

The expression of the *von Hippel-Lindau* gene product and its impact on invasiveness of human breast cancer cells

MOHAMMAD K. ZIA, KHALED A. RMALI, GARETH WATKINS,
ROBERT E. MANSEL and WEN G. JIANG

Metastasis and Angiogenesis Research Group, University Department of Surgery,
Cardiff University School of Medicine, Cardiff, UK

Received May 21, 2007; Accepted June 27, 2007

Abstract. The *von Hippel-Lindau* (*VHL*) gene is located on the short arm of chromosome 3, the mutations of which lead to the development of von Hippel-Lindau disease. The *VHL* gene is a putative tumour suppressor gene in VHL and a few other conditions, possibly by negative regulation of hypoxia-inducible factor-1 (*HIF-1*) and the stromal-derived factor-1 (*SDF-1*) receptor, *CXCR4*, via which the *VHL* protein negates angiogenesis and tumour cell migration. The current study investigated the expression of *VHL* at the mRNA and protein levels in clinical breast tumours and evaluated the impact of *VHL* on the invasion of human breast cancer cells *in vitro*. Primary breast cancer samples (n=124), adjacent non-cancerous breast tissues obtained from patients in cohort (n=33) and a panel of human breast cancer cells (n=12) were used. Tissue distribution of *VHL* protein in human breast cancer tissues was assessed using immunohistochemical analysis, and *VHL* transcript was determined using quantitative reverse transcription PCR. Breast cancer cell line MDA-MB-231 was transfected with a human *VHL* expression construct (pCR3-GFP/*VHL*) to allow forced overexpression of *VHL* in the cells. Invasiveness and migration of cancer cells were assessed using the Matrigel invasion and Cytodex-2 migration assays. Statistical analysis was performed using the Student's t-test. Our results showed that breast cancer cell lines MCF-7 and ZR-75-1 expressed very high levels of *VHL* transcripts, but the highly aggressive MDA-MB-231, MDA-MB-435 and MDA-MB-453 expressed either no *VHL* or a low level. The levels of *VHL* transcripts were significantly lower in grade 2 and grade 3 tumours (mean \pm SD, 1.36 ± 0.55 and 0.9 ± 0.37), compared with grade 1 tumours (12.3 ± 7.6 , $p < 0.002$). Node-positive tumours had lower levels of *VHL* than node-negative tumours. Although tumours from patients with metastasis

and from those who died of breast cancer had low levels of *VHL*, the most significant reduction in *VHL* was seen in tumours which developed local recurrence ($p = 0.03$). The staining of *VHL* protein was most abundant in mammary epithelial cells and moderate in endothelial cells. Tumour cells in breast tissues had low to moderate *VHL* staining. pCR3-GFP/*VHL*-transfected MDA-MB-231 (MDA-MB-231^{VHL+}) exhibited a reduced spontaneous *in vitro* invasiveness (14.8 ± 2.7) compared with the control cells (18.4 ± 1.4). MDA-MB-231^{VHL+} cells also lost their invasion response to HGF/SF, an invasion-inducing cytokine. The MDA-MB-231^{VHL+} cells had substantially reduced motility compared with that of the controls (14.8 ± 0.7 for MDA-MB-231^{VHL+} and 20.7 ± 1.2 for the control; $p < 0.001$). Thus, *VHL* exerts inhibitory effects on the invasive and migratory capacity of breast cancer cells *in vitro*. Low levels of *VHL* occur in most aggressive breast tumours. Taken together, *VHL* is a powerful putative tumour suppressor gene in human breast cancer.

Introduction

The *von Hippel-Lindau* (*VHL*) tumour suppressor gene encoding the von Hippel-Lindau protein (pVHL) contains mutations in patients with VHL syndrome (1), which is an autosomal dominant inherited multi-system cancer syndrome. A large number of different mutations have been identified so far, including single-base mutations, deletions, rearrangements and more complex mutations. So far germline mutations have been detected in ~75% of VHL families (2). These mutations may cause substitutions of specific amino acid residues and functional change of *VHL* protein (pVHL) (3). The hallmark lesions of VHL syndrome are retinal angiomas, haemangioblastomas of the cerebellum and spinal cord and renal cell carcinomas (4). Other features include pheochromocytoma, angiomatosis retinae, haemangioblastoma of the CNS, renal lesions, pancreatic cysts and epididymal cystadenoma (5-8). The estimated incidence of this syndrome is 1:36,000 live births (9), and the most common causes of death are metastases from renal cell carcinoma and neurological complications from cerebellar haemangioblastomas (10). The *VHL* gene was mapped to the region of chromosome 3p25 by linkage analysis in 1988 and was finally identified on that locus by positional cloning in 1993 (11-13). It controls cell cycle progression by the regulation of

