Transcriptional Regulation of Phenylethanolamine *N*-Methyltransferase in Pheochromocytomas from Patients with von Hippel–Lindau Syndrome and Multiple Endocrine Neoplasia Type 2

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ABSTRACT: Pheochromocytomas in multiple endocrine neoplasia type 2 (MEN-2) express phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that catalyzes conversion of norepinephrine to epinephrine, whereas those in von Hippel–Lindau (VHL) syndrome do not. Consequently, pheochromocytomas in MEN-2 produce epinephrine, whereas those in VHL syndrome produce mainly norepinephrine. This study examined whether transcription factors known to regulate expression of PNMT explain the different tumor phenotypes in these syndromes.

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Quantitative polymerase chain reaction (PCR) and Western blotting were used to assess levels of mRNA and protein for the glucocorticoid receptor, early growth response 1 (Egr-1), the Sp1 transcription factor (Sp1), and MYC-associated zinc finger protein (MAZ) in 6 MEN-2 and 13 VHL tumors. Results were cross-checked with data obtained using microarray gene expression profiling in a further set of 10 MEN-2 and 12 VHL tumors. Pheochromocytomas in MEN-2 and VHL syndrome did not differ in expression of the glucocorticoid receptor, Egr-1, Sp1, or MAZ as assessed by quantitative PCR and Western blotting. Microarray data also indicated no relevant differences in expression of the glucocorticoid receptor, Egr-1, MAZ, and the AP2 transcription factor. Thus, our results do not support a role for the above transcription factors in determining differences in expression of PNMT in pheochromocytomas from patients with VHL syndrome and MEN-2. Microarray analysis, however, did indicate differences in expression of genes involved in neural crest cell lineage and chromaffin cell development, consistent with differential survival of PNMT-expressing cells in the two syndromes.

KEYWORDS: pheochromocytoma; transcription factors; phenylethanolamine N-methyltransferase; catecholamines; microarray

INTRODUCTION

Pheochromocytomas are rare neuroendocrine tumors arising from chromaffin cells and characterized by excessive production of catecholamines. Pheochromocytomas in patients with multiple endocrine neoplasia type 2 (MEN-2) and von Hippel–Lindau (VHL) syndrome have distinctly different gene expression profiles, patterns of catecholamine production, and clinical presentations.[1–3] Pheochromocytomas in MEN-2 patients express phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine, whereas VHL tumors do not express PNMT. Consequently, pheochromocytomas in MEN-2 produce norepinephrine and epinephrine, and are characterized by an adrenergic biochemical phenotype, whereas those in VHL syndrome produce only norepinephrine and are characterized by a noradrenergic phenotype.

The glucocorticoid dependence of adrenal medullary expression of PNMT, originally described by Wurtman and Axelrod,[4] is mediated by binding to nuclear glucocorticoid receptors and subsequent activation of the glucocorticoid response element in the promotor region of the PNMT gene.[5] Numerous other transcription factors, including early growth response 1 (Egr-1),[6] the Sp1 transcription factor (Sp1),[7] the MYC-associated zinc finger protein (MAZ),[8] and the AP2 transcription factor[9] are now also identified as participating in the regulation of PNMT gene expression.

On the basis of the above findings, we hypothesized that differences in catecholamine phenotypes in pheochromocytomas from patients with MEN-2 and

VHL syndrome might be on account of differences in expression of transcription factors known to regulate PNMT. We therefore used quantitative polymerase chain reaction (PCR) and Western blotting to assess levels of mRNA and protein for the glucocorticoid receptor, Egr-1, Sp1, and MAZ in pheochromocytomas from patients with MEN-2 and VHL syndrome. Differences in gene expression were further examined using our cDNA and oligonucleotide microarray gene expression databases.

MATERIALS AND METHODS

Patients

The study population included 31 patients with pheochromocytoma, 13 on account of MEN-2 and 18 on account of VHL syndrome. The diagnosis of MEN-2 or VHL syndrome was confirmed by identification of germline mutations of the *RET* proto-oncogene or the *VHL* tumor-suppressor gene. Blood and tumor tissue samples were obtained under studies approved by the appropriate institutional review boards, with informed consent obtained from all patients.

