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Identification of Cyclin D1 and Other Novel Targets for the von Hippel-Lindau Tumor Suppressor Gene by Expression Array Analysis and Investigation of Cyclin D1 Genotype as a Modifier in von Hippel-Lindau Disease[1]

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ABSTRACT

Germ-line mutations in the von Hippel-Lindau (VHL) tumor suppressor disease are associated with a high risk of retinal and cerebellar hemangioblastomas, renal cell carcinoma (RCC), and, in some cases, pheochromocytoma (PHE). In addition, somatic mutation or epigenetic inactivation of the *VHL* gene occurs in most clear cell RCCs. VHL protein (pVHL) has a critical role in regulating proteasomal degradation of the HIF transcription factor, and VHL inactivation results in overexpression of many hypoxia-inducible mRNAs including vascular endothelial growth factor (VEGF). To identify novel pVHL target genes we investigated the effect of wild-type (WT) pVHL on the expression of 588 cancer-related genes in two VHL-defective RCC cell lines. Expression array analysis identified nine genes that demonstrated a >2-fold decrease in expression in both RCC cell lines after restoration of WT pVHL. Three of the nine genes (*VEGF*, *PAI-1*, and *LRP1*) had been reported previously as pVHL targets and are known to be hypoxia-inducible. In addition, six novel targets were detected: cyclin D1 (*CCND1*), cell division protein kinase 6, collagen VIII α 1 subunit, CD59 glycoprotein precursor, integrin β8, and interleukin 6 precursor *IFN-β2*. We found no evidence that *CCND1*, cell division protein kinase 6, CD59, and integrin β8 expression was influenced by hypoxia suggesting that pVHL down-regulates these targets by a HIF-independent mechanism. A type 2C pVHL mutant (V188L), which is associated with a PHE only phenotype (and had been shown previously to retain the ability to promote HIF ubiquitylation), retained the ability to suppress *CCND1* expression suggesting that loss of pVHL-mediated suppression of cyclin D1 is not necessary for PHE development in VHL disease. Other studies have suggested that: (*a*) genetic modifiers influence the phenotypic expression of VHL disease; and (*b*) polymorphic variation at a *CCND1* codon 242 A/G single nucleotide polymorphism (SNP) may influence cancer susceptibility or prognosis in some situations. Therefore, we analyzed the relationship between CCND1 genotype and phenotypic expression of VHL disease. There was an association between the G allele and multiple retinal angiomas ($P = 0.04$), and risk of central nervous system hemangioblastomas ($P = 0.05$). These findings suggest that a variety of HIF-independent mechanisms may contribute to pVHL tumor suppressor activity and that polymorphic variation at one pVHL target influences the phenotypic expression of VHL disease.

INTRODUCTION

VHL[4] disease (MIM 193300) is a dominant familial cancer syndrome characterized by susceptibility to HABs of the retina and CNS, clear cell RCC, and PHE (1–3). Somatic inactivation of the *VHL* tumor suppressor by loss, mutation, or promoter hypermethylation occurs in most sporadic clear cell RCC, and reintroduction of WT pVHL in *VHL*-null RCC cells suppresses tumor formation *in vivo* in nude mice (4–8). VHL disease displays complex genotype-phenotype correlations such that allelic heterogeneity can result in four clinical subtypes with different tumor-specific susceptibilities (2, 9). Phenotypic variability in VHL disease also results from modifier effects (10).

The complex genotype-phenotype correlations in VHL disease suggest that pVHL has multiple functions. Although the sequence of pVHL did not provide clues to the mechanism of tumor suppressor function, after the identification of elongins B, C, and Cul2 as pVHL-interacting proteins, the recognition of sequence and structural similarities between the pVHL/elongin C/elongin B/Cul2 complex and the yeast Skp1-Cdc53/Cul1-F-box complex led to suggestions that pVHL may have a role in targeting oncogenic proteins for ubiquitin-dependent proteolysis (11, 12). Subsequently, pVHL was demonstrated to target the regulatory α-subunits of hypoxia inducible factors, HIF-1 and HIF-2, for oxygen-dependent proteolysis (13). HIF-1 is a heterodimeric transcription factor with a critical role in cellular responses to hypoxia. Under normoxic conditions, the HIF-α subunits are degraded rapidly by the proteasome in a pVHL-dependent ubiquitylation process (14–16). Constitutively high HIF-α levels are observed in *VHL*-defective RCC lines, causing up-regulation of an extensive range of hypoxia-inducible mRNAs including those involved in energy metabolism, angiogenesis, and apoptosis (*e.g.*, glucose transporter 1 [GLUT-1] and VEGF).

The exact relationship between pVHL-dependent HIF-α subunit regulation and tumor suppressor activity is not well defined, but analysis of mutant pVHLs associated with different RCC susceptibilities suggests that HIF dysregulation is not sufficient for RCC tumorigenesis. Other functions of pVHL have been reported including a role in cell cycle exit control (17), fibronectin binding, and extracellular matrix assembly (18) and post-transcriptional regulation of target gene expression through mRNA stability effects (19–21).

To identify novel pVHL targets we analyzed differences in gene expression profile after restoration of WT pVHL expression into two pVHL-defective clear cell RCC cell lines. *CCND1* and five other novel target genes (plus three targets identified previously) were

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[4] The abbreviations used are: VHL, von Hippel-Lindau; HAB, hemangioblastoma; RCC, renal cell carcinoma; pVHL, von Hippel-Lindau protein; VEGF, vascular endothelial growth factor; WT, wild-type; CCND1, cyclin D1; CDK6, cell division protein kinase 6; COL8A1, collagen VIII α 1 subunit; ITGB8, integrin β8; IL, interleukin; PHE, pheochromocytoma; HIF, hypoxia-inducible factor; nt, nucleotide; HA; hemagglutinin; HR, hazard ratio; LRP1, lipoprotein receptor-related protein 1; PAI-1, plasminogen activator inhibitor 1 precursor; CNS, central nervous system.

up-regulated at least 2-fold in both pVHL-defective RCC cells lines analyzed. Cyclin D1 is a key cell cycle regulatory protein. The *CCND1* gene contains a common A/G polymorphism at nt 870 (codon 242) that modulates mRNA splicing to produce two transcripts (22). Although both transcripts encode proteins that contain the functional cyclin box (amino acids 55–161), the unspliced transcript does not contain the exon 5 sequence encoding a PEST rich destruction box responsible for the rapid turnover of the protein. The *CCND1* 870 polymorphism has been reported to influence susceptibility to colorectal cancer in hereditary nonpolyposis colon cancer syndrome (23, 24). In view of the evidence for unidentified genetic modifiers in VHL disease (10), we proceeded to investigate whether CCND1 genotype might modify the phenotypic expression of VHL disease.