Correspondence to: Dr Wen G. Jiang, Metastasis and Angiogenesis Research Group, University Department of Surgery, Cardiff University School of Medicine, Cardiff CF14 4XN, UK
E-mail: jiangw@cf.ac.uk

Key words: von Hippel-Lindau, breast cancer, tumour suppressor gene, tumour progression

p27Kip1 at both the mRNA and protein levels (14). Aberrant expression of tumour suppressor genes has been reported in some human malignancies, including kidney, thyroid and gastric cancer. 3p allelic losses have been reported in early-stage invasive breast cancer (15). However, allelic loss at the VHL gene region (3p25-26) has not been found in sporadic cases of human breast cancer (16). Gene inactivation by promoter hypermethylation has been demonstrated. Abnormal methylation of the CpG islands located in the promoter region of the genes leads to transcriptional silencing (17). The preclusion of the formation of the transcription complex occurs due to the recruitment of MBP, MeCPs and histone deacetylase. This results in the deacetylation of histone and thus the formation of a compact chromatin complex unfavourable for the initiation of transcription (18).

Breast cancer occurs in 1 in 10 women in the US and the UK, and the prognosis is largely affected by the presence and development of metastasis (19). The VHL gene is a putative tumour suppressor gene in VHL disease and a few other conditions, possibly by negative regulation of hypoxia-inducible factor-1 (HIF-1) and the stromal-derived factor-1 (SDF-1) receptor, CXCR4, via which it exerts an inhibition of angiogenesis and tumour cell migration. VHL inactivation acquired by incipient tumour cells early in tumorigenesis confers, not only a selective survival advantage, but also the tendency to home to selected organs.

Previous studies have suggested that the VHL condition is associated with tumours which are hypervascular and overexpress hypoxia-inducible mRNAs (5), indicating that the tumour suppressor acts as an angiogenic regulator in cancer. Hypervascularisation is induced by overexpression of vascular endothelial growth factor (VEGF) which is a key factor in tumour angiogenesis (20-22). Hypoxia is a major stimulus for VEGF production (20).

In the current study we aimed to investigate the expression of VHL at the mRNA (using Q-RT-PCR) and protein level (using the immunohistochemistry approach) in breast cancer cells as well as in a cohort of breast tissues (cancer and normal background tissues). We also correlated the level of the expression with clinical/pathological parameters. Furthermore, we examined the effect of the knocked-out VHL gene from breast cancer cells on invasiveness and migratory properties of breast cancer cells.

Materials and methods

Cell culture conditions. Breast cancer cell lines MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453, BT-474, BT-549, MCF-7, ZR-75-1 and control cells [European Collection of Animal Cultured Cells (ECACC), Salisbury, UK] were grown in DMEM F12 medium (supplemented with 10% foetal calf serum and antibiotics). VHL-transfected breast cancer cells (MDA-MB-231^{VHL+}) were grown in medium containing a neomycin derivative (G418; Sigma, Poole, Dorset, UK) to which MDA-MB-231 transfected cells carried the resistance marker. Cell cultures were routinely incubated in a humidified incubator maintained at 37°C.

Collection and storage of breast tissues. Frozen breast cancer tissues (124) together with 33 normal background tissues

with a median follow-up of 120 months were collected immediately after mastectomy, and stored at -80°C until ready for use. All specimens were collected with the approval of the local ethics committees. A pathologist (ADJ) verified the normal background and cancer tissues, and confirmed that the background tissues were free from tumour deposits. Details of the clinical and pathological information of the patients have been previously documented by Jiang *et al* (23) and Ye *et al* (24).

