VHL inactivation is an important pathway for the development of malignant sporadic pancreatic endocrine tumors

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Abstract

A small subset of familial pancreatic endocrine tumors (PET) arises in patients with von Hippel–Lindau syndrome and these tumors may have an adverse outcome compared to other familial PET. Sporadic PET rarely harbors somatic VHL mutations, but the chromosomal location of the VHL gene is frequently deleted in sporadic PET. A subset of sporadic PET shows active hypoxia signals on mRNA and protein level. To identify the frequency of functionally relevant VHL inactivation in sporadic PET and to examine a possible prognostic significance we correlated epigenetic and genetic VHL alterations with hypoxia signals. VHL mutations were absent in all 37 PETs examined. In 2 out of 35 informative PET (6%) methylation of the VHL promoter region was detected and VHL deletion by fluorescence in situ hybridization was found in 14 out of 79 PET (18%). Hypoxia inducible factor 1α (HIF1-α), carbonic anhydrase 9 (CA-9), and glucose transporter 1 (GLUT-1) protein was expressed in 19, 27, and 30% of the 152 PETs examined. Protein expression of the HIF1-α downstream target CA-9 correlated significantly with the expression of CA-9 RNA ($P < 0.001$), VHL RNA ($P < 0.05$), and VHL deletion ($P < 0.001$) as well as with HIF1-α ($P < 0.005$) and GLUT-1 immunohistochemistry ($P < 0.001$). These PET with VHL alterations and signs of hypoxia signalling were characterized by a significantly shortened disease-free survival. We conclude that VHL gene impairment by promoter methylation and VHL deletion in nearly 25% of PET leads to the activation of the HIF-pathway. Our data suggest that VHL inactivation and consecutive hypoxia signals may be a mechanism for the development of sporadic PET with an adverse outcome.

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Introduction

The molecular pathogenesis of the sporadic pancreatic endocrine tumors (PET) is still poorly understood (Gumbs et al. 2002). Mutations in the MEN1 gene are the most common finding in hereditary PET (Chandrasekharappa et al. 1997) and this gene is also mutated in a subset of sporadic PET (Perren et al. 2007). Patients with the von Hippel–Lindau disease develop PET in 15% (Lubensky et al. 1998), and these tumors tend to be malignant (Corcos et al. 2008). Malignant PET also occur rarely in neurofibromatosis type 1 (NF1; Perren et al. 2006) and tuberous sclerosis 1 (TSC1; Francalanci et al. 2003) syndromes. On a functional level, all three genes predisposing to PET with an increased risk of malignancy, namely VHL, NF1, and TSC1, are linked to hypoxia signalling via upregulation of hypoxia inducible factor 1 (HIF1; Brugarolas & Kaelin 2004, Semenza 2007, Johannessen et al. 2008). The pVHL protein plays a key role in this regulation: a protein complex
including pVHL marks HIF for proteasomal degradation and loss of pVHL function which leads to an accumulation of HIF1-α in the nucleus (del Peso et al. 2003). The resulting activation of HIF responsive elements leads to the increased transcription of a wide range of hypoxia-inducible genes, among them carbonic anhydrase 9 (CA-9) and glucose transporter 1 (GLUT-1; Behrooz & Ismail-Beigi 1999, Zhang et al. 1999, Semenza 2007). HIF may also be activated by a loss of function of the NF1 and the TSC1 genes via activation of mammalian target of rapamycin (mTOR) (Brugarolas & Kaelin 2004, Dutcher 2004).

In sporadic PET, somatic mutations of the VHL gene are rare (Chung et al. 1997, Moore et al. 2001) and a minor role of the VHL gene in the genesis of these tumors has been deduced from this fact (Lindberg et al. 2007). However, there is evidence that hypoxia signalling plays an important role in sporadic PET: expression array data comparing benign and malignant signalling in sporadic PET (Schmitt et al. 2007) was analyzed mRNAs (Couvelard et al. 2001). As these 3p deletions often comprise large parts of the short arm of chromosome 3, it is unknown whether the loss of the VHL gene or the other genes is of relevance for tumor development. At last, hypoxia is a well-known driving force of growth in several tumors (Brizel et al. 1996, Hockel et al. 1996).