MATERIALS AND METHODS

Cell Lines and Preparation of Stable Transfectants. Stable transfectants were obtained in the following VHL-defective cell lines: 786−0 (containing a 1 nt deletion at VHL nt 523, codon 104FS), RCC4 (C407G, Ser65Trp), and UMRC2(G458C, Arg82Pro). RCC4 stably transfected with plasmid expressing either empty vector (pcDNA3.1; Invitrogen), WT pVHL (pcDNA 3.1-VHL (1–213).HA), mutant pVHL (pcDNA 3.1VHL (1–213; Leu188Val)HA) have been described previously (9). UMRC2 cells transfected with the same vectors were a gift from Dr. P. Ratcliffe. 786−0 cells transfected with either empty vector (pRc) or WT pVHL (pRc HA.VHL (1–213)) containing an NH_2-terminal HA epitope tag were a gift from Dr. W. Kaelin, Jr. The expression of the VHL transgene was tested by Western blotting with anti-HA antibodies and normalized against β-actin expression (data not shown). Three additional cell lines with intact VHL were used to test for the oxygen-dependent regulation of pVHL target genes: EJ-28 (bladder cancer), HBL-100 (breast cancer), and 293 (kidney embryonal cell line).

Cell Culture. All of the stable transfectants were maintained in DMEM medium supplemented with 10% FCS and G418 (1 mg/ml). EJ-28 cells were grown in RPMI 40/10% heat inactivated FCS, HBL-100 in DMEM/10% heat inactivated FCS, and 293 in DMEM/10% FCS. Cells were plated onto 150-mm cell culture dishes 24 h before transferring cells to hypoxic conditions. Hypoxic conditions were achieved by growing cells for 16 h in chambers containing a mixture of 1% oxygen and 5% CO2 in nitrogen (BOC Gases).

Expression Array Analysis. The Atlas Human Cancer cDNA expression array (containing 588 cancer-related genes; Clontech) was used to identify candidate genes differentially expressed between VHL (−/−) cells stably transfected with either: (*a*) WT pVHL; or (*b*) backbone vector only. Briefly, stably transfected cells were grown to 70% confluence under normoxic conditions as described, before harvesting by scraping in ice cold PBS, pelleting, and storage at −70°C. Total RNA was extracted from the cell pellets using Tri-Reagent (Sigma) according to the manufacturer's instructions. Before analysis, RNA integrity was confirmed by agarose gel electrophoresis and quantified spectrophotometrically. ^{32}P-labeled cDNA probes were then prepared from 5 μg of each RNA sample and hybridized to the arrays according to the manufacturer's instructions. Probe hybridization to each array was quantified and analyzed using a PhosphorImager and ImageQuant software (Molecular Dynamics). Normalized signal data are available as supplementary information.[5] Differences in gene expression were compared between arrays, after standardization of results by: (*a*) subtraction of background signal from each array; and (*b*) normalization of results between arrays according to the average expression levels of nine housekeeping genes contained on the array.

Northern Blot Analysis. Total RNA was extracted with RNeasy columns (Qiagen) from cell lines and separated on standard agarose formaldehyde gel at 100V for 3 h. Fifteen μg of RNA was loaded per lane. It was transferred overnight onto Hybond N+ membrane (Amersham-Pharmacia Biotech) by capillary transfer in Northern transfer buffer (10× SSC; Sigma) and fixed to the membrane by exposing to UV light for 4 min. Templates for probe synthesis were prepared from RNA by reverse transcription (Promega) followed by amplification with gene-specific primers (Clontech). PCR fragments were cloned into pGEM vector (Promega), and their identity was confirmed by sequencing. EcoRI fragments from pGEM-based clones were purified from agarose gels and used directly in labeling reactions by the random priming method (Roche) with [α-32P]dCTP (Amersham). The probes were purified from unincorporated nucleotides on Sephadex g-50 columns (Roche). Hybridization was performed overnight at 68°C in PerfectHyb Plus hybridization buffer (Sigma). Membranes were washed according to manufacturer instructions, exposed in phosphorimager cassettes, and analyzed on PhosphorImager program ImageQuant (Molecular Dynamics). Northern blot analysis was performed at least twice to ensure that results were reproducible.

Whole-Cell Protein Extraction and Immunoblot Analysis. Cells were grown to ~70% confluence, washed with ice-cold PBS, and harvested by scraping. Cell pellets were homogenized in extraction buffer (Mammalian cell lysis kit; Sigma) and incubated on ice for 10 min. Lysates were centrifuged for 15 min at 14,000 rpm/4°C and stored at −20°C. Protein samples (20 μg each) were separated on sodium dodecyl sulfate-10.5% polyacrylamide gel and electroblotted to transblot polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences). Anticyclin D1 antibodies (287; kindly provided by G. Peters, Cancer Research UK, London, United Kingdom) diluted 1/2000, and anti-HIF-1α (Transduction Laboratories) at 1 μg/ml were applied followed by goat antirabbit and rabbit antimouse immunoglobulin-peroxidase conjugate, respectively, and visualization by the enhanced chemiluminescence detection system (ECL-plus; Amersham Bioscience). The filter was stained with India ink for standardization, and quantification was performed using a Bio-Rad imaging densitometer with Quantity One software. Immunoblots from seven primary renal cell cancers with VHL inactivation were prepared as described previously (25).

***CCND1* Genotyping and Modifier Gene Analysis.** *CCND1* genotyping studies were performed on 118 VHL disease patients for whom detailed phenotypic information had been collected and from whom sufficient DNA was available (see Ref. 10 for details on assessment of phenotypes). The A/G *CCND1* polymorphism at nt 870 (codon 242) in exon 4 was detected using PCR restriction fragment length polymorphism. A 167-bp fragment of the *CCND1* was amplified by PCR using the primers: C26F, GTGAAGTTCATTTCCAATCCGC and C27R, GGGACATCACCCTCACTTAC (22) at an annealing temperature of 57°C, and the product identity was confirmed by sequencing. The PCR product was cut with ScrF1. The A allele at position 870 is refractory to cleavage, whereas the G allele is cleaved into 145- and 22-bp fragments. Cleavage products were visualized on a 10% polyacrylamide gel.

Statistical Analysis. Statistical analyses were performed using the statistical software program STATA Version 6 (Stata Corporation, College Station, TX). To test for a trend in the distribution of non-normally distributed continuous measures between groups of unequal sizes the nonparametric test proposed by Cuzick (26) was used. For analysis of survival data Kaplan-Meier curves were constructed and HR estimates obtained together with corresponding 95% confidence limits and associated *P*s derived. A *P* of 0.05 was considered statistically significant.

