Genetic Characteristics of Pheochromocytomas: An Analysis of p16 Tumor Suppressor Gene Inactivation

A Senior Honors Thesis

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by

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Background

Epidemiology

Pheochromocytoma is a rare endocrine neoplasm which, if untreated, is most often fatal, but if diagnosed, can be safely excised in 90% of cases. Up to 10% of pheochromocytomas are familial, inherited by itself, as part of a MEN syndrome, or in association with a neuroectodermal dysplasia (Manger et al. 1993). Familial transmission is thought to be autosomal dominant, with varying degrees of penetrance (Cushman et al. 1962). MEN syndromes were first described in 1961, when Sipple characterized a syndrome of concomitant pheochromocytoma and medullary carcinoma of the thyroid (Sipple et al. 1961). This was later renamed as multiple endocrine neoplasia (MEN) type IIA, with the addition of parathyroid adenoma. In 100% of patients in families with the MEN IIA allele, medullary carcinoma of the thyroid develops; in 33%, pheochromocytoma is diagnosed (bilaterally in the majority), and 34% become hyper parathyroid (Cance et al. 1985). Other studies of MEN II families have shown genetic abnormalities. Eight of 10 MEN II kindreds were shown to have a point mutation of the RET proto-oncogene in chromosome 10, near its centromere, whereas the remaining two families were found to have mutations in the von Hippel-Lindau gene, present on chromosome 3 (Neumann et al. 1995).

These MEN II-associated pheochromocytomas tend to secrete substantially more epinephrine than norepinephrine. Thus, with the current management of screening MEN II family members at risk after undergoing mutational analysis, an elevated urinary epinephrine level may be the only abnormality suggesting the tumor in an asymptomatic patient. The neuroectodermal dysplasia is also associated with pheochromocytoma. Von Recklinghausen's disease, or neurofibromatosis, has one of the strongest relationships. The disease is characterized by multiple neurofibromas of peripheral nerves, presenting as subcutaneous or intradermal nodules. Other features include vascular and hairy nevi, cutaneous polyps, and café au lait spots. The prevalence of pheochromocytoma in patients with von Recklinghausen's disease is 1% to 2%. Conversely, 5% of pheochromocytoma patients have neurofibromatosis. Other neuroectodermal dysplasia linked to pheochromocytoma include Sturge-Weber syndrome, consisting of familial

hemangiomas, angiomatous brain malformations, seizures, tuberous sclerosis, mental deficiency, epileptiform seizures, and multiple adenoma sebaceum (Bonzani et al. 1999).

Pheochromocytomas can occur at any age, from infancy to old age, being quite uncommon after age 60. They account for only 0.1% to 0.2% of all cases of diastolic hypertension (Manger et al. 1997), and they have an incidence of 0.005% to 0.1% in the general, unscreened population. Pheochromocytomas occur at a rate of about 1 in 1,000,000 to 1 in 50,000 persons per year and about 1 in 1,000 hypertensive individuals (Cryer et al. 1985). As many as 800 persons may die annually in the United States as a result of associated complications (Graham et al. 1951). One review cites a slight female predilection (Chong et al. 1974); others show no difference in incidence (Melmon et al. 1965). Additionally, another study has shown a slightly more common incidence in the right adrenal gland (Samaan et al. 1988). Ten to twenty percent of all patients with pheochromocytomas are children. These tumors display some different characteristics than those of adults. Roughly two-thirds of the pediatric pheochromocytoma populations are prepubertal boys; the tumors have a much higher likelihood of being multiple (32%), bilateral (24%), and extraadrenal (15%-31%) than in adults (Stackpole et al. 1963).

Traditionally, 10% of all pheochromocytomas were believed to lie outside the adrenal medulla. A recent review by Whalen and colleagues summarized the literature to date and reported the incidence to be 18%. Eighty-five percent of extraadrenal pheochromocytomas are infradiaphragmatic; 98% of all pheochromocytomas lie below the diaphragm, with 1% residing in the cervical region and the remaining 1% being located intrathoracically (Whalen et al. 1992).

To better delineate the nature and location of these extraadrenal tumors, the term *paraganglion system* was developed as a replacement for the term *pheochromocytoma*. It is defined as a widely dispersed collection of specialized neural crest cells, arising in association with the segmental or collateral autonomic ganglia, which migrate to one of three final destinations (Bonzani et al. 1999):

1. Adjacent to arterial vessels and cranial nerves of the head and neck
2. Alongside the sympathetic plexus and chains, extending from the neck to the pelvis
3. Adrenal medulla

Thus, these extraadrenal tumors can occur anywhere along the sympathetic nervous system. Certain variants are specifically named. A *chemodectoma* arises from paraganglia of the carotid body. A *glomus jugulare* tumor has its origins in paraganglia of the intracranial branches of the ninth and tenth cranial nerves. *Ganglioneuromas* arise from postganglionic sympathetic neurons. All extraadrenal chromaffin tissues behave similarly to those within the adrenal medulla. Even though trends are emerging to support use of these anatomic descriptions, the label of "pheochromocytoma" is still very much ingrained into the medical literature, and because of its universal recognition, it is used here to apply to all variants of these neural crest tumors (Bonzani et al. 1999).

Symptoms

The clinical presentation of pheochromocytoma is attributable to the physiologic effects of its circulating catecholamines. The predominant sign is hypertension. Half of cases yield sustained hypertension, whereas approximately 45% of patients experience paroxysms of high blood pressure. The remaining 5% are normotensive. It is highly unusual to correctly diagnose pheochromocytoma in the absence of hypertension, except in multiple endocrine neoplasia II (MEN II) family members at known risk, who have biochemical screening performed. Hypotension can occur, and some patients may even alternate between hypertensive and hypotensive episodes. Another important diagnostic clue is the evaluation for pheochromocytoma in postural hypotension. When seen in a hypertensive patient not on hypertensive medication, it raises strong clinical suspicion for the presence of pheochromocytoma. Probable mechanisms for this include autonomic insensitivity, resulting from excessive circulating catecholamines, and hypovolemia. Postural hypotension is more common in sustained hypertension (Bonzani et al. 1999).