RNA extraction and RT-PCR for VHL detection in breast cancer. Total cellular RNA was isolated from either homogenised breast tissues, or cell line monolayers using RNA-zol reagent (ABgene, Epsom, UK) according to the manufacturer's instructions. RNA was extracted using the standard guanidine isothiocyanate method by following the manufacturer's protocol. cDNA was generated from 1 µg RNA using an AMV-reverse transcription kit (ABgene). Conventional PCR primers were designed using Beacon Designer software (Palo Alto, CA, USA), to allow amplification of regions that have no overlap with known genes and span at least one intron. PCR was carried out using the following primer pairs (VHLF4, ATGCCCGGAGGGCGG AGAACTG and VHLR2, TCAATCTCCCATCCGTTGAT) (Life Technologies, Inc., Paisley, Scotland, UK). The expression of VHL in different breast cancer cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453, BT-474, BT-549, MCF-7 and ZR-75-1) were examined by using RT-PCR.

PCR reactions were performed using a Gene Amp PCR system 9700 (PE Biosystems, Warrington, UK) thermal cycler. Amplification of cDNA samples was carried out at the following conditions: 94°C for 15 sec, 56°C for 15 sec, 72°C for 45 sec followed by a final extension phase of 7 min at 72°C for 36 cycles. The PCR products, including a 1-kb ladder were separated on 0.8% agarose gels. PCR products were then visualised using ethidium bromide (10 mg/ml) and a UV transilluminator (UVItect, Cambridge, UK). Agarose gels were photographed using a Unisave camera (Wolf Laboratories, York, UK), and subsequent images were documented using Adobe Photoshop Elements software.

Quantitative-real time-PCR analysis of VHL in breast cancer. The quantity of VHL transcripts was assessed using Q-RT-PCR as we recently reported (23-24). Quantitative-real time-PCR was carried out using an iCycler IQ™ system (Bio Rad, Camberley, UK), which incorporated a gradient thermocycler and a 96-channel optical unit. A Universal probe system (UniPrimer™) was used in the current study (Intergen, Oxford, UK). The internal standards used in the study were specific plasmids generated using Pcr2.1-cloning vector (Invitrogen, Paisley, Scotland, UK). Conditions for Q-RT-PCR were as follows: enzyme activation at 95°C for 12 min, 1 cycle; followed by 60 cycles of denaturing at 95°C for 15 sec; annealing at 55°C for 40 sec; and an extension at 72°C for 25 sec.

Immunohistochemical detection of VHL in cancer and normal breast tissues. As recently reported by us (25), briefly, immunohistochemical staining was performed on

paired frozen-sectioned tissues (cancer tissue paired with normal background tissue from the same patient). Frozen sections were cut, air-dried and fixed in 50% methanol and 50% acetone for 15 min. The sections were then air-dried once more for 10 min and stored at -20°C in foil-wrapped slide trays. Immediately before staining, specimens were placed in PBS (Optimax wash buffer) for 5 min. The slides were incubated with primary rabbit polyclonal antibodies against VHL (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) or positive control at 1:200 dilution for 1 h. After 4 washes with PBS, the slides were placed in universal multi-link biotinylated (Vector BA-1300) secondary antibody at a 1:100 dilution and incubated for 30 min. This was followed by 4 washes with PBS. Slides were then placed in avidin biotin complex (ABC-Vector Labs) for 30 min. The bound antibody complex was detected using diaminobenzidine tetrahydrochloride (3,3'-diaminobenzidine)-DAB chromogen (Sigma) for 5 min. The slides were washed with H_2O for 5 min and placed in Mayer's haematoxylin for 1 min, followed by differentiation in H_2O for 10 min. This was followed by dehydration in methanol (3 times) and clearing in 2 changes of xylene before mounting under a coverslip and examination under a microscope. Negative controls (using PBS buffer instead of the primary antibody) and positive controls were used in this study. The complete procedure was carried out at room temperature.

Construction of VHL expression cassettes and transfection of breast cancer cells. Eight human breast cancer cell lines were screened for endogenous expression of tumour suppressor gene VHL receptor. An invasive cell line, MDA-MB-231 which was VHL negative was chosen for introduction of the VHL gene. The gene, after amplification from normal breast tissue cDNA (using primer sets VHLEXF1, ATGCCCCGGAGGGCGGAGAACTG and VHLEXR1, TCAATCTCCATCCGTTGAT) was T-A cloned into pCR3-GFP-TOPO (Invitrogen) plasmid before electroporation into the breast cancer cells, using a method previously reported (26). Expression of the gene was confirmed by RT-PCR, with those cells positive for VHL designated MDA-MB231^{VHL}. In total, four independent clones expressing the VHL gene were constructed to verify *in vitro* assays assessing the effects of this protein in breast cancer cells. Two *in vitro* assays analyzing the VHL gene-transformed breast cancer cells were used to determine what, if any, effect over-expression of VHL would have on the human breast cancer cell line MDA-MB231^{VHL}. These cells [MDA-MB-231^{WT}, MDA-MB231^{CP} (transfected with control plasmid) and MDA-MB231^{VHL}] were used later after incubation with/ without HGF at 25 ng/ml which was used as a stimulator of motility and invasion.