RESULTS

Expression Array Analysis of Paired VHL+ and VHL Null Renal Carcinoma Cell Lines. To identify novel pVHL targets we used the Atlas Human Cancer cDNA Expression Array (Clontech) to analyze gene expression in VHL-positive and VHL-defective RCC cell lines. The array includes 588 cancer-related cDNAs arranged into functional groups, *e.g.*, cell cycle regulators, growth regulators, filament markers; apoptosis, oncogenes, and tumor suppressors; DNA damage response repair and recombination, cell fate, and developmental receptors; cell adhesion and motility, angiogenesis; invasion regulators; and growth factors and cytokines. Gene expression patterns were analyzed in two RCC cell lines, 786−0 and RCC4, shown previously to be defective for pVHL (13). Each was stably transfected with a WT VHL gene or empty vector (see "Materials and Methods"). Although many genes demonstrated pVHL-dependent alterations in expression, we prioritized nine genes for additional investigation because they demonstrated at least a 2-fold difference in expression between VHL+ and VHL-null transfectants in both host lines (see Table 1 and Fig 1).

[5] http://www.bham.ac.uk/ICH/genetics.htm.

Table 1 *Analysis of candidate VHL target genes*

For array analysis, a set of housekeeping genes was used for standardisation of the results (as recommended by manufacturer). For Northern analysis fold downregulation was determined using β-actin as an internal control. Each Northern was done at least twice.

Gene	Accession no.	786-0 FD[a]		UMRC2 FD	RCC4 FD	
		Arrays	Northern	Northern	Arrays	Northern
VEGF	M32977/M27281	+	++	+	++	++++
Cyclin D1	X59798/M64349	++	++	+	+	++++
Cell division protein kinase 6	X66365	++	++	+	++	+
Collagen type VIII-α1	X57527	+	+	ND[b]	++	+
CD59	M34671	++	+		+	+
Integrin β 8	M73780	++	++	−	++	+++
Plasminogen activator inhibitor 1 precursor (PAI1)	X04429	+	++	ND	+	+++
IL-6 precursor (interferon β 2)	X04602/M14584	+	+++	ND	++	−
α-2 macroglobulin receptor (LRP1)	X13916	+	++	+	+++	+

[a] FD, fold downregulation (FD) in RCC cells with WT VHL transgene compared with VHL-null cells. Key: − = <2 FD; + = 2–3.9 FD; ++ = 4–7.9 FD; +++ = 8–15.9 FD; ++++ = >16 FD.

[b] ND, not detected by Northern analysis.

The nine genes selected for additional study were all overexpressed in the absence of functional pVHL and included three genes of which the expression had been demonstrated previously to be down-regulated by pVHL: (*a*) VEGF (19, 27); (*b*) low density LRP1 precursor = α-2 macroglobulin receptor (28); and (*c*) PAI-1 (29). In addition six novel candidate VHL target genes were detected: (*a*) *CCND1*; (*b*) *CDK6*; (*c*) *COL8A1*; (*d*) *CD59* glycoprotein precursor; (*e*) *ITGB8*; and (*f*) *IL-6* precursor (*IFN-β2*).

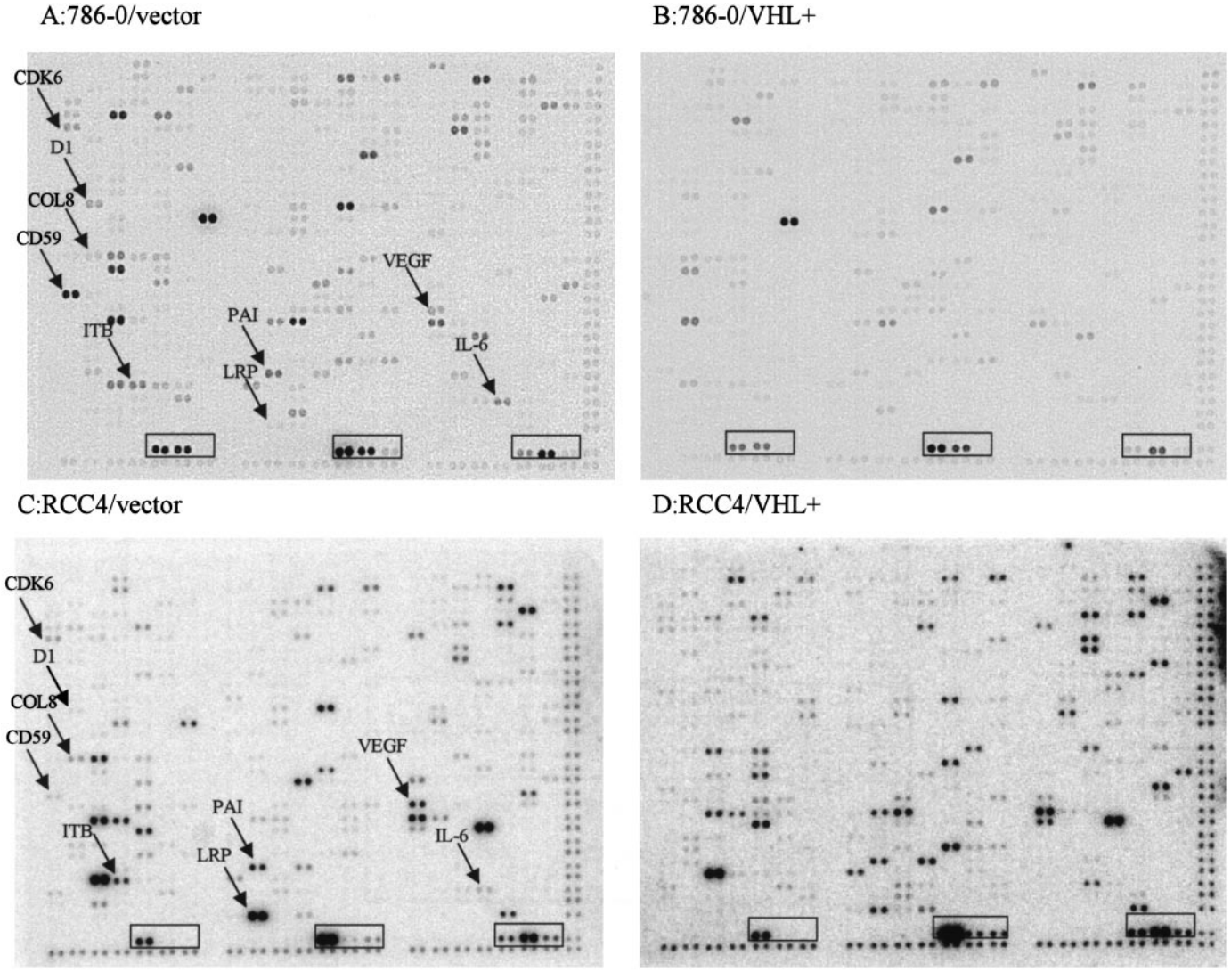

Fig. 1. Expression array analysis. cDNA array analysis showing differential expression of genes in VHL-positive and VHL-deficient cell lines. The panels show gene expression in the following cell lines: *A*, 786−0 stably transfected with empty vector; *B*, 786−0 stably transfected with *VHL* gene; *C*, RCC4 stably transfected with empty vector; and *D*, RCC4 stably transfected with *VHL* gene. *Arrows* on *A* and *C* indicate the position of identified target genes, the *boxes* show the location of housekeeping genes (from *left to right*: ubiquitin, phospholipase A2, hypoxanthine-guanine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase, α-tubulin, HLA class I histocompatibility antigen, β-actin, M_r 23,000 highly basic protein, and ribosomal protein S9).