Headache is by far the most common symptom in pheochromocytoma, occurring in 92% of paroxysmal patients and 72% of those with stable hypertension (Manger et al. 1993). Hyperglycemia is seen commonly. In patients with preexisting diabetes mellitus, impaired glucoregulation develops, sometimes presenting with diabetic ketoacidosis. This impaired glucose metabolism may be due to beta-adrenergic receptor desensitization as a modulator of insulin resistance (di Paulo et al. 1989).

Hypocalcaemia is noted, even in the absence of the MEN syndromes, which resolves after surgical resection of the tumor. In most cases, this is apparently related to the secretion of parathyroid-related peptide, which does not cross-react with any of the parathyroid hormone (PTH) assays currently used. Thus, PTH levels will be suppressed if measured. Nausea and vomiting may occur, especially in conjunction with severe headache. Weakness and weight loss are present, despite a normal or increased appetite. Chest or abdominal pain may mimic coronary artery disease or an acute abdomen. Excessive catecholamines decrease intestinal mobility and result in severe constipation, whereas the rare VIP-secreting tumor predisposes the patient to diarrhea. These paroxysmal "spells" are elicited by multiple stimuli, including pain, tumor manipulation (such as an abdominal examination), exercise, anxiety, bladder distention or voiding (in the case of bladder pheochromocytomas), constipation, trauma, sexual intercourse, diagnostic procedures, radiographic contrast media, surgery, and anesthesia. Additionally, certain foods, for example, tyramine-containing cheeses, beer, and wines, as well as medications, such as phenothiazines, tricyclic antidepressants, ACTH, histamine, glucagons, beta-blockers, metoclopramide, nicotine, angiotensin II analogues, and epinephrine, can all promote an attack (Bonzani et al. 1999).

Diagnosis

Biochemical confirmation of pheochromocytoma is crucial before proceeding to definitive treatment, and the cornerstone of diagnosis remains the measurement of 24-hour urinary catecholamine levels. Major substances that are currently used for pheochromocytoma detection include plasma catecholamine, urinary free catecholamine, urinary metanephrine, and normetanephrine, as well as urinary vanillylmandelic acid (VMA) levels. Metanephrine and VMA represent two of the main metabolites of norepinephrine and epinephrine (Bonzani et al. 1999). The usefulness of these assays arises from the fact that 95% to 99% of patients with pheochromocytoma have elevated levels of catecholamines in their blood or urine (Sjoerdsma et al. 1966).

A finding of elevated urinary catecholamines does not eliminate other diseases. The differential diagnosis of increased urinary catecholamines includes intracranial lesions, carcinoid syndrome, acute porphyria, factitious hypertension, clonidine

withdrawal, and hypoglycemia. Provocative and suppressive testing may be used for the minority of patients with equivocal findings, although their role in the diagnosis of pheochromocytoma has diminished sharply (Bonzani et al. 1999).

Following the biochemical confirmation of pheochromocytoma, radiologic localization of the tumor is necessary to plan definitive surgical therapy. Not only does imaging delineate location, but it also aids in defining singularity, bilaterality, and possible multicentricity. Unsuspected metastatic lesions in bone, liver, or the lymph nodes may also be visualized. The three major modalities currently in use are computed tomography (CT) scans, magnetic resonance imaging (MRI), and 131-I-metaiodobenzlguanidine-labeled nuclear scanning (MIBG). Additionally, imaging with positron emission tomography scans using isotope-labeled glucose or ephedrine, and scintiscans, with indium-labeled octreotide, are being evaluated in a number of endocrine centers. A CT scan is the most commonly used imaging test for pheochromocytoma. A CT scan can identify approximately 95% of all pheochromocytomas – it has the capacity to detect 1-cm tumors within the adrenal gland and 2-cm extraadrenal lesions. Several disadvantages to the CT scan have prompted some centers to adopt MRI as their first imaging modality. The CT scan entails radiation exposure, and it does require intravenous contrast material, with its risk of inducing a paroxysm (Bonzani et al. 1999). Not only does MRI define the anatomy but, on T2-weighted images, pheochromocytomas and paragangliomas show a characteristic and nearly unique high-intensity signal. Other advantages that may cause MRI to challenge CT scanning as the optimal localization test include its lack of radiation exposure, the clear definition of surrounding vascular structures, and the lack of interference from pre-existing metal clips. MIBG scan uses an agent that concentrates in the adrenergic vesicles. It has a sensitivity of 80% to 90%. Some centers have used MIBG as the primary modality to identify pheochromocytomas and rule out synchronous lesions or metastatic disease. Unfortunately, the iodine isotope is not readily available because of its short half-life and expense. The advantage of MIBG localization is offset by its limited availability and the cumbersome protocol for its use. The thyroid must be blocked with iodine before and after administration of the radioactive iodine (Roman et al. 2001).

Pathology

Pheochromocytomas are of variable size, ranging from 1 cm to several kilograms, but are normally between 50 and 200 g. In sporadic pheochromocytomas, even though lobulated, the tumor is actually a single neoplasm. In MEN 2, even though synchronous bilateral pheochromocytomas may not be present, the adrenal medulla of the contralateral adrenal will be hyperplasic and is thought to represent pretumor change analogous to C-cell hyperplasia and medullary thyroid carcinoma. Fed by a rich blood supply, pheochromocytomas often have a purplish-gray hue when resected and frequently show demarcated areas of cystic necrosis. As stated previously, nearly 90% of pheochromocytomas are located within the adrenal glands. The remaining 10 to 15% are found from the neck to the bladder, typically along the course of the sympathetic chain (GRANT et al. 1997).