Invasion assay. Trans-well chambers equipped with 6.5-mm diameter polycarbonate filters (pore size $8\ \mu\text{m}$) (Becton Dickinson Labware, Oxford, UK) were pre-coated with $50\ \mu\text{g}/\text{membrane}$ ($100\ \mu\text{l}$) of solubilised basement membrane in the form of Matrigel (Collaborative Research Products, Bedford, MA) and dried overnight. After membrane rehydration ($100\ \text{ml}$ of complete medium), 30,000 cells were aliquoted into each insert with/without HGF (25 ng/ml).

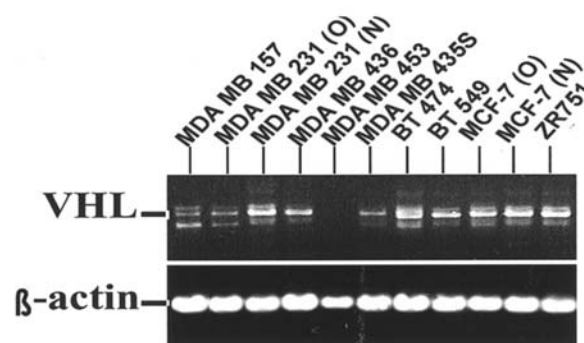


Figure 1. Expression of VHL in human breast cancer cells MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453, MDA-MB-435S, BT-474, BT-549, MCF-7 and ZR-75-1. β -actin acted as a housekeeping gene.

After 96 h of co-culture, non-invasive cells were removed from the inner chamber with a cotton swab. Invaded cells on the underside of the insert were then fixed (4% formaldehyde) and stained with crystal violet. The cells were then counted microscopically (20 fields/insert).

Cytodex-2-bead motility assay. Cells were pre-coated onto cytodex-2 carrier beads (Sigma) for 2 h in complete medium as previously reported by us (27). After the medium was aspirated and the cells washed ($\times 2$ in complete medium), they were aliquoted into wells of a 96-well plate in triplicate ($300\ \mu\text{l}/\text{well}$). HGF (25 ng/ml) was added, and the cells were incubated overnight. The beads were washed off in medium, and the cells that had migrated onto the floor of the well were fixed (4% formaldehyde) and stained with crystal violet. The cells were then counted microscopically on $\times 20$ objective lens.

Data analysis. Statistical analyses were performed using the Student's t-test, and Chi-square test, where appropriate (Minitab and Microsoft Excel). Differences were considered to be statistically significant at $p < 0.05$.

Results

Determination of the presence of VHL transcript in human cancer cell lines using RT-PCR. Breast cancer cell lines MCF-7 and ZR-75-1 expressed high levels of the VHL transcript, but the highly aggressive MDA-MB-231, MDA-MB-435 and MDA-MB-453 cell lines expressed either no VHL or low levels of VHL transcript (Fig. 1).

Levels of expression of VHL transcript in breast cancer tissues and its correlation with nodal status. The levels of VHL transcript in tumour and normal tissues were compared. In the entire cohort, tumour tissues ($n=124$) had 3.0 ± 1.32 (mean \pm SD) copies of transcript per 50 ng total RNA, and normal tissues ($n=33$) had 1.9 ± 1.3 . The difference was not statistically significant ($p=0.59$). In the cohort, 33 patients had paired normal and tumour tissues; we further sub-analysed the transcript levels in these paired samples. Tumour tissues showed a marginally low level of the VHL transcript (1.48 ± 0.52 copies of transcript per 50 ng total RNA) and the