Investigation of Candidate VHL Targets by Northern Analysis. To confirm the expression array results we proceeded to repeat the investigations by Northern blot analysis. Thus, total RNA was prepared from three VHL-defective clear cell RCC cell lines (786–0, RCC4, and, in addition, UMRC2), stably transfected with either a WT VHL-expressing plasmid or empty vector. Representative results of Northern analysis are shown at Fig. 2. For standardization of the results each membrane was reprobed with β-actin. Quantification performed by a PhosphorImager (using the ImageQuant). The results of quantification are presented in Table 1. Northern analysis confirmed that all nine of the candidate genes were down-regulated by pVHL in the 786–0 cell line. For RCC4, eight of nine candidate VHL targets genes demonstrated pVHL-induced down-regulation when analyzed by Northern analysis. Thus, in a RCC4 background, pVHL strongly down-regulated *VEGF*, *CCND1*, *ITGB8*, and *PAI-1* (>8-fold), and down-regulated *CDK6*, collagen type VIII, *LRP1*, and *CD59* less markedly. However, an effect on *IL-6* expression was not confirmed (see Table 1).

Northern analysis of the nine candidate VHL target genes in a UMRC2 cell line (which had not been investigated by array analysis) did not demonstrate detectable transcripts for *COL8A1, PAI-1,* and *IL-6*. However, *VEGF, CCND1, CDK6,* and *LRP1* were down-regulated at least 2-fold. *CD59* and *ITGB8* showed no significant change. Generally the effects of pVHL on target gene expression were less pronounced in UMRC2 than in RCC4 or 786–0 cell lines (Table 1). Western blotting demonstrated that these differences did not correlate with levels of pVHL transgene expression (which was highest in 786-0 and lowest in RCC4, although the difference in pVHL expression was <2-fold).

Regulation of VHL Target Genes by Oxygen. In view of the well-established effect of pVHL on hypoxia-inducible gene (*e.g.,* VEGF) expression, by regulating the proteasomal degradation of HIF1α, (13), we proceeded to investigate whether expression of the confirmed pVHL-target genes demonstrated oxygen-dependent regulation. As assessment of hypoxia responsiveness in RCC cell lines transfected with WT VHL may produce false-negative results, we analyzed hypoxic gene induction in three cell lines with intact endogenous VHL: the 293 kidney embryonal cell line, the EJ-28 bladder, and HBL-100 breast cancer cell lines. In 293 cells, only *CCND1*, *CD59*, and *VEGF* transcripts were detectable by Northern analysis. As expected, *VEGF* expression was up-regulated by hypoxia but *CCND1* and *CD59* expression was unchanged. In HBL-100 cells, *CDK6*, *CD59*, and *ITGB8* expression was not influenced by hypoxia, but *VEGF*, *LRP*, and *PAI-1* all >2-fold demonstrated hypoxic induction (we did not detect measurable levels of CCND1, COL8A1, and IL-6 transcripts). Analysis of the EJ-28 bladder cell line demonstrated no effect of hypoxia on CCND1, CDK6, ITGB8, and CD59 mRNA levels; however, the expected hypoxic-inducibility of VEGF and PAI-1 was confirmed. IL-6 and COL8A1 could not be assessed (see Fig. 3 and Table 2).

Additional Investigation of Cyclin D1 as a pVHL Target. Although cyclin D1 has not been described previously as a target for the VHL tumor suppressor gene, immunohistochemical studies of sporadic RCC demonstrated cyclin D1 protein overexpression in 50% of tumors (30, 31), and a recent study by Stassar *et al.* (32) showed overexpression of cyclin D1 mRNA in >70% of RCC tumors. To confirm that pVHL can down-regulate cyclin D1 protein level, Western blots of paired VHL+ and VHL-null protein extracts were probed with anticyclin D1 antibody for each of the three RCC cell lines. WT pVHL down-regulated cyclin D1 protein levels in each of the cell lines. The down-regulation was >20-fold in UMRC2 and RCC4, and ~6-fold in 786–0 cell lines (Fig. 4*A*). To exclude the possibility that regulation of cyclin D1 by VHL was a feature of RCC cell lines and not primary tumors we analyzed cyclin D1 expression in seven primary clear cell RCC with known *VHL* gene mutations. Cyclin D1 protein overexpression was detected in six of seven tumors, but none of the adjacent normal renal tissue samples demonstrated detectable cyclin D1 protein expression (see Fig. 4*D*).

Northern analysis of 293 and EJ-28 cell lines had demonstrated no significant effect of hypoxia on cyclin D1 mRNA levels (see above). Consistent with these findings, we found that cyclin D1 protein levels were not up-regulated by hypoxia in EJ-28 (0.6-fold) and HBL-100 (0.8-fold) cell lines, although HIF1 protein expression was induced at least 8-fold, respectively, in the same cells (see Fig. 4*B*). (We could not detect cyclin D1 protein in 293 cells.)

The observations that cyclin D1 mRNA and protein levels were down-regulated by pVHL but were not up-regulated by hypoxia suggested a HIF-independent mechanism for pVHL regulation of cyclin D1. Recently, we and others have demonstrated that the L188V pVHL missense mutation [which is associated with a PHE only (type 2C) phenotype] retains the ability to regulate HIFα levels, suggesting HIF-independent mechanisms may play a role in L188V-mediated PHE development (9, 33). Therefore, we investigated the ability of the L188V mutant pVHL to regulate cyclin D1 in a RCC4 cell line. We found that the L188V pVHL down-regulated cyclin D1 expression on both mRNA (11-fold; Fig. 2*B*) and protein level (Fig. 4*C*; as expected, L188V also down-regulated VEGF expression).

Evaluation of *CCND1* Genotype as a Modifier for VHL Disease. To assess the influence of variation in *CCND1* on the retinal, renal, and CNS manifestations of VHL we genotyped 118 patients for the codon 242 polymorphism. Thirty of the patients (25%) possessed the AA genotype, 56 (47%) the AG genotype, and 32 (27%) the GG genotype.

