L adrenal					Sx	Norepi	1.5	8	Y		9y4mo
VI-16	1	9	R pararenal	Y				Sx		4		Implied	3	
VI-16	2	12	R adrenal					Sx		3.2		Implied	4	
VI-16	3	16	L adrenal		Y			Sx	Norepi	1.6		Y		4y4mo
VI-16	3B	16	L adrenal		Y			Sx	Norepi	0.7		Y		4y4mo
VI-16	3C	16	L extra-adrenal	Y	Y			Sx	Norepi	1.5		Y		4y4mo
VII-1	1	17			Y									
VII-1	1B	17			Y									
VII-4	1	12	L adrenal					Scrn	Norepi, normet	3.9	7.9	Y		3y1mo
VII-5	1	11	R adrenal		Y			Sx	Norepi, epi	2.0	6.0	Y		3y1mo
VII-5	1B	11	L adrenal		Y			Sx	Norepi, epi	2.5	5.0	Y		3y1mo
Family 2														
IV-1	1	47						Scrn				Y		21y0mo
IV-2	1	29	L adrenal							2.5	6.75	?		No f/u
IV-5	1	30												31y0mo
IV-6	1	28	R adrenal					Sx				Implied		
IV-6	2	40	R Paravertebral	Y		Y		Scrn				?		19y0mo
IV-10	1	41	L adrenal					Sx						23y0mo-deceased
IV-13	1	37	R adrenal					Sx				Implied	31	
IV-13	2	68	R adrenal bed		Y	Y		Sx	Norepi, VMA	5.0	89.5	Y		2mo
IV-13	3	68	L adrenal		Y			Sx	Norepi, VMA	1.2	40.0	Y		2mo
IV-13	4	68	Thoracic	Y	Y			Sx	Normet			Awaiting rsxn		
V-1	1	9						Sx				Implied	3	
V-1	2	12										Implied	6	
V-1	3	18												No f/u
V-2	1	28										?		

V-4	1	11	R adrenal					Sx	Norepi	3.0	200.0	Implied	17	
V-4	2	29	L adrenal					Sx	Norepi	3.0	13.3	Y		10y0mo
V-9	1	16										Implied	11	
V-9	2	27												11y0mo
V-14	1	15	L adrenal		Y			Scrn	VMA, norepi, epi	2.8		Implied	12	
V-14	1B	15	R adrenal		Y			Scrn	VMA, norepi, epi	3.0		Implied	12	
V-14	2	27	R paravertebral thoracic	Y				Sx	Norepi, met	4.0	35.0	no f/u tests		20y0mo
V-18	1	14	R adrenal		Y			Sx	Norepi, met?, VMA?	4.0	20	Y		32y0mo
V-18	1B	14	L adrenal		Y			Sx	Norepi, met?, VMA?	5.0	18	Y		32y0mo
V-18	1C	14	L adrenal LN		Y		Y-regional	Sx	Norepi, met?, VMA?	1.5		Y		32y0mo
V-19	1	11	L adrenal									Implied	22	
V-19	2	33	R adrenal					Scrn	VMA, norepi	2.8	30.0	Y	4	11y0mo
V-19	2B	33	L thoracic				Y	Scrn		6.5	60.0	Y	4	11y0mo
V-19	3	37	L lung metastasis				Y	Scrn				Y		11y0mo
V-20	1	9	L adrenal		Y			Sx	VMA, norepi	1.0	0.8	Y		34y0mo
V-20	1B	9	Zuckerkandl	Y	Y			Sx	VMA, norepi	1.3	1.3	Y		34y0mo
V-20	1C	9	R adrenal		Y			Sx	VMA, norepi	7.0	57.0	Y		34y0mo
V-21	1	8	R adrenal									Implied	21	
V-21	2	29	R adrenal bed		Y	Y				6.2	49.0	Y		
V-21	2B	29	L adrenal		Y					4.0	42.0	Y		10y9mo
V-22	1	10						Scrn				Y		29y0mo
V-23	1	14	R adrenal									Implied	13	
V-23	2	27	L adrenal					Scrn	Normet, met, VMA	3.3	8.2	Y		8y5mo
V-25	1	23	R adrenal					Sx	Norepi	4.5	10.1	Y		10y2mo
V-26	1	8	R adrenal		Y			Sx	Norepi, VMA	5.6	25.0	Implied	13	
V-26	1B	8	Zuckerkandl	Y	Y			Sx	?	2.0	4.4	Implied	13	
V-26	2	21	L adrenal					Scrn	Norepi	2.4	16.8	Y		10y4mo
V-29	1	14	R periaortic	Y	Y			Scrn	Norepi	3.0	8.0	?	1	
V-29	1B	14	L adrenal		Y			Scrn	Norepi	4.0	42.0	?	1	
V-29	2	15	R adrenal		Y			Sx	Norepi	1.5		Y		9y6mo
V-29	2B	15	R inferior renal hilum	Y	Y			Sx	Norepi	3.2		Y		9y6mo
V-30	1	12	L adrenal					Sx						2y0mo

V-31	1	16	Thoracic	Y	Y			Scrn	Norepi	3.0		Y		2y2mo
V-31	1B	17	Zuckerkandl	Y	Y			Scrn	?	1.5	2.4	Y		2y2mo
V-35	1	41	R adrenal		Y			Sx	Norepi, VMA	1.8	12.0	Y		3y0mo
V-35	1B	41	L adrenal		Y			Sx	Norepi, VMA	1.5	3.0	Y		3y0mo
V-36	1	31	L adrenal					Sx	Norepi, VMA	2.3	18.47	Y		6mo
V-37	1	24	R adrenal					Sx	Norepi	3.0	12.8	Y		4y0mo
V-40	1	36	Zuckerkandl	Y				Sx	Norepi	4.0		Y		1mo
VI-4	1	15										?		12y0mo
VI-6	1	18	R adrenal					Sx	Norepi	3.1		Y		2y9mo
VI-7	1	11	R adrenal		Y			Sx		5.5		Y		9y0mo
VI-7	1B	11	Zuckerkandl	Y	Y			Sx		3.5	10.0	Y		9y0mo
VI-7	1C	11	Zuckerkandl	Y	Y			Sx		1.4		Y		9y0mo
VI-7	1D	11	Zuckerkandl	Y	Y			Sx		0.9		Y		9y0mo
VI-9	1	10	R adrenal		Y							?		
VI-9	1B	10	L adrenal		Y							?		No f/u
VI-10	1	11			Y							?		
VI-10	1B	11	Periaortic	Y	Y							?		No f/u
VI-12	1	14	L adrenal		Y			Scrn	Norepi	4.2	37.5	Y		1y3mo
VI-12	1B	14	L adrenal		Y			Scrn	Norepi	1.8		Y		1y3mo
VI-16	1	9	Mid retroperitoneum (aortocaval space)	Y				Sx	Norepi	2.6		Y		5mo

Pheo, pheochromocytoma; Paragang, paraganglioma; Synch, synchronous; Recurr, recurrence; Mets, metastasis; Dx, diagnosis; Scrn, screening; Sx, symptoms; F/U, follow-up; LN, lymph nodes; Norepi, norepinephrine; Epi, epinephrine; Met, metanephrine

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