Collection of Tissue and Blood Samples

Samples of tumor tissue were obtained within 90 min of surgical removal of pheochromocytomas. Dimensions of tumors were recorded, and small samples (50–400 mg) were dissected away, placed on dry ice, and then stored at −80°C. Blood samples were obtained from patients using an intravenous catheter inserted into a forearm vein, with patients supine for at least 20 min before blood collection. Blood samples were transferred into tubes containing heparin as anticoagulant and immediately placed on ice until centrifuged (4°C) to separate the plasma. Plasma was stored at −80°C until assayed.

Tissue and Plasma Catecholamines

Tissue and plasma concentrations of catecholamines (norepinephrine, epinephrine, and dopamine) were quantified by liquid chromatography with electrochemical detection.[10] Samples of tumor tissue were weighed frozen and homogenized in 5–10 volumes of 0.4-M perchloric acid containing 0.5-mM EDTA. Homogenates were centrifuged and supernatants collected for catecholamine determinations. Plasma concentrations of metanephrines (normetanephrine and metanephrine) were determined using a different liquid chromatography procedure after extraction onto solid-phase ion exchange columns.[11]

Quantitative PCR

RNA was extracted from frozen samples of pheochromocytoma tissue after homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA) followed by RNeasy Maxi (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Total RNA (2 μg) was reversibly transcribed to cDNA using random hexamers. Real-time quantitative PCR (TaqMan PCR), using a 7000 Sequence Detector (Applied Biosystems, Foster City, CA), was used for quantification of mRNA for the glucocorticoid receptor, Egr-1, Sp1, and MAZ. The primers and TaqMan probes were designed from the human glucocorticoid receptor, Egr-1, Sp1, and MAZ gene sequences using the Primer Express program from Applied Biosystems (TABLE 1).

Expression of mRNA for the glucocorticoid receptor, Egr-1, Sp1, and MAZ was normalized for differences in total RNA using 18S ribosomal RNA (Human 18S rRNA Pre-Developed TaqMan Assay Reagents, Applied Biosystems). PCR amplifications of 18S rRNA and the glucocorticoid receptor, Egr-1, Sp1, and MAZ were carried out in the same tubes. Reaction tubes contained 20 ng cDNA product as template, 1 × TaqMan Universal PCR Master Mix, 0.9 μmol/L for each of the forward and reverse primers for the glucocorticoid receptor, Egr-1, Sp1, and MAZ, 0.2 μmol/L for TaqMan probes for the glucocorticoid receptor, Egr-1, Sp1, and MAZ, and 1 × human 18S rRNA primers and probe, all to a final volume of 50 μL with H_20. PCR involved 40 cycles at the following temperature parameters: 15 s at 95°C and 1 min at 60°C. Input RNA amounts were calculated manually using the comparative Ct method for the target genes and 18S rRNA.

Western Blot Analysis

Nuclear proteins were prepared according to the procedure of Andrews and Faller.[12] Samples of pheochromocytoma tissue (approximately 15 mg)

TABLE 1. Oligonucleotide primers and probes used in PCR

Primers	Oligonucleotide sequence
Egr-1	
Forward	5′-TTT GCC AGG AGC GAT GAA C
Reverse	5′- TTT GTC TGC TTT CTT GTC CTT CTG
TaqMan	5′ (FAM)- CAA GAG GCA TAC CAA GAT CCA CTT GCG-(TAMRA)
Sp1	
Forward	5′-TGG CAC AGT CAC TGT GAA TGC
Reverse	5′- GTA CCC AAT GCA CTG AGG TTA ATG
TaqMan	5′ (FAM)- CTC TCC TCC ATG CCA GGC CTC CA-(TAMRA)
MAZ	
Forward	5′-CCA TGC CTG CGA GAT GTG T
Reverse	5′- TCC GAG TGC GAC AGC TTG T
TaqMan	5′ (FAM)- CGC GAC GTC TAC CAC CTG AAC CGA-(TAMRA)

were homogenized using a Dounce homogenizer in 0.5 mL of 10 mmol/L HEPES-KOH buffer (pH 7.9) containing 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride together with protease inhibitors and incubated on ice for 10 min. Nuclear fractions pellets were collected by centrifugation at 17, 000 *g* for 10 s (4°C). The pellets were lysed in 0.1 mL of 20 mM HEPES-KOH (pH 7.9) buffer containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF together with protease inhibitors and incubated on ice for 20 min. Supernatant nuclear fractions were collected by centrifugation at 17,000 *g* for 2 min (4°C) and stored at −80°C.