Pheochromocytomas arise from chromaffin cells, which are of neuroectodermal origin. At about the fourth week of gestation, this neuroectodermal tissue separates away from the adjacent, newly formed neural tube. It eventually becomes positioned between this neural tube and the true ectoderm. This tissue is then neural crest, and cells from this crest begin migrating throughout the developing embryo, ultimately differentiating into the peripheral sympathetic nervous system, paraganglionic system, and adrenal medulla. These embryologic relationships help one better understand the coexistence of other diseases, such as the von Hippel-Lindae disease, with pheochromocytoma; affected tissues in these disorders are derived from adjacent neuroectodermal areas. Synthesis of norepinephrine begins in the cytoplasm of the cells. This metabolic pathway continues until the compound dopamine forms and is then taken up by intracellular granules and converted to norepinephrine by the enzyme dopamine beta-hydroxylase. Granular concentration of norepinephrine establishes equilibrium with free norepinephrine that freely diffuses into the cytoplasm of the cell; free norepinephrine can be converted into epinephrine via the action of phenyl ethanolamine n-methyl transferase. This newly formed epinephrine can be readily stored in separate intracellular granules within the cell (BONZANI et al. 1999).

Microscopically, tumor cells closely resemble normal adrenal medulla. They are arranged in alveolus-shaped clusters, separated by endothelium-lined spaces. Numerous

vessels are present, reflecting the abundant vascularity of these tumors. Tumor cell cytoplasm may be granular, basophilic, or eosinophilic. Nuclei are round or oval, with prominent nucleoli. Mitotic figures are scarce. Nuclear gigantisms and hyperchromatasia are seen but are not indicators of malignancy. As a rule, histologic appearance alone cannot distinguish between benign and malignant tumors (SCHLUMBERGER et al. 1992). Currently, the only definitive criterion for malignancy is the spread of the tumor to distant sites: lymph nodes, bone, lung, liver, brain, and the central nervous system are the most common sites. Because histologic examination cannot diagnose malignancy, some investigators have tried to identify any prognostic factors that may imply a malignant course. Tumor size and local tumor extension at the time of surgical exploration have been suggested, as has DNA ploidy. In a study of 184 pheochromocytoma patients by Nativ and colleagues, 84% of those with vascular endothelial invasion and 100% of patients with regional or distant metastases had either aneuploid or tetraploid DNA content. All 12 patients dying of their disease had abnormal DNA ploidy, whereas no patient with diploid DNA died (NATIV et al. 1992).

Treatment

Once the diagnosis of pheochromocytoma is firmly established, the patient must be prepared for surgical resection. Optimization of the patient requires adequate blood pressure control and correction of intravascular fluid deficits. Sometimes this is not feasible; conditions mandating emergent surgical removal include malignant hypertension, acute cardiovascular complications (sporadic ventricular arrhythmias, impending heart failure), and abdominal morbidity (tumor hemorrhage). In most cases, however, preoperative management can be instituted. The mainstay of preoperative treatment is alpha-adrenergic blockade. Instituted 1 to 3 weeks before surgery, this normalizes blood pressure, controls paroxysms, and allows for intravascular volume expansion. Phenoxybenzamine (Dibenzyline) is the drug of choice because of its irreversible binding and its 100-fold greater potency at the alpha-1, rather that the alpha-2, receptor. Phentolamine (Regitine) is a competitive norepinephrine antagonist that also may be used preoperatively. This agent is less effective than phenoxybenzamine, as its effects are transient (requiring more frequent dosing intervals). Side effects are prominent

and include nausea, vomiting, nasal congestion, and ejaculatory dysfunction, all to a greater degree than that of the other alpha-blockers. For these reasons, phentolamine is essentially reserved for intraoperative hypertensive crises. Other pharmacologic management will center around beta-adrenergic blockade. Treatment with beta-blockade is indicated for the appearance of tachycardia (greater than 140 beats per minute) and arrhythmias while on alpha-blocker therapy, as well as for purely epinephrine-secreting tumors. The standard agent is propranolol, a competitive beta-receptor blocker. Beta-blockade is to be instituted only after alpha-blockade has been achieved. Unopposed beta-blockade can cause worsening hypertension by blocking the vasodilatory effects of epinephrine, resulting in enhanced pressor response to norepinephrine. More cardioselective beta-blockers such as metoprolol and atenolol may decrease the risk. Beta-blockers must be used with caution in elderly patients and those with proven or suspected heart disease, because it may promote profound bradycardia, myocardial depression, and congestive heart failure. When used, they have been commonly instituted 3 to 7 days before surgery (Bonzani et al. 1999).

Surgery

The treatment of pheochromocytoma is surgical extirpation. Close intraoperative monitoring with central venous and arterial pressure monitoring, an experienced anesthesiologist, and careful use of anesthetics with minimal myocardial depressant effects are important for safe surgery. The use of hemocynamic stabilizing drugs such as nitroprusside, phentolamine, and esmolol are commonly required at different stages of the procedure. Regardless of the location of the pheochromocytoma, the most critical part of the surgery is the ligation of the adrenal vein. The patient may become hypotensive because of the short half-life of catecholamines. The anesthesiologist needs to be warned when this point of the surgery has been reached. A variety of incisions and approaches have been used for either unilateral adrenal resection or bilateral excision with wide retroperitoneal lymphadenectomy or extraadrenal tumor resections. The open anterior approach allows access to both adrenal glands and the intraabdominal and retroperitoneal areas. The open approach enables the resection of other organs as necessary in genetic syndromes such as VHL or NF1. The open lateral or retroperitoneal approach may use

30- to 45-degree lateral decubitus positioning of the patient and subcostal/flank incision, and the open posterior approach has patients in the prone position, and a "J" or "hockey stick" incision is made curving along the 12th rib, which is then resected for better exposure. This approach is more difficult for larger tumors and involves more manipulation of the tumor than the anterior approach. The open thoracoabdominal approach is seldom employed and is most often used with very large, malignant tumors when en bloc resection is necessary. The patient is positioned in a slight lateral angle, and the abdominal cavity is not necessarily entered, unless needed (ROMAN et al. 2001).

