Overexpression of the natural antisense hypoxia-inducible factor-1α transcript is associated with malignant pheochromocytoma/paraganglioma

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Abstract

Paragangliomas (PGLs) have widely different metastastic potentials. Two different types of PGLs can be defined by expression profiling. Cluster 1 PGLs exhibit VHL and/or succinate dehydrogenase (SDH) mutations and a pseudohypoxic phenotype. RET and neurofibromatosis type 1 (NF1) mutations occur in cluster 2 tumors characterized by deregulation of the RAS/RAF/MAP kinase signaling cascade. Sporadic PGLs can exhibit either profile. During sustained hypoxia, a natural antisense transcript of hypoxia-inducible factor 1 (aHIF) is expressed. The role of aHIF in the metastatic potential of PGL has not yet been investigated. The aim was to test the hypothesis that genotype-specific overexpression of aHIF is associated with an increased metastatic potential. Tumor samples were collected from 87 patients with PGL. Quantitative PCR was performed for *aHIF*, vascular endothelial growth factor (*VEGF*), *aquaporin 3*, *cytochrome b561*, *p57Kip2*, *slit homolog 3*, and *SDHC*. Expression was related to mutation status, benign versus malignant tumors, and metastasis-free survival. We found that both aHIF and VEGF were overexpressed in cluster 1 PGLs and in metastatic tumors. In contrast, slit homolog 3, p57Kip2, cytochrome b561, and SDHC showed overexpression in non-metastatic tumors, whereas no such difference was observed for aquaporin 3. Patients with higher expression levels of aHIF and VEGF had a significantly decreased metastasis-free survival. Higher expression levels of SDHC are correlated with an increased metastasis-free survival. In conclusion, we not only demonstrate a higher expression of VEGF in cluster 1 PGL, fitting a profile of pseudohypoxia and angiogenesis, but also of aHIF. Moreover, overexpression of aHIF and VEGF marks a higher metastatic potential in PGL.

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Introduction

Paragangliomas (PGLs) are catecholamine-producing tumors that originate from chromaffin cells of the adrenal medulla (pheochromocytoma proper) or from sympathetic neuronal tissue in extra-adrenal locations of the abdomen or chest (DeLellis *et al*. 2004). PGLs in the head and neck usually derive from parasympathetic tissues. Metastases occur in 10–15% of patients with PGL (Lenders *et al*. 2005). It was observed that 25–30% of the PGLs are caused by germline mutations

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of one of the susceptibility genes (Gimenez-Roqueplo *et al*. 2008), i.e. the proto-oncogene *RET* and the tumor suppressor genes von Hippel–Lindau (*VHL*), neurofibromatosis type 1 (*NF1*), and succinate dehydrogenase subunits A–D (*SDHA/B/C/D*) (Baysal 2008). Mutations in the *VHL* and/or *SDH* genes result in a phenotype known as pseudohypoxia (Gimenez-Roqueplo *et al*. 2001, Kaelin 2007), leading to uncontrolled expression of *HIF-1*-regulated genes, such as vascular endothelial growth factor (*VEGF*) which in turn facilitates angiogenesis. The signature of hypoxia and angiogenesis is not limited to *VHL*- and/or *SDH*-related tumors and is also prominent in a subset of sporadic PGLs, as shown by tumor gene expression profiling (Dahia *et al*. 2005). These 'cluster 1' tumors are distinct from 'cluster 2' tumors, which include *RET*- and *NF1*-related and another subset of sporadic PGLs (Dahia *et al*. 2005). This latter cluster is characterized by the deregulation of the RAS/RAF/MAP kinase signaling cascade.

High expression of HIF-1α and VEGF is associated with higher tumor aggressiveness and an unfavorable prognosis in many types of solid cancers (Manders *et al*. 2003, Hoogsteen *et al*. 2007). Hypoxia initially induces increased expression of HIF-1α, but during sustained hypoxia, the amount of *HIF-1α* mRNA is reduced (Wenger *et al*. 1998). Instead, there is considerable overexpression of the HIF-1α natural antisense transcript (aHIF), which encodes the antisense template of the 3′-untranslated region of *HIF-1α* mRNA (Thrash-Bingham & Tartof 1999). This represents a negative feedback loop, in which aHIF inhibits the translation of *HIF-1α* mRNA after chronic hypoxia (Uchida *et al*. 2004). A 20% of the human genome consists of these naturally occurring antisense transcripts, which regulate gene transcription, RNA splicing, polyadenylation, editing, stability, transport, and translation (Yelin *et al*. 2003, Chen *et al*. 2004, Katayama *et al*. 2005). Importantly, besides acting as a transcription factor for hypoxia-inducible genes, HIF-1α also stabilizes the tumor suppressor gene *p53* under early hypoxic conditions (An *et al*. 1998). This situation is reversed during later stages of hypoxia, when HIF-1α translation is inhibited by aHIF (Uchida *et al*. 2004), resulting in the loss of p53 followed by deregulated cell proliferation. Thus, aHIF expression also allows tumor cells to survive hypoxia without inducing the tumor suppressor p53 (Thrash-Bingham & Tartof 1999). Theoretically, aHIF expression is not mainly a phenotypical sign of chronic hypoxia but also in itself mechanistically associated with more aggressive tumor behavior and increased metastatic potential (Cayre *et al*. 2003).