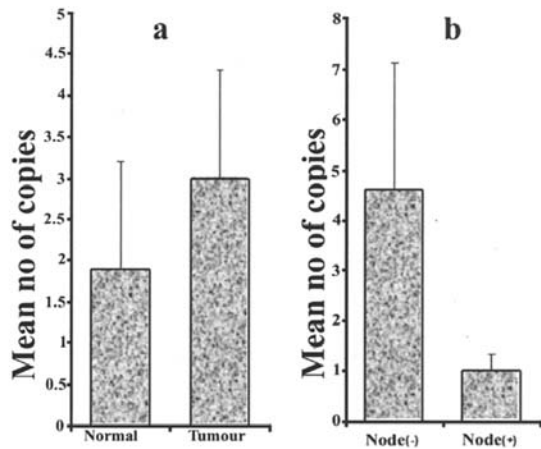


Figure 2. (a) No significant differences were shown in the level of expression of VHL between normal and breast cancer tissues ($p=0.59$). (b) The level of VHL transcripts in tumours with different nodal statuses. mRNA levels of VHL were lower in node(+) (1.0 ± 0.35 copies) tumours, as compared with node(-) (4.6 ± 2.5 ; $p=0.16$) tumours.

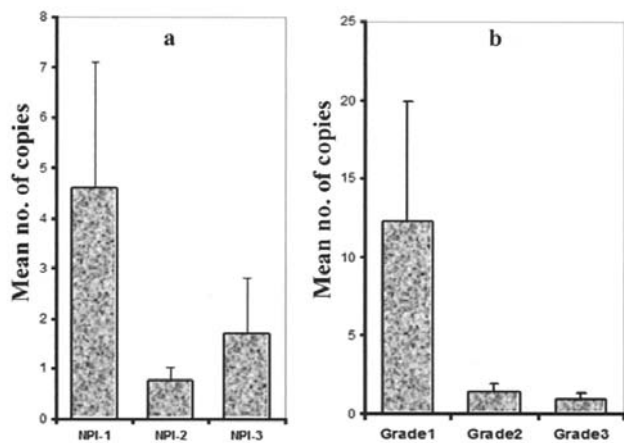


Figure 3. (a) Levels of VHL transcript in patients with NPI-1, NPI-2 and NPI-3 tumours. Patients with NPI-2 and NPI-3 who had moderate and poor prognoses had low levels of VHL as compared with NPI-1 patients. mRNA levels of VHL were lower in NPI-3 (1.7 ± 1.1 ; $p=0.29$) tumours, as compared with NPI-1 (4.6 ± 2.5) tumours. (b) Levels of VHL transcripts were significantly lower in grade 2 (1.36 ± 0.55 copies; $p=0.002$) and grade 3 (0.9 ± 0.37 ; $p<0.002$) tumours, as compared with grade 1 (12.3 ± 7.6 ; $p<0.002$).

matched normal tissues had 2.08 ± 1.4 . The difference was not statistically significant ($p=0.24$) (Fig. 2a). Low levels of the VHL transcripts were seen in node-positive tumours as compared with node-negative tumours (1.0 ± 0.35 copies vs 4.6 ± 2.5 ; $p=0.16$). (Fig. 2b).

VHL expression in relation to breast tumour grade and prognosis. Here, we used the Nottingham Prognostic Index (NPI) as a tool to predict the prognosis of the patients. An NPI <3.4 was regarded as having a good predicted prognosis (NPI-1), 3.4-5.4 indicated moderate (NPI-2) and >5.4 poor prognosis (NPI-3). Patients with NPI-2 and NPI-3 who had moderate and poor prognoses had low levels of VHL. Levels of VHL transcripts were lower in NPI-2 (0.76 ± 0.27 copies;

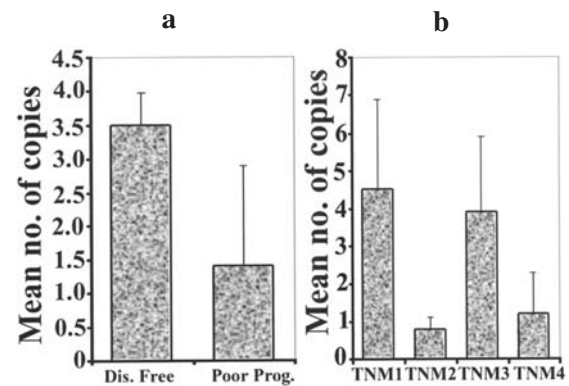


Figure 4. (a) The level of VHL transcripts were lower in patients with poor prognosis (1.4 ± 0.7 copies; $p=0.28$) as compared with disease-free (3.5 ± 1.8 ; $p<0.28$) patients. (b) mRNA levels of VHL transcripts were lower in TNM2 (0.78 ± 0.32 copies; $p=0.13$), TNM3 (3.9 ± 2.0 ; $p=0.86$) and TNM4 (1.2 ± 1.1 ; $p=0.21$) stage patients, as compared with TNM1 (4.5 ± 2.4 ; $p<0.13$) stage patients.