We have shown previously that the number of retinal angiomas in VHL patients is readily quantifiable, but they are not normally distributed, and there is no relationship between age and number (*i.e.*, number of retinal angiomas is likely to be determined early in life; Ref. 10). The number of retinal angiomas was significantly higher in individuals harboring the G-allele compared with AA-homozygotes (95% percentiles: 0–6, 0–10, and 0–9 in patients with AA, AG, and GG genotypes, respectively; $P_{trend} = 0.04$). There was evidence that *CCND1* genotype influences the onset of CNS HAB (Fig. 5). Possession of one or more G-alleles was associated with earlier diagnosis of tumor by almost 2-fold, although the difference did not attain formal statistical significance (HR = 1.9; 95% confidence interval, 1.0–3.9; $P = 0.05$). A similar analysis for onset of RCC showed no evidence of an association with *CCND1* genotype (HR = 1.6; 95% confidence interval, 0.6–4.4; $P = 0.3$).

DISCUSSION

The identification of the VHL tumor suppressor gene and the subsequent investigation of pVHL function have provided important insights into the molecular pathology of sporadic clear cell RCC. To date, the role of pVHL in the regulation of HIFα proteolysis has been the most intensively investigated aspect of pVHL function (9, 13–16). However genotype-phenotype correlations suggest that pVHL has multiple functions (2). To provide clues to pVHL function we investigated the effect of WT pVHL on the expression of 588 cancer-related genes in two VHL-defective RCC cell lines (786–0 and RCC4). To reduce the chances of false positives, we concentrated on investigating further the nine genes that demonstrated at least a 2-fold difference in expression between VHL+ and VHL-null in both 786–0 and RCC4 cell lines. Although these selection criteria might falsely exclude some VHL targets genes that were not expressed in both cell lines, they would be expected to provide a high specificity. Three of

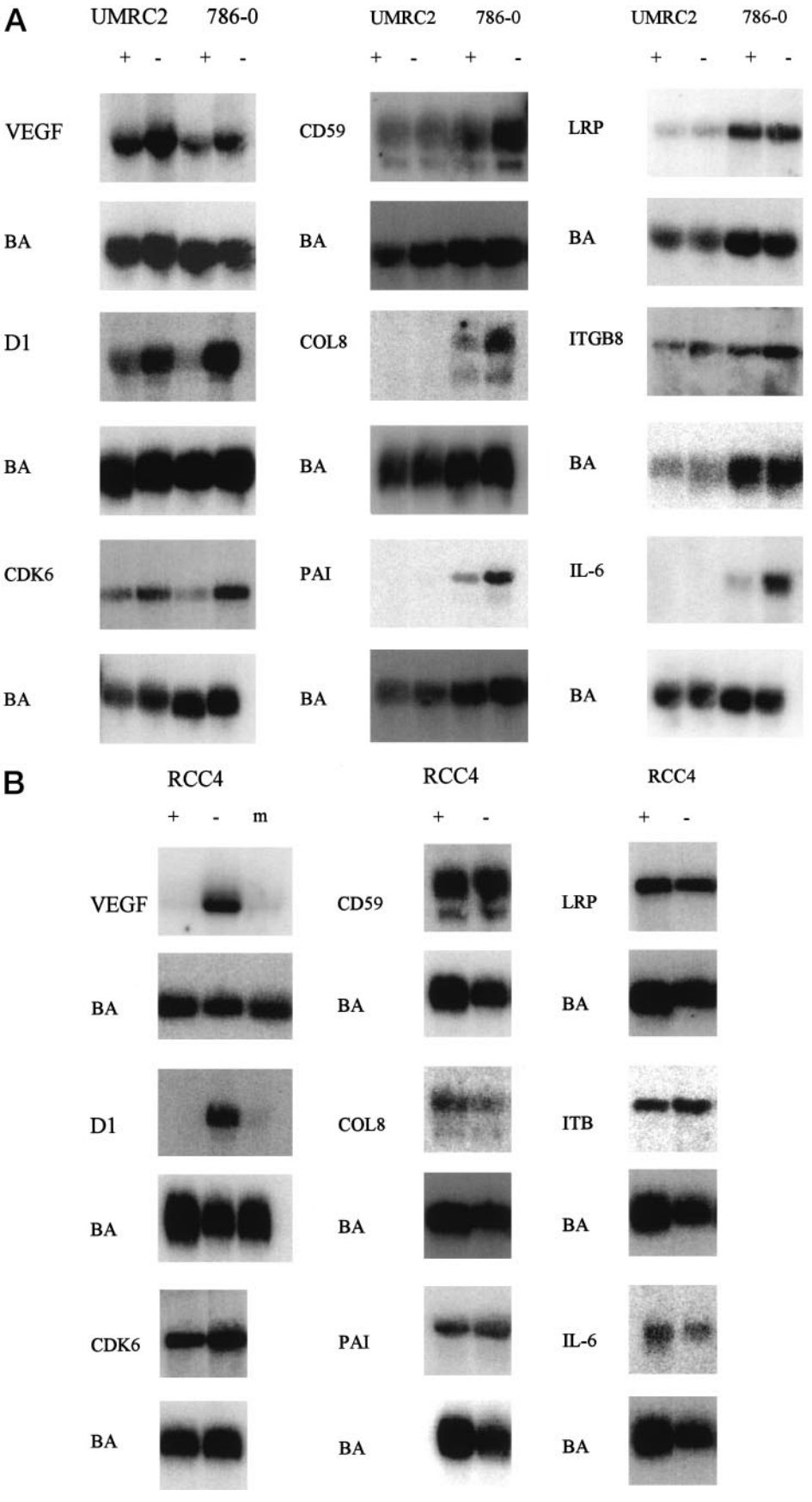

Fig. 2. Northern blot analysis of genes down-regulated by VHL tumor suppressor in three renal carcinoma cell lines under normoxic conditions. *A*, UMRC2 and 786−0 cell lines, which were stably transfected with either plasmid carrying WT *VHL* gene (+) or the empty vector (−). *B*, RCC4 cell line stably transfected with either plasmid carrying VHL WT (+) or empty vector (−). For cyclin D1 and VEGF RCC4 stably transfected with mutant pVHL (L188V) also included (*m*). Below each Northern the same membrane reprobed with β-actin is shown.