Since the early 1990s, laparoscopic adrenalectomy has become the procedure of choice for tumors without overt signs of malignancy, such as locoregional invasion, adenopathy, or metastases. It is clearly superior to the open approaches by reducing postoperative hospital stays, return to normal physical activity, and wound complications. The most commonly employed approach is the transabdominal lateral approach. The patient is placed in flexed 90-degree decubitus position, allowing gravity to help in organ dissection. For bilateral adrenalectomy, the patient may be turned from side to side for respective adrenal gland resection with excellent results. In patients with MEN 2 syndrome who present with bilateral adrenal pheochromocytomas, a bilateral laparoscopic total adrenalectomy will be curative in most patients, yet it will render the patients anadrenal. The laparoscopic retroperitoneal approach may be used if the patient has significant intraabdominal adhesions from prior surgery, which may preclude a laparoscopic transabdominal approach. It is difficult for tumors larger than 4 to 5 cm (ROMAN et al. 2001).

Post-op

Most patients will have an uneventful postoperative course, yet rebound hypotension may occur in some patients if phenoxybenzamine is used, given its long half-life. Fluid and occasional vasopressor support is necessary. In patients who may have developed catecholamine excess cardiomyopathy, treatment of postoperative hypotension with fluid resuscitation can be hazardous. This complication can be avoided if adequate alpha 1-specific blockade is achieved preoperatively. Approximately half of all patients will remain hypertensive long term. This may be because of chronic vascular

or metabolic changes from prolonged exposure to catecholamines, or to preexisting essential hypertension. Because pheochromocytomas may recur or have metastatic development many years after extirpation of the primary tumor, patients should have lifelong follow-up with yearly or biannual biochemical screening. Imaging studies are warranted only if there is evidence of catecholamine excess. The lifetime rate of recurrence or metastatic development is 6.5% to 15%. Surgical extirpation is curative in up to 85% to 90% of patients. The development of laparoscopic surgical approaches has had a significant impact on the recovery and wound complications of surgery (ROMAN et al. 2001).

Introduction

Pheochromocytoma is a rare neuroendocrine tumor histologically characterized by chromaffin tissue and is comprised of catecholamine-containing neurosecretory granules. Pheochromocytomas are primarily located in the adrenal medulla, but may also arise in the dorsal root ganglia of the sympathetic nervous system. Pheochromocytomas cause endocrine hypertension by oversecretion of catecholamines. Such hypertension can be sustained, or paroxysmal, and may lead to death from cardiovascular or cerebrovascular disease (KOCH et al. 2001). Although pheochromocytomas are known to be associated with several hereditary syndromes, relatively little is known about other genetic abnormalities involved in their tumorigenesis.

Abrogation of the *Rb/p16* tumor-suppressive pathway is thought to be an important mechanism in the development of many human cancers (KAMB et al. 1994; SERRANO et al. 1993; NOBORI et al. 1994). This pathway is a well described component of global cell cycle regulation and inactivation may occur through alterations of *Rb*, *CDK4*, *cyclin D*, or *p16*. These alterations occur almost universally exclusively of each other, indicating that only one event is necessary for abrogation of the pathway. The *p16* gene product is a 16 Kd protein that inhibits formation of cyclin D/CDK4 complexes. Consequent phosphorylation of the Rb protein results in the release of activated transcription factors and progression of the cell cycle through the G1/S checkpoint. Inactivating *p16* gene alterations occur frequently in a number of human cancer cell lines and primary tumors (NOBORI et al. 1994). Mechanisms of inactivation include

homozygous deletion, mutation, and aberrant methylation of 5' CpG islands (HERMAN et al. 1995; MERLO et al. 1995; GONZALEZ-ZULUETA et al. 1995). Research previously performed in the laboratory demonstrates that inactivating alterations of *p16* occur frequently in chemically induced hamster pancreatic tumors (LI et al. 2004) and human pancreatic endocrine tumors (MUSCARELLA et al. 1998). Pheochromocytomas are a rare endocrine tumor and data regarding the role of *p16* inactivation in these tumors is currently limited. The purpose of this study was to determine the frequency of *p16* inactivation in pheochromocytoma specimens obtained at the time of surgical resection at The Ohio State University Medical Center.

All tissue specimens were obtained from patients undergoing surgical resection of pheochromocytomas, and foci of tumor cells were identified by a pathologist under light microscopy after staining with hematoxylin and eosin. To test the described mechanisms of inactivation, the genetic status of p16 gene in pheochromocytoma specimens was analyzed. The techniques of multiplex real-time PCR, direct DNA sequencing, and methylation-specific PCR were used to evaluate tumor specimens for homozygous deletions, point mutations, and abberant methylation of 5' CpG islands, respectively. In order to determine whether potentially inactivating alterations were associated with loss of *p16* expression, immunohistochemical analysis of tissue sections was performed using p16 monoclonal antibodies.

Materials and methods

Tumor specimens and genomic DNA extraction

Seventeen pheochromocytoma tumors from 15 patients (there were two patients with bilateral tumors) were obtained at the time of surgical resection at The Ohio State University Medical Center. Following resection, the tissues were fixed in formalin and paraffin-embedded according to routine protocol. The diagnosis of pheochromocytoma was made preoperatively based on clinical presentation, urine biochemical analysis, and radiologic imaging for tumor localization. Histological analysis confirmation of the tumor type was made by staff pathologists after hematoxylin and eosin staining. The study was approved by the Institutional Review Board.