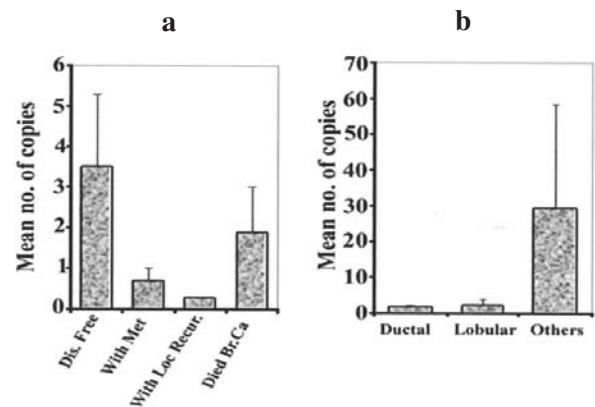


Figure 5. (a) Levels of VHL transcript in relation to clinical history. Patients who were disease-free post treatment for breast cancer had the highest levels of VHL transcript (3.5 ± 1.8 copies). Tumours from the patients with metastatic disease and from those who died from breast cancer had low levels of VHL mRNA (0.67 ± 0.33 ; $p=0.13$ and 1.9 ± 1.1 ; $p=0.48$). However, the most significant reduction in VHL was observed in tumours that had developed local recurrence (0.0027 ± 0.002 ; $p=0.03$). (b) Ductal and lobular breast cancers had low VHL transcript (1.76 ± 0.47 and 2.34 ± 1.5) compared with other breast cancer types (29.3 ± 29).

$p=0.13$) and NPI-3 (1.7 ± 1.1 ; $p=0.29$) tumours compared with NPI-1 (4.6 ± 2.5 ; $p\leq0.13$) tumours (Fig. 3a). Significant low levels were seen in grade 2 (1.36 ± 0.55 copies) and grade 3 (0.9 ± 0.37 copies) tumours compared with grade 1 (12.3 ± 7.6 copies) tumours ($p=0.002$ and $p=0.001$ respectively) (Fig. 3b).

Level of the VHL expression and its correlation to the poor prognosis and tumour-node metastatic classification of breast cancer patients. Patients with poor prognosis had low levels of VHL transcript compared with patients who were disease-free and had good prognosis. mRNA levels of VHL were lower in poor prognosis (1.4 ± 0.7 copies; $p=0.28$) patients compared with disease-free (3.5 ± 1.8 ; $p\leq0.28$)

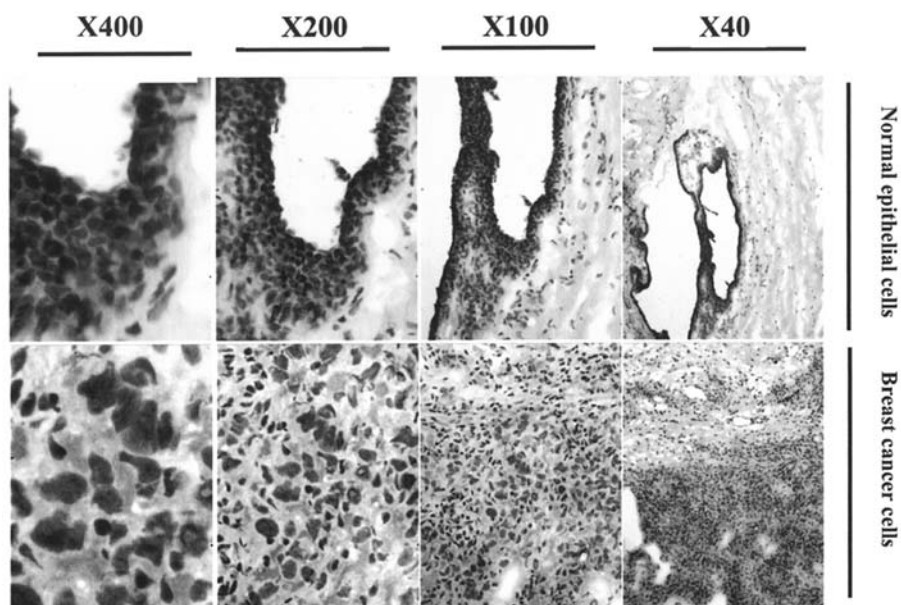


Figure 6. Immunohistochemical staining of VHL in human breast cancer. Shown are photos taken from breast tumour tissue with residual normal epithelial cells. A clear difference was observed between the residual normal epithelial cells which stained strongly for VHL and breast cancer cells which showed weaker VHL staining.