The aim of this study was to investigate if hypoxia signalling in sporadic PET is associated with genomic VHL alterations. In order to answer this question, we analyzed a well-characterized series of sporadic PET (Schmitt et al. 2007)

a) on DNA level by VHL mutation, deletion, and VHL promoter methylation analysis
b) on mRNA level by analyzing the expression of the VHL gene and of hypoxia-associated target genes
c) on protein level by determining the expression of the hypoxia target proteins HIF1-α, CA-9, and GLUT-1.

Materials and methods

Tumor specimens

Tissue specimens of 152 patients were included in the study (Schmitt et al. 2007). The available paraffin specimens comprised 139 primary tumors and 13 metastases. According to the WHO 2004 classification, 51 out of the 139 primary tumors (37%) were well-differentiated PET (wd PET), 37 tumors (27%) were well-differentiated PET of uncertain behavior (wd PET ub), 34 tumors (24%) were well-differentiated pancreatic endocrine carcinomas (wd PEC), and 3 tumors (2%) were poorly-differentiated PEC (pd PEC). In 14 tumors (10%), not all information for the classification was available. According to the recently proposed tumor, node, metastasis (TNM) classification (Rindi et al. 2006) primary tumors were classified as follows: 54 tumors (39%) were classified as T1, 39 tumors (28%) as T2, 25 tumors (18%) as T3, and 2 tumors (1%) as T4. In 19 tumors (14%), not all information for the classification was available.

Fresh frozen tissue of 37 out of the above mentioned tumor samples (28 primary tumors and 9 metastases) were available for liquid-based DNA and RNA analysis. Seven out of these 37 fresh frozen tissue samples (19%) were classified as wd PET, 13 (35%) as wd PET ub, 14 (38%) as wd PEC, and 3 (8%) as pd PEC.

Immunohistochemistry

Sections of 4 μm from a tissue microarray (TMA; Schmitt et al. 2007) were stained with antibodies against HIF1-α, CA-9, and GLUT-1. The immunohistochemical staining for all antigens was performed on automated staining systems (GLUT-1 on Ventana BenchMark, Ventana Medical Systems, Tucson, AZ, USA and HIF1-α and CA-9 on Bond Refine, Vision BioSystems Ltd, Newcastle Upon Tyne, UK). The following antibodies were used: HIF1-α clone mgc3 ab16066 (Abcam, Cambridge, UK), dilution 1:500; CA-9 polyclonal antibody ab15086 (Abcam), dilution 1:200; GLUT-1 polyclonal antibody AB1341 (Chemicon International, Temecula, CA, USA), dilution 1:1000. Antigen retrieval was performed by heating (H2(60) (EDTA-based pH 9.0 Bond Epitope Retrieval Solution, 60 min), for HIF1-α, H2(30) (EDTA based pH 9.0 Bond Epitope Retrieval Solution, 30 min) for CA-9 and CC1 mild (Tris based buffer, 30 min) for GLUT-1). Visualization was performed using the avidin–biotin complex method leading to a brown staining signal. For HIF1-α glioblastoma tissue and for both CA-9 and GLUT-1 normal liver tissue was used as controls.

For all stainings the intensity (negative, weak positivity, strong positivity) as well as the percentage of positive tumor cells was recorded.
VHL deletion analysis