A ChargeSwitch gDNA mini tissue extraction kit was used for genomic DNA extraction. Two cores of tumor (10 mg) were separated from archived paraffin blocks and confirmed by the pathologist (WLF). These were digested with lysis buffer (L13) and proteinase K at 65˚C overnight. RNaseA was added to each of the microcentrifuge tubes containing these tissue samples after cooling. This liquid was transferred to a fresh tube and centrifuged at maximum rpm. Supernatants were placed into fresh tubes and treated with purification buffer (N5) by tip-mixing. Magnetic beads were added and evenly suspended for a short incubation period at room temperature. The target DNA was bound to these beads at this point. The beads were separated on the ChargeSwitch MagnaRack, allowing the remaining supernatant to be removed with pipettes and discarded. The beads were fully redispersed in wash buffer (W12), and then separated on the MagnaRack two additional times. Resuspension in elution buffer (E5) by tip-mixing was performed in order to separate magnetic beads from the target DNA. Overnight incubation in a 55˚C water bath was performed in order to improve the efficiency of this detachment. The microcentrifuge tubes were separated on the MagnaRack and the solution containing purified genomic DNA was transferred into fresh tubes and stored at -20˚C until needed for further analyses. The amount of DNA yielded from purification was determined by spectrophotometry.

Real-time PCR to detect homozygous deletions in human p16 exon 2

Amplification reactions were carried out using the Cepheid Smart Cycler. Forward (5'-GGCTCTACACAAGCTTCCTTTCC-3') and reverse (5'-TCATGACCTGCCAGAGAGAACA-3') primers (Life Technologies) were used at a final concentration of 0.2 μM. The dual-labeled fluorogenic probe (5'-FAM-CCCCCACCCTGGCTCTGACCA-TAMRA-3') was used at the final concentration of 0.1 μM (CARTER et al. 2001). Each reaction was performed in the presence of 200 uM dNTPs, 3 mM MgSO4, 1.25 units of Taq polymerase (Stratagene), 1X Additive (1 mg/ml BSA, 750 mM Trehalose and 1 % Tween 20), and 2.0 μl of genomic DNA (containing about 5 to 30 ng DNA). The sample was subjected to 96°C for 1 min, followed by 55 cycles of 30 s at 95°C, 30 s at 62°C, 45 s at 68°C. A final extension step was performed at 68°C for 5 min. A housekeeping gene, β-actin was co-amplified as an internal control.

The primers and fluorogenic probe for the internal controls are: forward primer, 5'-AGCGCGGCTACAGCTTCA-3'; reverse primer, 5'-CGTAGCACAGCTTCTCCTTAATGC-3'; and the probe, 5'-TET-ATTTCCCGCTCGGCCGTGGT-TAMRA-3'. All experiments were performed in duplicate.

p16 gene deletion analysis

The accuracy of the above real-time PCR assay was verified using a series of mixtures of genomic DNA from human Hela cells (+/+) and Mia PaCa-2 cells (-/-) at various ratios (Hela:Mia PaCa-2 = 100:0, 75:25, 50:50, 25: 75, 0:100). Multiplex real-time PCR was performed and the resulting cycle threshold (Ct) values were normalized against those of Hela using the following equation:

$$\Delta\Delta Ct = \Delta Ct_{p16} - \Delta Ct_{\beta\text{-}actin},$$

where ΔCt_{p16} is equal to Ct_{p16} of a sample minus Ct_{p16} of Hela, and $\Delta Ct_{\beta\text{-}actin}$ is the net Ct $_{\beta\text{-}actin}$ of a sample deducted by Ct $_{\beta\text{-}actin}$ of Hela (Li et al. 2004). While $\Delta Ct_{\beta\text{-}actin}$ is correlated with the difference in total genomic DNA concentrations between the sample and Hela, ΔCt_{p16} is ascribed to the "total" difference in *p16* gene concentrations between the sample and Hela (wild type), including the difference in total genomic DNA concentrations and the difference in the *p16* gene dosage. Therefore, $\Delta\Delta Ct$ reflects the difference in the *p16* gene dosage. When the $\Delta\Delta Ct$ values were plotted against the relative ratio of normal *p16* DNA in the mixtures (in exponential form), a linear graph with a correlation coefficient of 0.992 was obtained, indicating that the relative concentration of *p16* gene can be accurately measured using this technique (Poi et al. 2001). On the basis of this observation, the *p16* gene dosage in 17 pheochromocytoma tumor specimens was determined using the above real-time PCR assay, and the results were interpreted as follows (Carter et al. 2001): relative concentration <25%, *p16* homozygous deletion (-/-); relative concentration >25% and <75%, *p16* hemizygous deletion (+/-); and relative concentration >75%, *p16* wild type (+/+).

Methylation-specific PCR of a CpG island inhuman p16 promoter

Genomic DNAs from tumor tissues were bisulfate-modified using the CpGenome DNA modification kit (Cat. # S7820, Serologicals, GA), and methylation at the promoter region of *p16* was analyzed using a CpG WIZ® *p16* Amplication Kit (Cat. # S7800, Serologicals, GA). The PCR mixture contained 1 X PCR buffer, 1 X enhancer, 1.5 mM $MgCl_2$, dNTPs (each at 1.25 mM), 1 unit of Platinum Taq DNA polymerase (Invitrogene), primers (0.2 μM each) and 10 μl of bisulfide-modified DNA in a final volume of 50 μl. Amplification was performed in a GeneAmp 9700 Thermal Cycler (PE Applied Biosystems) with PCR conditions of 95°C for 2 min followed by 40 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 60 s, and a final elongation step of 72°C for 5 min. Of note, the primer sequences in this kit are not disclosed due to proprietary reasons. The PCR products were analyzed by electrophoresis on a 10% PAGE gel. Fragments amplified from methylated and unmethylated *p16* gene were 145 and 154 bp, respectively, and both negative and positive controls were provided by the manufacturer.