Nuclear proteins (20 μg) were electrophoresed on a 10% SDS-polyacrylamide and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were incubated in blocking buffer (50 mM Tris, pH 7.4, 0.9% NaCl, 0.05% Tween-20, and 10% dry milk) overnight at 4°C. Membranes were then washed three times for 10 min each with Tris buffer saline (50 mM Tris, pH 7.4, 0.9% NaCl) containing 0.05% Tween-20, and then incubated with either rabbit anti-Egr-1 polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-Sp1 polyclonal antibody (1:1000, Santa Cruz Biotechnology) or mouse anti-MAZ monoclonal antibody (1:400, kind gift from Dr. Kenneth Marcu, Stony Brook, New York) or glucocorticoid receptor antibody for 1 h at room temperature. Membranes were then washed again three times for 10 min each with Tris buffer saline containing 0.05% Tween-20 and incubated for 1 h with horseradish peroxidase conjugated anti-rabbit IgG or anti-mouse IgG, respectively at 1:2000. Membranes were again washed three times for 10 min each with Tris buffer saline containing 0.05% Tween-20. Proteins were visualized using an enhanced chemiluminescence method (SuperSignal West Pico Chemiluminescent kit, Pierce, Rockford, IL).

Microarray Databases

Gene expression databases were generated from cDNA and oligonucleotide microarrays of tumor samples from patients with MEN-2 and VHL syndrome as described elsewhere.[2,13] Databases were explored for differences in expression of transcription factors known to be involved in regulation of PNMT as well as other genes associated with neural crest cell lineage development and chromaffin cell differentiation.

Statistical Analysis

Differences in tissue and plasma catecholamines and expression of transcription factors and the glucocorticoid receptor were examined using the Student's *t*-test. A P value 0.05 was considered significant.

TABLE 2. Concentrations of catecholamines in tumor tissue and of catecholamines and metanephrines in plasma

	VHL ($n = 18$)	MEN-2 ($n = 13$)
Tumor catecholamines		
Norepinephrine (μmol/g)	15.0 ± 2.7	32.0 ± 8.1*
Epinephrine (μmol/g)	0.3 ± 0.1	27.8 ± 6.9**
Plasma catecholamines		
Norepinephrine (nmol/L)	5.8 ± 1.1	4.8 ± 1.3
Epinephrine (nmol/L)	0.1 ± 0.0	1.0 ± 0.3**
Plasma metanephrines		
Normetanephrine (nmol/L)	2.4 ± 0.4	5.6 ± 1.9
Metanephrine (nmol/L)	0.2 ± 0.0	3.5 ± 1.7**

All values are means ± SEM.
*$P < 0.05$, **$P < 0.001$.

RESULTS

Tumor and Plasma Catecholamines

Norepinephrine accounted for 98% of the total catecholamine contents in pheochromocytomas from VHL patients, whereas in MEN-2 patients, norepinephrine accounted for only 53% and epinephrine 47% of total tumor catecholamine contents (TABLE 2). Pheochromocytoma tissue concentrations of epinephrine were 93-fold higher ($P < 0.001$) in tumors from MEN-2 patients than in those from VHL patients, while norepinephrine concentrations in MEN-2 tumors were twofold higher ($P < 0.04$) than in VHL tumors. Plasma concentrations of epinephrine were ninefold higher ($P < 0.001$) and those of metanephrine 18-fold higher ($P < 0.001$) in MEN-2 patients than in VHL patients.