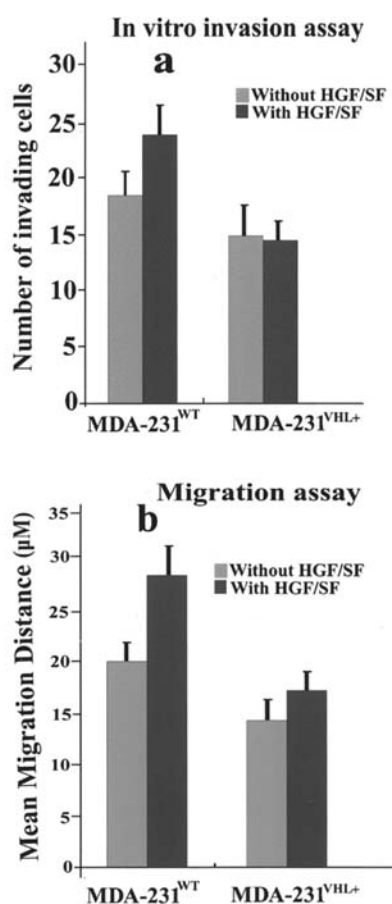


Figure 7. (a) Forced expression of the VHL gene and its impact on the invasion of breast cancer cells. VHL-transfected MDA-MB-231 cells (MDA-MB-231^{VHL+}) showed reduction in invasion in addition to losing their response to HGF to a certain degree. (b) The expression of the VHL gene and its impact on cellular migration of breast cancer cells. VHL-transfected MDA-MB-231 cells (MDA-MB-231^{VHL+}) showed reduction in migration and lost their response to HGF to a certain degree.

patients (Fig. 4a). mRNA levels of VHL were lower in TNM2 (0.78 ± 0.32 copies; $p=0.13$), TNM3 (3.9 ± 2.0 ; $p=0.86$) and TNM4 (1.2 ± 1.1 ; $p=0.21$) stages, compared with TNM1 (4.5 ± 2.4 ; $p \leq 0.13$) stage patients (Fig. 4b).

Expression of VHL transcript and its correlation with clinical outcome and survival. Patients who were disease-free post treatment for breast cancer had the highest levels of the VHL transcript (3.5 ± 1.8 copies). Although tumours from the patients with metastatic disease and those who died from breast cancer had low mRNA levels of VHL (0.67 ± 0.33 ; $p=0.13$ and 1.9 ± 1.1 ; $p=0.48$), the most significant reduction in VHL was observed in tumours that had developed local recurrence (0.0027 ± 0.002 ; $p=0.03$) (Fig. 5a).

More malignant ductal and lobular breast cancers had low VHL transcript (mean \pm SD, 1.76 ± 0.47 and 2.34 ± 1.5) compared with less malignant breast cancer types (29.3 ± 29) (Fig. 5b).

Immunohistochemical staining of VHL protein in human breast cancer. On immunohistochemical staining of VHL protein in human breast cancer, normal epithelial cells stained strongly for pVHL in the cytoplasm whereas cancer cells displayed much weaker staining. The staining of VHL protein was most abundant in normal mammary epithelial cells and moderate in endothelial cells. Breast cancer cells had low to moderate VHL staining (Fig. 6).

Overexpression of VHL and its impact on migration and invasion of breast cancer cells. VHL-transfected MDA-MB-231 highly aggressive breast cancer cells (MDA-MB-231^{VHL+}) exhibited reduced invasion (MDA-MB-231^{VHL+}, 14.8 ± 2.7 vs. MDA-MB-231^{WT} wild-type, 18.4 ± 1.4 ; $p < 0.001$). In addition, we tested the response of the cells to an invasion-enhancing cytokine HGF. HGF significantly increased the

invasiveness in wild-type cancer cells MDA-MB-231^{WT} (with HGF vs without HGF; $p < 0.05$). However, MDA-MB-231^{VHL+} cells completely lost their response to HGF (Fig. 7a).