Sections of 7 μm of the TMA were used for fluorescence in situ hybridization (FISH) analysis to detect copy number losses. Deletion analysis was performed by double-target FISH using a chromosome 3-specific centromere probe in combination with a 3p25-specific probe containing the VHL gene. The DNA probes were labeled with fluorescein-12-dUTP (spectrum green) and Alexa-647-aha-dUTP (spectrum red) by nick translation (Roche) and precipitated in ethanol in the presence of Cot-1 DNA, sodium acetate, and glycogen. The DNA pellet was re-suspended in the hybridization buffer (55% formamide, 10% dextran sulfate). After dehydration in an ethanol series, the slides were denatured in 0.2 M HCl at room temperature for 20 min and in 1 M NaSCN for 30 min at 80 °C. Proteinase treatment was performed by incubation in 0.4 g/l pepsin in 10 mM Tris–HCl solution for 10 or 20 min at 37 °C, followed by post-fixation in 4% formalin for 10 min at room temperature. After sequential washings in 2×SSC and distilled water and dehydration in an ethanol series, both probes were applied to the tissue specimens, denatured at 80 °C for 5 min and then incubated at 37 °C overnight in a humidified chamber. Post-hybridization washes were performed twice for 5 min at 60 °C in 0.4×SSC and once for 5 min at room temperature in 4×SSC. Blocking was performed by incubating the slides with each 100 μl of a solution of 4×SSC and non-fat-dried-milk 5% at 37 °C in a humidified chamber for 20 min. After washing the slides in 4×SSC/0.05% Tween for 5 min, the hybridized fluorescein-labeled probes were detected using a cascade of rabbit anti-FITC immunoglobulins (1:1000, Dako, Glostrup, Denmark), FITC-conjugated swine anti-rabbit immunoglobulins (1:100, Dako) and FITC-conjugated rabbit anti-swine immunoglobulins (1:1000, Dako, Glostrup, Denmark). The Alexa-647-aha-dUTP-labeled probes were detected using a mouse anti-cyanine 5 monoclonal antibody (1:1000, Kreatech, Amsterdam, The Netherlands). The slides were washed in 4×SSC/0.05% Tween, air-dried and then mounted in Vectashield (Vector, Burlingame, CA, USA) containing 0.5 μg/ml 4',6-diamidino-2-phenylindole-antifade (Sigma) for nuclear counterstaining. Images were recorded with Analysis software (Olympus Biosystems, Hamburg, Germany).

VHL mutation analysis

In 37 tumors, mutation analysis for the VHL gene was performed. DNA from fresh frozen tissue was extracted using the Purgene-kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer’s recommendations. Frozen sections stained by hematoxylin–eosin were used for the morphological control of tumor tissue quality and microdissection. PCR using genomic DNA as template was carried out in a 50 μl mixture of 1×PCR buffer containing 400 ng of template DNA, 200 μM dNTP (Roche), 1 μM of each intron-based primer, and 1 μl Taq Polymerase (Ampli Taq Gold, Perkin Elmer, Europe, UK). Mutation analysis was performed by denaturing gradient gel electrophoresis and direct sequencing of samples with aberrant banding patterns as described (Anlauf et al. 2006).

Methylation-sensitive PCR

In 35 tumors, a methylation sensitive PCR of the VHL promoter region was informative. DNA from fresh frozen tissue was modified by bisulfite treatment (Qiagen), converting all unmethylated cytosine residues to uracil. This bisulfite treatment was followed by amplification with primers specific for methylated and unmethylated DNA (Table 1). PCR amplification of template DNA was performed as follows: 99 °C for 5 min, 60 °C for 25 min, 99 °C for 5 min, 60 °C for 85 min, 99 °C for 5 min, 60 °C for 175 min, and a final extension step of 20 °C for at least 5 min. For each set of DNA modification and PCR, a cell line of human kidney cancer with known hypermethylation of the VHL promoter (769-P) was included as a positive control. The PCR-products were additionally cycle sequenced and the methylation status of all the CpG residues was analyzed to assess the possibility of incomplete CpG methylation.