Quantitative PCR and Western Blot Analyses

Expression of glucocorticoid receptor (alpha and beta) protein tended to be lower in VHL than MEN-2 tumors, while mRNA expression tended to the opposite direction (FIG. 1A). These differences, however, did not reach significance. Similarly, quantitative PCR and Western blot analyses indicated no consistent differences in expression of Egr-1, Sp1, and MAZ mRNA or protein between pheochromocytomas in MEN-2 and VHL syndrome (FIG. 1B, C, and D).

Microarray Databases

Both cDNA and oligonucleotide microarray analyses indicated higher ($P < 0.0001$) levels of expression of PNMT in MEN-2 tumors than in VHL tumors.

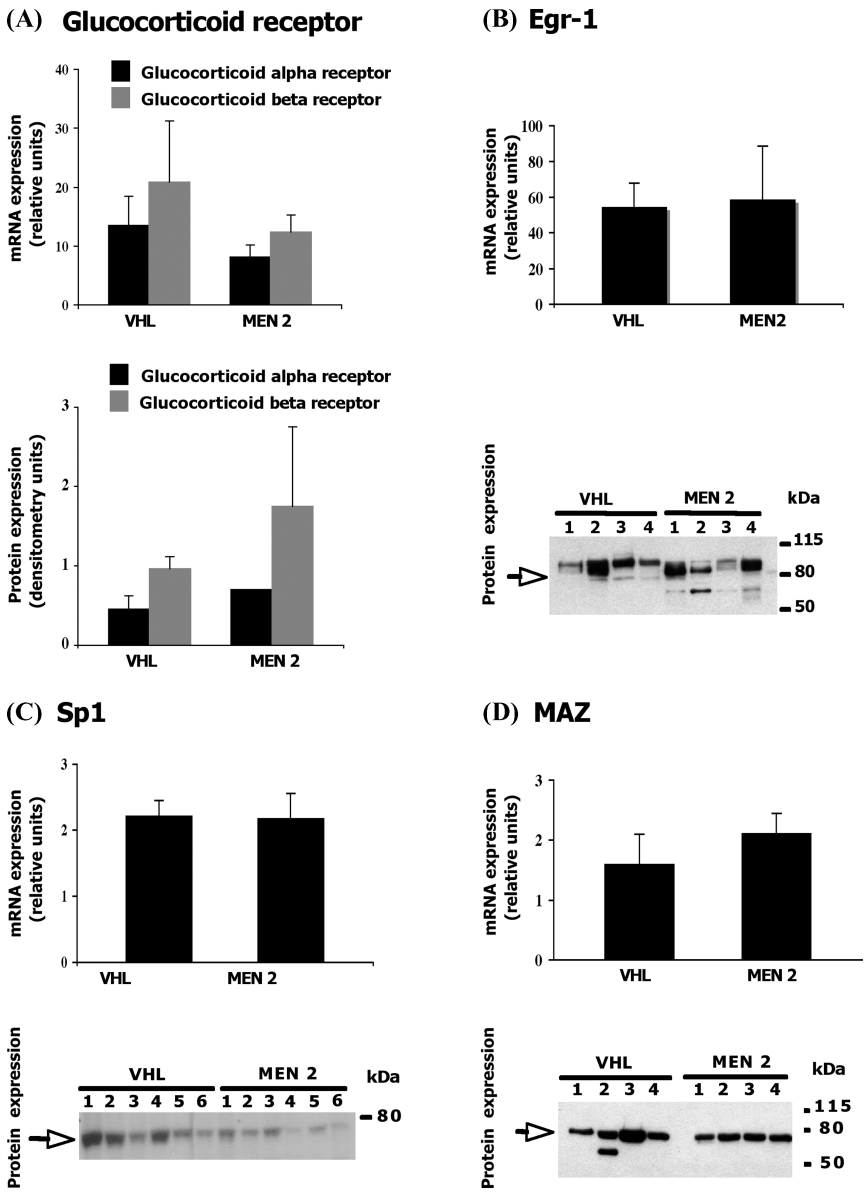

FIGURE 1. Expression of the glucocorticoid receptor, Egr-1, Sp1, and MAZ mRNA and protein in pheochromocytomas from patients with MEN-2 and VHL syndrome. Expression of mRNA was determined by TaqMan PCR, with expression shown relative to that of 18S ribosomal mRNA, as described in the "Methods" section. Expression of glucocorticoid receptor protein in Western blots was determined by densitometry using NIH image 1.62 software. Levels of expression of Egr-1, Sp1, and MAZ protein are shown in the Western blot images.