Automatic sequencing of p16 exons 1 and 2

Human *p16* exon 1 was amplified by PCR using the following intron-based primers: for exon 1, 5'-GCT GCG GAG AGG GGG AGA GCA GGC A-3' (forward) and 5'-GCG CTA CCT GAT TCC AAT TC -3' (reverse) (Poi et al. 2001). Exon 2 was amplified in two fragments (2a and 2b) with at least one of the primers in a set located within an intron to exclude coamplification of a similar gene family member or potential pseudogenes. The primers for 2a are 5-ACA AGC TTC CTT TCC GTC ATG CCG-3' (forward) and 5-CCA GGC ATC GCG CAC GTC CA-3 (reverse), while the 2b primers are 5-TTC CTG GAC ACG CTG GTG GT-3 (forward) and 5-TCT GAG CTT TGG AAG CTC TCA G-3 (reverse). Potential mutations at exon 3 were not analyzed due to the following fact that exon 3 represents only 3% (12 bp) of *p16* gene and mutations at this region do not cause significant changes in the structure and function of P16 protein. The PCR mixture contained 1 X PCR buffer, 1.5 mM $MgCl_2$, dNTPs (each at 1.25 mM), 1.0 μM of each primer, 1 unit of Taq DNA polymerase (Invitrogene), 1.5 X enhancer, and 4 μl of genomic DNA in a final volume of 50 μl. The whole procedure included: 96°C for 2 min (1 cycle); 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C (50 cycles); 5 min

at 72°C (1 cycle). The PCR products were purified using a PCR Product Purification kit (Qiagen), and subsequently sequenced using an ABI 377A automated DNA sequencer. Both strands of the PCR-amplified fragments were sequenced to confirm the mutations.

Tissue Microarray (TMA)

The methods for TMA creation have been described previously (*Arch Pathol Lab Med* 2005, 129: 1100-5). Briefly, formalin-fixed, paraffin-embedded tissues were obtained from the archival files at the Ohio State University Department of Pathology. Two tissue cores (1.5 mm diameter each) were punched out of each donor paraffin block and transferred to each of the recipient TMA blocks using a precision instrument (Beecher Instruments, Silver Spring, MD). Paraffin embedded tissue was cut at 4 microns and placed on positively charged slides then heated to 40°C for 30 minutes. After leveling paraffin and cores, the array was cooled to 4°C for 15 minutes. Cores were also obtained from adjacent normal adrenal tissue.

Immunohistochemistry (IHC) for p16

TMA slides were placed in a 60 °C oven for 1 hour, cooled, and deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 minutes in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase. Antigen retrieval was performed by a heat method in which the specimens were placed in a citric acid solution (Target Retrieval Solution, pH 6.1, DakoCytomation, Carpinteria, CA) for 25 minutes at 94°C using a vegetable steamer and cooled for 15 minutes in solution. Slides were placed on a DakoCytomation Autostainer immunostaining system for use with immunohistochemistry. The primary antibody was added using Cell Marque's (Hot Springs, Ark) monoclonal anti-p16 (clone 16PO4) at a dilution of 1:20 and incubated for 60 minutes. A labeled streptavidin-biotin complex was used for detection and visualization occurred after the application of a 3,3'-diamino-benzidine chromogen. Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions and cover-slipped. Positive and negative controls stained appropriately. All TMA slides were read by a senior pathologist (WLF).

The percentage of cells with convincing nuclear p16 staining was determined for each sample.

Results

Seventeen tumor samples obtained from fifteen patients were analyzed for *p16* genetic alterations and altered expression by the techniques described above. The genetic data are summarized in Table 1. Real-time PCR-based deletion analysis indicated that two specimens harbored hemizygous deletions (+/-) and two specimens harbored homozygous deletions (-/-) for a total frequency of 23.5%. There were no *p16* mutations identified by direct sequencing. Methylation-specific PCR analysis indicated 5' CpG island methylation in 47.1% of specimens. Overall, 64.7% of specimens demonstrated evidence of potentially inactivating *p16* deletions or methylation. One tumor (specimen #1) demonstrated both methylation and hemizygous deletion, suggesting that the undeleted allele was suppressed by methylation. Genomic DNA extracted from histologically normal appearing adjacent adrenal tissue was assessed for each specimen, and no potentially inactivating *p16* abnormalities were identified.

Table 1. Genetic analysis of p16 alterations in pheochromocytoma specimens.

Patient/Specimen	Deletion Analysis	Methylation Status	Mutation Analysis	Inactivation
1	+/-	+	-	+
2	-/-	-	-	+
3	+/+	-	-	-
4R	+/+	+	-	+
4L	+/+	-	-	-
5	+/+	-	-	-
6	+/+	+	-	+
7	+/+	+	-	+
8	+/-	-	-	+
9	+/+	-	-	-

10	+/+	-	-	-
11R	+/+	-	-	-
11L	+/+	+	-	+
12	+/+	+	-	+
13	-/-	-	-	+
14	+/+	+	-	+
15	+/+	+	-	+

The IHC data were difficult to interpret because of lack of sufficient cells or tissue in 52.9% of specimens. In general, lack of nuclear *p16* IHC staining corresponded with the data from the DNA analysis (Figure 1**)**. Two specimens lacking identifiable inactivating events demonstrated loss of *p16* expression (>50% decrease in nuclear staining) when compared to matched-normal tissues. It is possible that *p16* expression may have been inactivated by alternative mechanisms in these patients. When this is considered, decreased *p16* expression and/or potentially inactivating events occurred in 76.5% of the specimens analyzed. Patient #11 was one of two patients noted to have bilateral pheochromcytomas. The left-sided tumor was positive for *p16* methylation while the right-sided tumor demonstrated no potentially inactivating alterations. In this instance, the genetic data correlated nicely with the IHC data (5% vs. 25% nuclear staining). No *p16* abnormalities were identified in either tumor in the only other patient with bilateral tumors (patient #4), and both tumors demonstrated relatively high nuclear expression of *p16* by IHC. All tumors demonstrating relatively high expression of p16 (>25%) did not contain potentially inactivating p16 alterations.