RNA analysis

In 32 tumors, high quality RNA could be extracted from fresh frozen tissue. RNA was analyzed for the expression of the VHL gene and of the hypoxia-associated target gene CA-9. RNA from fresh frozen tissue was extracted using the RNasy MiniKit (Qiagen) according to the manufacturer’s recommendations. After extraction, DNase digestion, NanoDrop measurement, and quality control via gel electrophoresis 1 μg RNA was transcribed into cDNA using oligo dT primers. Quantitative TaqMan analysis was

Table 1 Primers used for methylation-sensitive PCR

<table>
<thead>
<tr>
<th>VHL</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>MF</td>
<td>5'-TGGAGGATTTTTTGTGYTGAYG-3'</td>
<td>5'-GTGGAGGATTTTTTGTGYTGAYG-3'</td>
</tr>
<tr>
<td>UF</td>
<td>5'-RAACRAACCRCCCA-3'</td>
<td>5'-CCCRACACACCRCCCA-3'</td>
</tr>
<tr>
<td>MR</td>
<td>5'-RAACRAACCRCCCA-3'</td>
<td>5'-CCCRACACACCRCCCA-3'</td>
</tr>
<tr>
<td>UR</td>
<td>5'-RAACRAACCRCCCA-3'</td>
<td>5'-CCCRACACACCRCCCA-3'</td>
</tr>
</tbody>
</table>
performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using the probes CA-9 (hCG37623) and VHL (hCG1994953; Applied Biosystems, Foster City, CA, USA). Data were normalized using the expression data of normal pancreatic islet RNA. \textit{GAPDH} was referred to as a housekeeping gene. The cut-off for overexpression (CA-9) and underexpression (\textit{VHL} RNA) was defined as at least twofold difference to normal islet RNA respectively.

\textbf{Statistical analysis}
Fisher’s exact test (SPSS software, Chicago, IL, USA) was used to examine the relationship between the results obtained from the DNA, RNA, and immunohistochemical investigations. Level of significance was defined as \( P < 0.05 \).

\textbf{Ethics}
The study was approved by the local ethical committee (Stv 40-2005).

\textbf{Results}

\textbf{HIF1-\( \alpha \), CA-9 and GLUT-1 immunohistochemistry}
HIF1-\( \alpha \) was scored as positive if a nuclear staining was detected in at least 5\% of the tumor cells (Fig. 1). Twenty-four out of 127 (19\%) tumors expressed HIF1-\( \alpha \). In wd PET, HIF1-\( \alpha \) was expressed in 2 out of 33 cases (6\%). In wd PET ub, HIF1-\( \alpha \) was expressed in 5 out of 37 cases (13.5\%), whereas in wd PEC and pd PEC HIF1-\( \alpha \) was expressed in 9 out of 31 (29\%) and in 1 out of 3 (33\%) primary tumors and in 4 out of 11 (36\%) of metastases thereof.

CA-9 was scored as positive if a cytoplasmic staining was detected in at least 5\% of the tumor cells (Fig. 1). Thirty-six out of 134 (27\%) tumors expressed CA-9. In wd PET, CA-9 was expressed in only a minority of the tumor cells (5\% each) in 2 out of 40 cases (5\%). In wd PET ub, CA-9 was expressed in 10 out of 34 cases (29\%), whereas in wd PEC and pd PEC, CA-9 was expressed in 14 out of 32 (44\%) and in 1 out of 3 (33\%) primary tumors, respectively and in 7 out of 12 (58\%) of their metastases.

GLUT-1 was scored as positive if a membranous and/or cytoplasmic staining was observed in at least 25\% of the tumor cells and/or if the staining was strong (Fig. 1). All tumors with \(< 25\% \) positive tumor cells showed only a faint intensity and were scored as negative.

Forty out of 133 (30\%) tumors expressed GLUT-1. In wd PET, GLUT-1 was expressed in 6 out of 40 cases (15\%). In wd PET ub, GLUT-1 was expressed in 12 out of 34 cases (35\%), whereas in wd PEC and pd PEC, GLUT-1 was expressed in 11 out of 32 (34\%) and in 3 out of 3 (100\%) primary tumors, respectively and in 6 out of 12 (50\%) of their metastases.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{A PET (A, H&E) with nuclear positivity for HIF1-\( \alpha \) (B), cytoplasmic and membranous positivity for CA-9 (C), and cytoplasmic positivity for GLUT-1 (D).}
\end{figure}
An example of a PET with immunohistochemical positivity for HIF1-α, CA-9 and GLUT-1 is shown in Fig. 1.