TABLE 3. Selection of differentially expressed genes from microarray expression profiles

	Gene symbols	*P* value
VHL > MEN-2		
Endothelial PAS domain 1	EPAS1, HIF 2α	<0.0001*
RE1-silencing transcription factor	REST	<0.0004*
Insulin-like growth factor 2 (somatomedin A)	IGF2	<0.0001
Glial cell-derived neurotrophic factor	GDNF	<0.0004
GDNF receptor alpha 1	GFRalpha1	<0.0003
neuregulin 1	NRG1, GGF	<0.0002
neuritin 1	NRN	<0.0003
MEN-2 > VHL		
Ret proto-oncogene	RET	<0.0001
Neural cell adhesion molecule 1	NCAM1	<0.0001
Neural cell adhesion molecule 2	NCAM2	<0.001
Astrotactin	ASTN	<0.001
Achaete-scute complex-like 1	ASCL1, MASH1	<0.0001
Bone morphogenetic protein 8b	BMP8B, OP2	<0.0001

**P* value from cDNA array database (all other *P*-values from oligonucleotide array database).

Consistent with the quantitative PCR results, there were no differences in expression of the glucocorticoid receptor, Egr-1, and MAZ by either of the microarray analyses. Expression of alpha, beta, and gamma isoforms of the AP2 transcription factor also showed no relevant differences consistent with higher expression of PNMT in MEN-2 than VHL tumors. However, microarray analyses indicated differences in expression of other genes involved in neural crest cell lineage and chromaffin cell development that might contribute to different noradrenergic and adrenergic catecholamine phenotypes of pheochromocytomas (TABLE 3).

DISCUSSION

Our study revealed no consistent differences in expression, at both mRNA and protein levels, of the glucocorticoid receptor, Egr-1, Sp1, and MAZ in pheochromocytomas from patients with MEN-2 and VHL syndrome. Microarray analyses also indicated no relevant difference in expression of the AP2 transcription factor. Differences in synthesis of epinephrine in pheochromocytomas from patients with MEN-2 and VHL syndrome are therefore unlikely to be on account of any continuing influence of these transcription factors in regulating expression of PNMT. As shown elsewhere,[14] the adrenergic phenotype of adrenal pheochromocytomas can be maintained after spread of tumor cells to sites distant from adrenal sites of steroid production. This observation is consistent with the view that expression of PNMT does not depend on continuing high local concentrations of glucocorticoids.

The above interpretation does not exclude the possibility that glucocorticoids and related transcription factors may be transiently involved in influencing expression of PNMT during differentiation of neural crest cells, either before or during development into tumor cells. Other levels of regulation that our methods did not identify, such as differences in posttranslational phosphorylation of those transcription factors, also remain possible.

Our findings in hereditary pheochromocytomas contrast with previous findings by others of greater expression of Egr-1 in sporadic adenergic than noradrenergic tumors.[15] Epinephrine production and the adrenergic phenotype in hereditary tumors may therefore reflect different mechanisms than in sporadic tumors.

Pheochromocytomas in MEN-2 and VHL syndrome have been suggested to develop from different populations of noradrenergic and adrenergic chromaffin cells.[2] However, while distinct populations of adrenergic and noradrenergic chromaffin cells are present in the adrenals of many mammalian species, almost all chromaffin cells in the adult human adrenal medulla express PNMT.[16] This observation suggests that development of adrenal pheochromocytomas producing almost exclusively norepinephrine, such as in VHL syndrome, might reflect dedifferentiation, with loss of expression of PNMT and other aspects of the adrenergic phenotype.