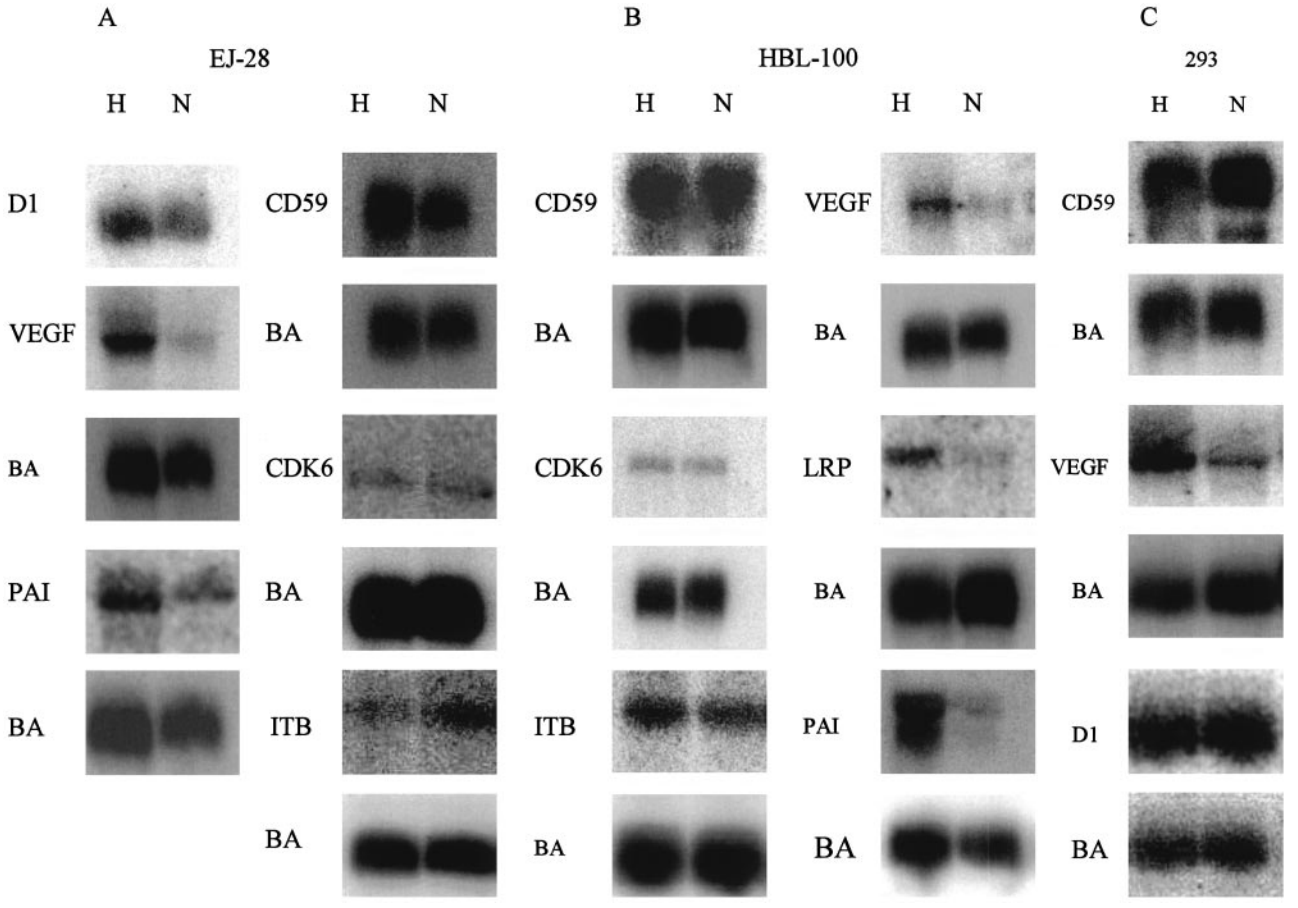

Fig. 3. Northern blot analysis of the induction by hypoxia of VHL target genes in cell lines with normal pVHL function. *A*, in EJ-28 bladder cell line; *B*, in HBL-100 breast cell line; *C*, in 293 kidney embryonal cells. Each membrane has been reprobed with VEGF to demonstrate that the culture was grown under hypoxic condition, but for clarity of the figure it is shown only for cyclin D1 in EJ-28 cells. Below each Northern, the same membrane reprobed with β-actin for standardization is shown.

the nine genes (*VEGF*, *PAI-1*, and *LRP1*) identified by expression arrays and confirmed by Northern analysis as pVHL targets had been reported previously to be down-regulated by VHL (19, 27–29). Both *VEGF* and *LRP1* were identified as pVHL targets in a similar study in which a glass chip microarray of 9182 genes was used to investigate gene expression patterns in VHL+ and vector only RCC4 cell line transfectants (28). *VEGF*, *PAI-1*, and *LRP1* each have a role in angiogenesis and are up-regulated in hypoxia (34). Thus, pVHL is likely to regulate these genes (at least in part) by a HIF-dependent mechanism.

The identification of three known pVHL targets provided some validation of our experimental approach, but the detection of six putative novel targets (*CCND1*, *CDK6*, *ITGB8*, *IL-6*, *COL8A1*, and *CD59*) was of particular interest. Northern analysis confirmed that pVHL down-regulated *CCND1* and *CDK6* mRNA expression in each of the three RCC cell lines analyzed, and *COL8* was down-regulated in two (it was not detectable in UMRC2 cells). *ITGB8* and *CD59* transcript levels were down-regulated in 786-0 and RCC4 cell lines but not in a UMRC2 cell line, and *IL-6* was confirmed to be down-regulated in 786-0 but not in RCC4 (and was not detectable in UMRC2).

Table 2 *Regulation of VHL target genes by hypoxia*

	Regulation by hypoxia		
Gene/cell lines	EJ-28	HBL-100	293
Cyclin D1	NI (1.1)[a]	NM	NI (1.0)
CDK6	NI (1.1)	NI (1.2)	NM
ITGB8	NI (1.0)	NI (1.2)	NM
CD59	NI (1.5)	NI (1.2)	NI (0.8)
COL8A1	NM	NM	NM
IL-6	NM	NM	NM
VEGF	+++	++	+
LRP1	ND	+	NM
PAI-1	+	+	NM

[a] Key. − = NI, not induced (fold change); + = 2–3.9-fold induction; ++ = 4–7.9-fold induction; +++ = 8–15.9-fold induction; NM, not detected at measurable level; ND, not done.

Each of the 588 genes represented on the Atlas array filters have been implicated in tumorigenesis. The hypoxia-responsive genes, *VEGF*, *PAI-1* and *LRP1*, are implicated in angiogenesis (and VHL-related tumors are notably hypervascular). ITGB8 is a member of the integrin family of transmembrane receptors, which, in addition to their structural role in supporting cell attachment, are also implicated in control of cell survival, differentiation, and proliferation. Signals from both growth factor receptors and integrins are required to stimulate progression through G_1 phase of cell cycle. In normal cells all of the important mitogenic signal transduction cascades are regulated by integrin-mediated cell adhesion, which results in their growth being anchorage dependent. In cancer cells constitutive activity of signal-transducing pathways reduces their dependence on mitogens and allows for anchorage-independent growth (35, 36).