**DNA analysis**

In 78 out of 152 cases (51%), FISH on the TMA was informative. In 14 out of 78 cases (18%), the deletion of the VHL gene was detected by FISH (Fig. 2). VHL deletion was defined as loss of one VHL signal in at least 20% of tumor cells. The percentage of tumor cells with a VHL deletion ranged from 20 to 90%.

VHL gene mutations were absent in all 37 tumors examined.

Methylation of the VHL promoter region was detected in 2 out of 35 cases (6%; Fig. 3). Sequencing showed a methylation of all CpG residues in the amplified promoter region. One of those two cases correlated with the highest overexpression of CA-9 (sevenfold) RNA observed and with a threefold underexpression of VHL mRNA. None of the 32 remaining samples showed any methylation including the methylation of single CpG residues.

Both PET with VHL promoter methylation showed two 3p signals by FISH.

**RNA analysis**

VHL RNA was underexpressed by factor 2 or more compared to normal islets in 8 out of 32 (25%) tumors, the remaining 24 PET (75%) showed an expression rate from 0.51- to three-fold. CA-9 RNA was regularly expressed in 22 out of 32 (69%) and overexpressed in 10 out of 32 (31%) tumors.

**Statistical analysis**

Results of HIF1-α, CA-9, and GLUT-1 immunohistochemistry correlated significantly with each other (*P* < 0.005 and < 0.001 respectively). CA-9 immunohistochemistry correlated significantly with CA-9 RNA overexpression (*P* < 0.001) and VHL underexpression (*P* < 0.05). Importantly, a positive correlation was found between CA-9 immunopositivity and VHL deletion (*P* < 0.001) and if CA-9 protein expression was correlated to VHL methylation and deletion counted as one ‘impaired VHL function’ group (*P* < 0.001). Moreover, HIF1-α protein expression correlated significantly with VHL underexpression (*P* < 0.05). Genomic VHL alteration
and CA-9 protein expression both correlated with recurrence and poor survival (Fig. 4). An overview over the correlation results is given in Table 2 and further details given in (Supplementary Table 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

**Discussion**

Somatic VHL mutations are a rare event in sporadic PET (Chung et al. 1997, Moore et al. 2001). However, several results from the recent literature suggest that VHL-associated hypoxia might nevertheless play a role in sporadic PET (Barghorn et al. 2001, Couvelard et al. 2005, 2006).

We analyzed a series of sporadic PET on DNA, RNA, and protein-expression levels to define the role of VHL and hypoxia. VHL mutations were absent in all 37 tumors, thus reflecting the low-mutation rate reported in the literature (Chung et al. 1997, Moore et al. 2001). In the present study, we extended the analysis to VHL deletion and promoter methylation. We found a hypermethylation in the VHL promoter in 6% of the investigated tumors. CpG island methylation of the VHL gene leading to gene silencing is frequent in renal cell carcinoma (Herman et al. 1994, Glavac et al. 1996, Dulaimi et al. 2004). CpG island methylation of other genes has been previously described in a subset of PET (Chan et al. 2003), which was associated with an adverse outcome (Arnold et al. 2007). VHL methylation has not yet been reported in PET. We found VHL gene deletion in 14 out of 78 tumors (18%) by FISH. This prevalence is comparable to the relative copy number changes detected by CGH (Perren et al. 2007) and is slightly lower than the LOH rates reported using the analysis of microsatellite markers (Chung et al. 1997, Barghorn et al. 2001), which might be explained by different sensitivities of the methods and different cut-off definitions. Interestingly, VHL deletions and VHL promoter methylation occurred as alternative events in our tumor set. Summing them up, we describe here nearly 25% of sporadic PET with genomic VHL alteration, making this a frequent genetic event in sporadic PET. To examine whether these alterations really do have an effect on VHL function, we examined VHL mRNA expression and a series of downstream targets of hypoxia signalling.