Findings by Lee *et al*.[17] now suggest an alternative explanation to the above for the development of pheochromocytomas with distinct mutation-dependent phenotypes. That study indicated that familial pheochromocytomas may be explained by a single pathway linking mutations in several disease-causing genes (i.e., *RET, VHL, NF1, SDHB,* and *SDHD*) to failure of apoptosis after withdrawal of growth factors during chromaffin cell development.

How would a single pathway lead to divergent phenotypes in different hereditary forms of pheochromocytoma? We speculate that there are different susceptibilities of the pathway to effects of mutations of specific genes at different stages of neural crest development and differentiation. According to this notion, pheochromocytomas in VHL syndrome would develop from neural crest progenitors before differentiation into PNMT-expressing and epinephrine-producing adrenal chromaffin cells, whereas epinephrine-producing tumors in MEN-2 would develop on account of susceptibility of fully differentiated PNMT-expressing chromaffin cells to the effects of activating *RET* mutations.

Preliminary support for the above view comes from our microarray data indicating differential expression in MEN-2 versus VHL tumors of a number of genes involved in the differentiation of neural crest progenitor cells. Endothelial PAS domain protein 1 (EPAS1), also known as hypoxia inducible factor 2α, is expressed in greater abundance in VHL and sporadic noradrenergic tumors than in MEN-2 and sporadic adrenergic tumors.[2] Apart from functions in endothelial cell hypoxia-induced signaling pathways, EPAS1 is also expressed in embryonic norepinephrine-producing sympathetic nerves and chromaffin cells, where it regulates the expression of tyrosine hydroxylase, the rate limiting

enzyme in catecholamine synthesis.[18,19] In such cells, impaired degradation of EPAS1, on account of defective VHL protein, might lead to failure of apoptosis at a critical period, with subsequent development of norepinephrine-producing chromaffin cell tumors

Other genes involved in neural crest cell lineage development indicated by expression profiling to be more highly expressed in VHL than MEN-2 tumors included the RE1-silencing transcription factor, insulin-like growth factor 2 (IGF2), glial cell-derived neurotrophic factor (GDNF), and GDNF receptor alpha 1. The RE1-silencing transcription factor, a repressor that silences transcription of neuron-specific genes in non-neuronal cells, has been implicated to regulate PNMT expression in chromaffin cells.[20] IGF2 is expressed in particularly high amounts in pheochromocytomas,[21,22] and acting through the IGF-I receptor substantially decreases catecholamine synthesis.[23] GDNF and other neurotrophin family members acting through a variety receptors (e.g., RET and GDNF receptor alpha 1) are important for the differentiation of neural crest cells, promoting survival of tyrosine hydroxylase positive cells,[24] and in chromaffin cells, a change from an endocrine to a neuronal morphology.[25]

Genes involved in neural crest cell lineage development indicated by expression profiling to be more highly expressed in MEN-2 than VHL tumors included the expected RET proto-oncogene, neural cell adhesion molecules 1 and 2, and achaete-scute homolog-1 (MASH1). The neural cell adhesion molecules have been previously implicated in the differentiation and morphological arrangement of PNMT-positive adrenergic and PNMT-negative noradrenergic cells of the developing adrenal medulla.[26,27] Furthermore, findings in transgenic mice lacking MASH1, show that this transcription factor is essential for the correct phenotypic differentiation of adrenal chromaffin cells.[28] In particular, chromaffin cells lacking MASH1 do not express catecholamine-synthesizing enzymes, including PNMT.

In summary, the present findings indicate that maintenance of PNMT expression and epinephrine production in pheochromocytomas from MEN-2 patients, and lack of PNMT in tumors from VHL patients, are unlikely to reflect influences of any continuing difference in expression of transcription factors known to regulate the PNMT gene (i.e., glucocorticoid receptor, Egr-1, Sp-1, MAZ, and AP2). Our microarray results lead to the alternative hypothesis that the differing catecholamine phenotypes in VHL and MEN-2 tumors reflect origins from developmentally distinct chromaffin cell progenitors, with presence or absence of PNMT maintained independently of the above transcription factors.

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