Discussion

Like many endocrine tumors, pheochromocytoma is rarely seen but commonly discussed. Hereditary syndromes have been known to be associated with pheochromocytomas for many years and the molecular basis for these syndromes is currently under investigation (LENDERS et al. 2005). There are currently four hereditary syndromes associated with pheochromocytoma development, including multiple

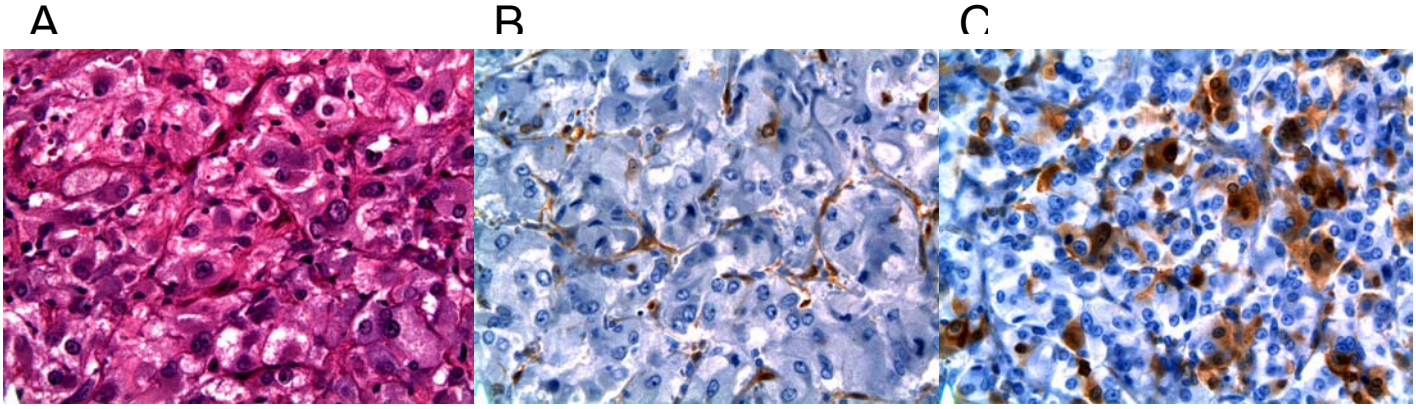

Figure 1. Pheochromocytoma immunohistochemical data. A) H&E stain; B) p16 negative. Note that there is some staining in the endothelial cells; C) p16 focally positive. (All images are 40x magnification).

endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau (VHL) disease, neurofibromatosis type 1 (NF 1), and hereditary paraganglioma and SDHD gene-related tumors (Koch et al. 2001). As yet unidentified genes are also felt to be responsible for an additional number of familial cases. In the past it has been accepted that 10% of pheochromocytomas are familial, but recent data indicate that up to 12-33% of patients with presumably sporadic pheochromcytomas harbor germline mutations of VHL, RET, SDHD, or SDHB (Neumann et al. 2002, Gimenez-Roqueplo et al. 2003, Amar et al. 2004). These findings have resulted in the establishment of less stringent criteria for genetic screening of affected individuals in the hope that surveillance will result in earlier detection of recurrent or associated tumors in patients and earlier detection of tumors in relatives.

Despite the advances that have been made in clinical cancer genetics for patients with familial pheochromocytomas, relatively little is known about the genetic changes that occur during the pathogenesis of sporadic pheochromcytomas. Surprisingly, RET, VHL, SDHB, and ADHD mutations do not appear to play a significant role in the development of sporadic pheochromocytomas (Maher et al. 2002). Furthermore, there is also a paucity of data regarding secondary molecular events contributing to pheochromocytoma tumorigenesis. Mutations in *c-mos*, *p53*, *endothelin B, RASSF1A, ras* and *MEN1* have not been identified in series of screened tumors (Dannenberg et al. 2003). Although increased expression of *c-myc* mRNA has been identified in malignant

pheochromocytomas, no amplifications or rearrangements of the encoding genes have been seen (GOTO et al. 1990).

Abrogation of the *Rb/p16* tumor-suppressive pathway is thought to be an important mechanism in the development of many human cancers (KAMB et al. 1994, SERRANO et al. 1993, NOBORI et al. 1994). This pathway is a well-described component of global cell cycle regulation and inactivation may occur through alterations of *Rb*, *CDK4*, *cyclin D*, or *p16*. These alterations occur almost universally exclusively of each other, indicating that only one event is necessary for abrogation of the pathway. The *p16* gene product is a 16 Kd protein that inhibits formation of cyclin D/CDK4 complexes. Consequent phosphorylation of the Rb protein results in the release of activated transcription factors and progression of the cell cycle through the G1/S checkpoint (Figure 2**)**. Inactivating *p16* gene alterations occur frequently in a number of human cancer cell lines and primary tumors (NOBORI et al. 1994). Mechanisms of inactivation include homozygous deletion, mutation, and aberrant methylation of 5' CpG islands (HERMAN et al. 1995; MERLO et al. 1995; GONZALEZ-ZULUETA et al. 1995). As stated previously, current data regarding alterations of *Rb/p16* tumor-suppressive pathway in pheochromocytomas are limited, although loss of *Rb* expression, as determined by IHC, has been identified in 40-70% of adrenal pheochromocytomas (LAM et al. 2001; GUPTA et al. 2000).