CD59 is a cell surface complement-inhibitory glycoprotein, which blocks the formation of the cytolytic and proinflammatory membrane attack complex. Many cancers express complement inhibitory proteins, such as CD59, and protect tumor cells from complement-mediated injury (37). Such protection helps tumor cells to escape

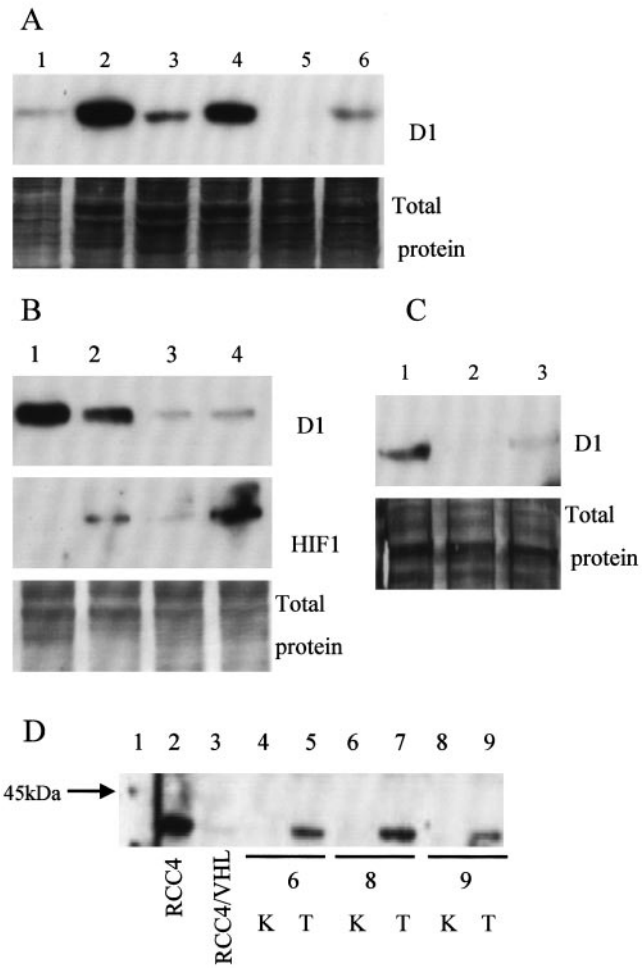

Fig. 4. Western blot analysis of cyclin D1 protein levels. *A*, down-regulation of cyclin D1 protein by VHL tumor suppressor gene in following cell lines: *Lane 1*, UMRC2/VHL+; *Lane 2*, UMRC2-null; *Lane 3*, 786–0/VHL+; *Lane 4*, 786–0-null; *Lane 5*, RCC4/VHL+; and *Lane 6*, RCC4-null. The protein extract stained with India ink for standardization is shown below the Western blot. *B*, regulation of cyclin D1 by oxygen. *Top panel*, cyclin D1; *Lane 1*, EJ-28/normoxia; *Lane 2*, EJ-28/hypoxia; *Lane 3*, HBL-100/normoxia; and *Lane 4*, HBL-100/hypoxia; *Middle panel*, the same membrane probed with antibody anti HIF1α. *Bottom panel*, total protein stained with India ink. *C*, down-regulation of cyclin D1 by mutated pVHL (Leu188Val). *Lane 1*, RCC4; *Lane 2*, RCC4/VHL; and *Lane 3*, RCC4/mutVHL(L188V). Below the Western total protein stained with India ink. *D*, overexpression of cyclin D1 protein in primary renal tumors with VHL inactivation. Cyclin D1 protein levels are up-regulated in the RCC4 cell line with VHL inactivation (*Lane 2*) and in three primary clear cell RCCs with VHL inactivation (*Lane 5*, T6; *Lane 7*, T8; *Lane 9*, T9) but not in RCC4 cell line transfected with WT VHL (*Lane 3*) or normal kidney samples adjacent to the tumors (K6, K8, and K9 in *Lanes 4*, *6*, and *8*, respectively). Tumors are numbered as in Neumann *et al.* (56).

immune surveillance mechanisms thus enhancing tumor cell survival and obstructing attempts to develop effective antibody-mediated immunotherapy for human cancers (38).

Although IL-6 (a multifunctional cytokine that regulates immune and inflammatory response; Ref. 39) was only confirmed to be down-regulated by pVHL in one RCC cell line, RCC cells have been reported to produce many cytokines, including IL-6, and serum IL-6 level appears to be an adverse prognostic factor in metastatic RCC (40). COL8A1 (type VIII collagen), a short chain collagen, is thought to play a key structural role in vasculature and is up-regulated in response to injury (41). It was shown recently that type VIII collagen deposited in vascular lesions functions to promote smooth muscle cell attachment and chemotaxis, and signals through integrin receptors to stimulate matrix metalloproteinase synthesis, which are all important mechanisms used in cell migration and invasion (42). Collagen VIII is consistently observed in human cell lines derived from several carcinomas, malignant astrocytoma and Ewing sarcoma, and there is an increased level of collagen VIII in human brain tumors compared with normal tissue. It is found around actively proliferating vessels of brain and in large fibrosed and more inert vessels of angiomas (reviewed in Ref. 43).

Previously, pVHL null RCC cells were reported to have a defect in cell cycle exit mechanisms (17). Additional evidence for a role for pVHL in cell cycle control is provided by the identification of cyclin D1 and CDK6 as novel VHL targets. Cyclin D1 and CDK6 have a pivotal role in linking growth regulatory signals to cell division and regulate G_1 to S phase transition by phosphorylation of the retinoblastoma protein (44). Abnormalities of the cyclin-cyclin dependent kinase machinery has been implicated in tumorigenesis in many cancer types. Overexpression of D-type cyclins can overcome G_1 arrest caused by deprivation of mitogens and allow abnormal entry into the cell cycle (45), and cooperates in oncogenic transformation with several oncogenes including *RAS*, *MYC*, and *E1A* (46). Cyclin D1 overexpression is a feature of many cancers including RCC cell lines and primary tumors (32, 47, 48). CDK6 overexpression has also been reported in a variety of cancers (49, 50) but has not been analyzed in RCC previously.

To date the best defined function of pVHL is the targeting of HIFα subunits for ubiquitylation and proteasomal destruction (13, 14). The ability of pVHL to bind and regulate fibronectin metabolism appears to be unrelated to regulation of HIF-1 and HIF-2, as mutant pVHL (*e.g.*, L188V), which is defective for fibronectin binding, can retain the ability to promote HIF-α subunit ubiquitylation (9, 33). The HIF-1 and HIF-2 transcription factors influence many aspects of cellular metabolism by regulating expression of a wide repertoire of hypoxia response genes. Three of the nine pVHL target genes (*VEGF*, *LRP1*, and *PAI-1*) identified by our expression array analysis were identified as hypoxia-inducible in a previous study in which the same Atlas arrays were used to identify gene responses to hypoxia (51). We investigated the hypoxia inducibility of our target genes in non-VHL-transfected cell lines, as RCC cells transfected with a VHL expression vector may not demonstrate normal hypoxic induction of known hypoxia-responsive genes because of pVHL overexpression. By Northern analysis using the 293 embryonal kidney cell line and two cancer cell lines we found no significant up-regulation ($\leq$1.5-fold) of *CCND1*, *CDK6*, *ITB8*, and *CD59* transcripts in response to hypoxia. However, *IL-6* and *COL8A1* transcripts were not detectable in any of the three cell lines tested, so we could not determine their response to