VHL is part of the E3 ligase marking hydroxylated HIF1-α for proteasomal degradation (Maxwell et al. 1999, Cockman et al. 2000). Loss of VHL function or hypoxia, therefore, increases HIF1-α levels, leads to nuclear translocation, and increases the transcription of hypoxia-inducible mRNAs and to an upregulation of several hypoxia response proteins such as CA-9 and GLUT-1 (Pastorekova et al. 1992, Opavsky et al. 1996, Loncaster et al. 2001, Hui et al. 2002). Here we show

**Table 2** Correlations of data obtained from immunohistochemistry, RNA, and DNA analysis

<table>
<thead>
<tr>
<th>Correlation</th>
<th>GLUT-1 IHC</th>
<th>HIF1-α IHC</th>
<th>CA-9 RNA</th>
<th>VHL RNA</th>
<th>VHL meth</th>
<th>VHL LOH</th>
<th>VHL mut/meth/del</th>
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<tbody>
<tr>
<td>CA-9 IHC</td>
<td>0.000</td>
<td>0.002</td>
<td>0.000</td>
<td>0.021</td>
<td>0.111</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
<td>GLUT-1 IHC</td>
<td>0.049</td>
<td>0.450</td>
<td>0.128</td>
<td>0.227</td>
<td>0.020</td>
<td>0.156</td>
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<tr>
<td>HIF1-α IHC</td>
<td>0.088</td>
<td>0.010</td>
<td>1.000</td>
<td>0.039</td>
<td>0.052</td>
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<tr>
<td>CA-9 RNA</td>
<td>0.217</td>
<td>0.083</td>
<td>0.009</td>
<td></td>
<td>0.001</td>
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<td>VHL RNA</td>
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Correlations indicated as $P$ values. Bold type indicates significant correlation.
for the first time that genomic alterations (deletions or promoter methylation) of the VHL gene are associated with a decreased level of VHL transcripts and with increased nuclear translocation of HIF1 and increased transcription of the hypoxia-inducible CA-9 RNA and expression of its protein. Expression of GLUT-1, another hypoxia-regulated protein, was also associated with genomic VHL alterations. Interestingly, this occurred although all tumors only showed either VHL deletion or promoter methylation. These findings suggest either VHL haploinsufficiency or another epigenetic inactivation of the second allele, such as histone acetylation, for example. Even though this correlation was statistically significant, the correlation was not 100%, indicating that other mechanisms than VHL-impairment might also lead to hypoxia signalling. Couvelard et al. (2005) described a cytoplasmic CA-9 staining pattern in tumors associated with VHL disease. In these familial tumors, both VHL alleles are inactive. In our study, only sporadic PET including four poorly-differentiated carcinomas (three primary tumors and one metastasis) were investigated. Nearly 70% of the CA-9 positive tumors in our study showed both a cytoplasmic and membranous staining pattern for CA-9 (Fig. 1).

As familial PET in the setting of VHL, NF-1, and TSC-1 disease have an adverse outcome compared to MEN1 tumors, we also analyzed the tumor-free survival of sporadic PET with regard to VHL status and hypoxia signals. Sporadic PET with genomic VHL alterations and increased hypoxia signalling were characterized by an adverse outcome in our series. We found a more frequent expression of CA-9 in metastatic PET than in non-metastatic PET (47 vs 16%), similar to previously reported results (Couvelard et al. 2005) and VHL alterations were found in 21% of benign and 69% of recurring tumors.

Our results link the upregulation of the HIF-pathway to alterations of the VHL gene and an adverse outcome in sporadic tumors. We additionally examined HIF1-α and the HIF1 downstream target GLUT-1 (Behrooz & Ismail-Beigi 1999), which were both expressed in significant correlation with CA-9.

In summary, we show that despite only rare mutations, the VHL gene is affected by promoter methylation and most frequently by deletion in sporadic PET (25%). Monoallelic changes of the VHL gene are associated with a decreased transcription and an activation of the HIF-pathway. This leads to an overexpression of HIF1-α and HIF-targets CA-9 and GLUT-1 both on mRNA and protein level.

We show that a subset of sporadic PET follows a genetic pathway similar to the VHL-associated familial PET and that these tumors have an adverse outcome.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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