We report here the first evidence of *p16* homozygous and hemizygous deletions in human pheochromocytoma specimens. Aguiar had previously used a semi-quantitative, standard multiplex PCR assay and found no homozygous deletions in 26 analyzed specimens (AGUIAR et al. 1996). In that study, tumors were not evaluated for mutations or hypermethylation, and the findings were not confirmed by IHC. Although an appropriately designed gene dosing curve was used as a control and appeared to confirm the validity of the findings, we believe that the real-time based multiplex PCR performed in the current study is more sensitive. In a more recent study of 25 pheochromocytoma tumor specimens, *p16* hypermethylation was identified in 24% of tumor specimens (DAMMAN et al. 2005). Hypermethylation was noted to be particularly associated with hereditary pheochromocytomas, as 42% of familial tumors exhibited *p16* hypermethylation compared to only 8% of sporadic tumors. These findings are consistent

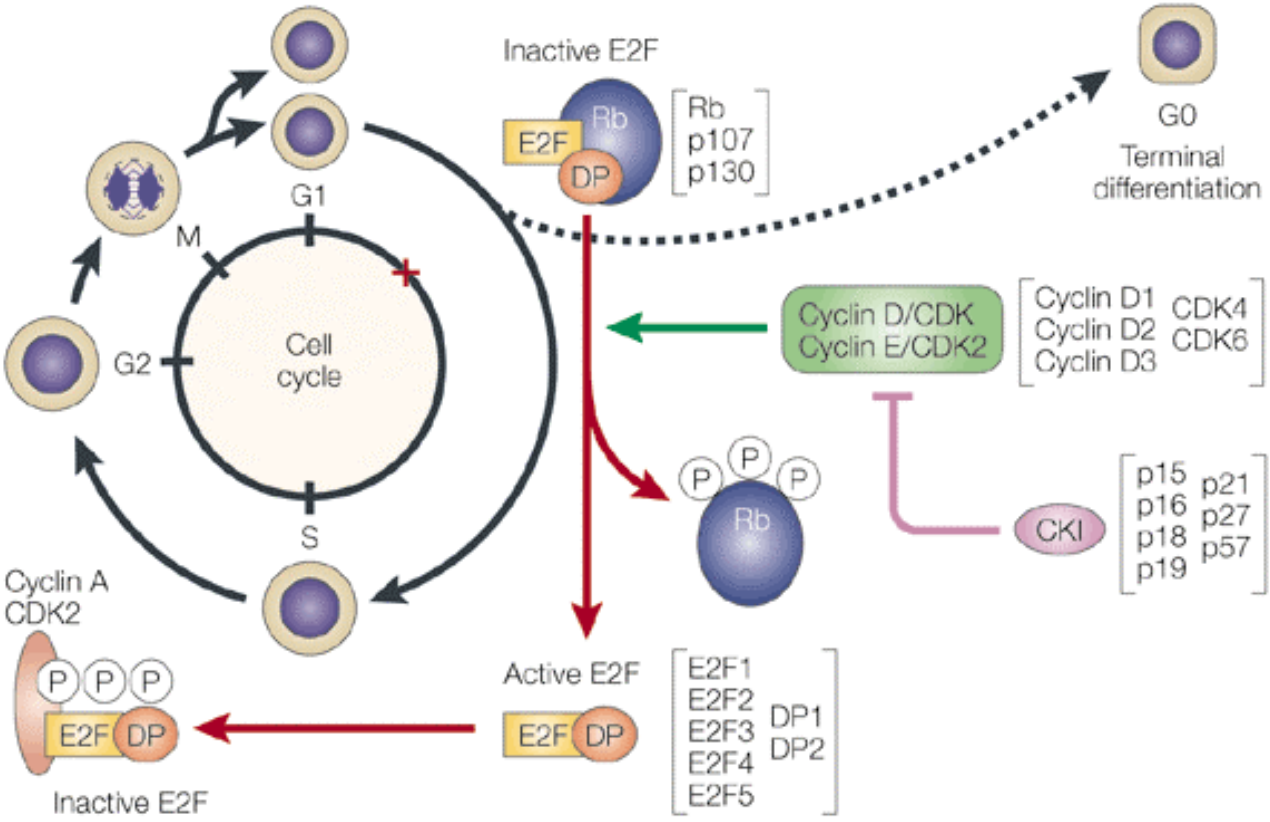

Figure 2. The p16/Rb pathway.

with our data, which show *p16* hypermethylation in 47.1% of specimens. Mutant mouse studies of *p16* +/- *PTEN* +/-, *p16* -/- *PTEN* +/- , and *p16* +/+ *PTEN* +/- mice indicate that there is functional synergy between *p16* and *PTEN* that is manifested most predominantly by an increased predisposition towards pheochromocytoma development in mice harboring hemizygous, and particularly homozygous, *p16* deletions (You et al. 2002). These data further support a role for *Rb/p16* pathway alterations in pheochromocytoma tumorigenesis.

In conclusion, we report here that *p16* alterations, by deletion (23.5%) and methylation (47.1%), occur commonly in human pheochromcytoma specimens with an overall total of 64.7% of specimens exhibiting some type of genetic abnormality. The data are generally supported by the IHC findings, which indicate that decreased

expression of *p16* might occur in up to 76.5% of specimens. These findings support further evaluation of *Rb/p16* pathway abnormalities in pheochromocytomas and contribute to our current understanding of pheochromocytoma pathogenesis.

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