Similarly, overexpression of VHL also resulted in significant reduction in cell motility potential (MDA-MB-231^{VHL+}, 14.8 ± 0.7 vs. wild-type MDA-MB-231^{WT}, 20.7 ± 1.2 ; $p < 0.001$) compared with MDA-MB-231^{WT} and control cells (MDA-MB-231^{CP}). MDA-MB-231^{VHL+} cells also showed a reduced response to HGF (Fig. 7b).

Discussion

The VHL protein (pVHL) has 213 amino acids and is widely expressed in human tissues having several distinct functions as a gatekeeper protein (3,28-30). As the principal function of VHL protein is the negative regulation of hypoxia-inducible mRNAs including VEGF mRNA, the inactivation of the *VHL* gene plays a critical role in the angiogenesis of VHL tumours. The VHL protein regulates VEGF expression at a post-transcriptional level, and VHL inactivation in target cells causes a loss of VEGF suppression which leads to the formation of a vascular stroma (21). In addition, since VHL protein is also required for the downregulation of transcription activity of certain genes for cell growth and cell cycle, the inactivation of the *VHL* gene may contribute to tumorigenesis (7).

Recently, a potentially important transcriptional regulatory network in which pVHL plays a key role has been revealed. In this network, the pVHL targets a cellular transcription factor called Elongin (SIII), which is a heterotrimer consisting of a transcriptionally active subunit (A) and two regulatory subunits (B and C) that activate transcription elongation by RNA polymerase II. The pVHL was shown to bind tightly and specifically to the Elongin B and C subunits and to inhibit Elongin (SIII) transcriptional activity *in vitro* (31). Mutation or deletion of the domain of pVHL with its partner proteins elongins B and C is associated with VHL disease (32). In addition to forming a stable complex with elongins B and C, pVHL also binds with Cullin-2 and Rbx1 and regulates target gene product levels, including vascular endothelial growth factor (VEGF) and glucose transporter 1 (33). This complex negatively regulates hypoxia-inducible mRNAs (34) and therefore controls the levels of hypoxia-inducible proteins (35) and is thought to function as an E3 ubiquitin ligase (36). Nearly 70% of naturally occurring cancer-predisposing mutations of VHL disrupts this interaction (37). The tumour suppressor activity function of pVHL may be linked to its ability to bind to Elongin B and C (30). Since the elongin-binding domain of VHL is frequently mutated in cancers, it is suggested that loss of elongin binding causes tumorigenesis by compromising pVHL stability and/or potential VHL ubiquitination functions (36).

In the current study, pVHL was found to be present in the cytoplasmic region of both normal and tumour cells of the breast, with the latter showing a lower degree of staining. VHL transcripts were found to be low in most aggressive breast cancers. The latter is particularly true in patients who developed local recurrence. Tumours from these patients had virtually no detectable amount of VHL transcript. In addition, poor prognosis breast cancer patients with axillary lymph

node metastases had low levels of VHL transcript when compared to node negative cancers.

Moreover, we endeavoured to gain an insight into the role of the VHL gene in breast cancer. We investigated the invasive and migratory capacity of the most aggressive breast cancer cells (MDA-MB-231) after they were transfected with VHL expression construct. Furthermore, we attempted to look at the potential alteration in propensity of these breast cancer cells to metastasize to distant organs.

VHL-transfected breast cancer cell line MDA-MB-231^{VHL+} exerted inhibitory effects on the invasive and migratory capacity of breast cancer cells *in vitro*, and low levels of VHL were found in most aggressive breast cancers. In addition, the transfected cells also lost some of their response to HGF. Equally interesting was the observation that the MDA-MB-231^{VHL+} cells tended to clump together in contrast to the breast cancer cells that acted as control cells [wild-type MDA-MB-231 (MDA-MB-231^{WT}) and GFP MDA-MB-231 (MDA-MB-231^{GFP}) cells] which showed no such propensity. This points towards a decrease in the metastatic potential of these cells. Also, the MDA-MB-231^{VHL+} cells were found to be less responsive to HGF.

Collectively, the study has shown that VHL had a low level of expression in aggressive breast cancers, as well as the negative impact of VHL on motile and invasive functions of breast cancer cells. These findings suggest that VHL is a powerful tumour suppressor gene in human breast cancer. It opens a new front for further research into the beneficial role of the *VHL* gene in breast cancer, especially regarding the development of potential advantageous therapeutic gene modalities.

Acknowledgements

The authors would like to thank Dr Anthony Douglas-Jones for his expert help in histology. The study was supported by Cancer Research Wales.

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