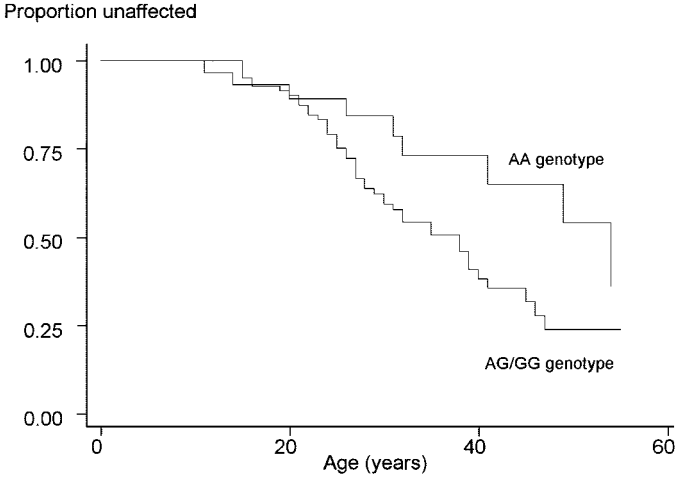

Fig. 5. Kaplan-Meier curves showing the relationship between CCND1 AA and AG/GG genotypes and risk of CNS HAB.

hypoxia. It would appear that pVHL down-regulation of *CCND1, CDK6, ITB8,* and *CD59* transcripts is mediated by a HIF-independent mechanism. Cyclin D1 protein expression is a feature of many cancer types. Changes in cellular cyclin D1 protein expression can result from a variety of mechanisms including alterations in transcription and proteolysis (52, 53). Although we cannot exclude the possibility that pVHL might also influence cyclin D1 protein levels by regulating proteasomal destruction (as described for HIF-α subunits and recently for an atypical protein kinase C; Ref. 54), we observed a clear effect on mRNA levels. This could result from regulation of transcription and/or mRNA stability. Recently Pioli and Rigby (55) have reported that pVHL down-regulates levels of the heterogeneous nuclear ribonucleoprotein A2 RNA binding protein (which enhances GLUT1 mRNA stability) via a proteasome-dependent mechanism. Thus, pVHL may influence expression of target genes by regulating the abundance of RNA-binding proteins. It might also regulate the transcription, *e.g.,* by controlling the abundance of a transcription factor(s). Thus far the only transcription factor described as a pVHL target was HIF, which seems not to affect the expression of cyclin D1 and other hypoxia-independent genes identified in our paper. Additional study is necessary to elucidate the mechanisms by which pVHL down-regulates mRNA levels of these genes. The L188V missense mutation is associated with a PHE only phenotype (type 2C VHL disease; Ref. 56). Functional analysis has demonstrated that the mutant L188V protein can suppress RCC cell line growth *in vivo*, and down-regulate HIF-1 and HIF-2, but is defective in promoting fibronectin matrix assembly (9, 33). Interestingly the L188V mutant pVHL retained at least partial ability to down-regulate cyclin D1. This finding suggests that complete loss of pVHL down-regulation of cyclin D1 is not necessary for PHE susceptibility, and is consistent with the hypothesis that up-regulation of cyclin D1 might contribute to RCC and HAB susceptibility in VHL disease. Additional studies of the ability of specific VHL mutations, which are associated with different tumor phenotypes, to down-regulate non-HIF-dependent pVHL targets will provide further insights into the relationships between specific pVHL functions and tumorigenesis in specific tissue types.

We have demonstrated previously that genetic modifiers influence the risk of RCC, and retinal and cerebellar HABs in VHL disease (10). The exon 4 A/G *CCND1* polymorphism at nt 870 has been reported to influence the risk of colorectal cancer in mismatch repair gene mutation carriers (23). However, in this report an earlier age at onset of colorectal cancer was associated with the A allele (whereas in our study the G allele was associated with more severe retinal and CNS HAB involvement). The codon 242 *CCND1* variant has been suggested to influence *CCND1* mRNA splicing. *CCND1* is alternately spliced between exon 4 and 5 to give two transcripts, which occur simultaneously in a number of tissues (22). Both the spliced and unspliced transcripts encode proteins that contain the functional cyclin box (amino acids 55–161), but the unspliced transcript does not contain the exon 5 sequence encoding a PEST destruction box responsible for the rapid turnover of the protein (22). We reasoned that if VHL gene inactivation resulted in cyclin D1 up-regulation the functional effects of this might be influenced by *CCND1* genotype. Our results suggest that the *CCND1* G allele may increase susceptibility to HABs but not RCC. The molecular pathology of sporadic HABs and clear cell RCC differ, and certain germ-line VHL mutations (*e.g.,* H98Y) may cause susceptibility to retinal and CNS HABs but not RCC (57). Thus, genetic modifiers that influence HAB but not RCC susceptibility are not be unexpected. Furthermore, although the A allele was reported to enhance colorectal cancer susceptibility (23), other studies have suggested that the GG genotype is associated with poorly differentiated tumors and reduced disease-free interval in patients with squamous cell carcinoma of the head and neck (58). Thus, the effect of polymorphic variation at *CCND1* codon 242 may differ between tumor types. Previously we were unable to demonstrate any genotype-phenotype correlations for retinal angiomatosis in VHL disease (59), and despite improvements in the clinical management of VHL disease retinal and CNS HABs remain an important cause of morbidity. Thus, confirmation of *CCND1* and the identification of additional genetic modifiers will enhance the management of VHL families by identifying high-risk individuals who might be targeted for increased surveillance or early entry into trials of antiangiogenic therapy.

In summary, we report the identification of six novel cancer related gene targets for the VHL tumor suppressor. The target genes additionally implicate the VHL tumor suppressor gene in control of cell cycle progression, extracellular matrix formation, tumor survival, cell migration, and invasion, and provide additional evidence for HIF-independent functions for pVHL. We focused our studies on genes that were down-regulated in both 786-0 and RCC4 cell lines. However, not all of these genes were expressed in UMRC2 suggesting that: (*a*) additional analysis of candidate targets that were regulated in only 786-0 or RCC4 is indicated, as studies of additional cell lines might demonstrate further pVHL targets; and (*b*) the precise functional consequences of pVHL inactivation will depend on the nature of other background genetic and epigenetic alterations. Analysis of the relationship between up-regulation of specific pVHL targets and tumor prognosis in sporadic RCC may provide additional insights into the role of VHL tumor suppressor in tumorigenesis and suggest which pVHL downstream pathways might be prioritized for therapeutic intervention.

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