So far, the role of aHIF in the pathophysiology of PGL had not been investigated. We hypothesize that there is a genotype-specific overexpression of aHIF expression in PGL, which is associated with an increased metastatic potential. To test this hypothesis, we performed quantitative RT-PCR of *aHIF* and other cluster-specific genes (*VEGF*, *aquaporin 3*, *cytochrome b561*, *p57Kip2*, *slit homolog 3*, and *SDHC*) in PGLs of different genotypes and investigated the relation between expression of these genes and metastasis-free survival (Dahia *et al*. 2005).

Materials and methods

Patients

Totally, 151 patients with histologically proven pheochromocytoma or extra-adrenal sympathetic PGL evaluated at the Departments of Endocrinology and General Internal Medicine at the Radboud University Nijmegen Medical Centre (RUNMC) underwent surgical resection of primary PGL between November 1967 and November 2008. Details on the post-surgical follow-up of part of this cohort were published elsewhere (Timmers *et al*. 2008). Frozen primary tumor tissue was obtained from 87 patients (41 males and 46 females), five of whom underwent surgery at the Erasmus MC in Rotterdam. Tumors were adrenal in 76 cases and extra-adrenal abdominal in 11 cases. The presence of germline mutations and large deletions of *RET*, *VHL*, *NF1*, and *SDHB/C/D* was investigated at the Department of Genome Diagnostics of the University Medical Centre in Utrecht according to standard procedures. Clinical characteristics and genotypes are listed in Table 1. In 44 patients with an apparently sporadic presentation, i.e. no family history and lack of syndromal features, full germline testing of all susceptibility genes was omitted. In these cases, SDHB immunostaining was carried out on tumor tissues to investigate the functional integrity of SDH (see below). Mutations in *SDHB*, *SDHC*, or *SDHD* genes lead to lack of SDHB immunoreactivity and thus can be used to reveal germline mutations in these genes (van Nederveen *et al*. 2009).

During a mean ± S.D. follow-up of 7.04 ± 5.07 (range: 0.05–20.83) years, seven (8%) developed metastatic disease (Table 2). The mean ± S.D. interval between surgery and the diagnosis of metastases was 5.50 ± 8.15 (range: 0.00–19.38) years. Of the total patients, 16 patients died, including five with metastatic PGL. Data were collected under conditions of regular clinical care, with the approval of ethics

Table 1 Clinical characteristics of paraganglioma (PGL) patients

Variable	PGL patients ($n=87$)
Age (years)	
Diagnosis mean ± S.D.	46.05 ± 16.17
Diagnosis range	7–77
Gender	
Male	41
Female	46
Primary tumor location	
Adrenal	76
Extra-adrenal	11
Tumor size (cm)	
Mean ± S.D.	6.40 ± 3.42
Range	1.4–18
Genotype	
SDHB	3
SDHD	1
VHL	3
MEN2A	10
MEN2B	1
NF1	7
Sporadic[a]	18 (16 adrenal, two extra-adrenal)
Apparently sporadic	44 (39 adrenal, five extra-adrenal; 37/37 SDH-negative[b])
Total follow-up (years)	
Mean ± S.D.	7.04 ± 5.07
Range	0.05–20.83
Metastases	
No	80
Yes	7

[a]SDHB, SDHC, SDHD, and MEN2 mutations and large deletions ruled out by germline testing.
[b]As assessed by positive SDHB immunohistochemistry.

committee obtained for the use of those data for scientific purposes.

SDHB immunohistochemistry

Of the 44 patients with apparently sporadic presentation, immunohistochemistry was performed in 37 patients to detect the expression of SDHB. Sections (4 μm) from paraffin-embedded tumor tissues were stained as described previously (van Nederveen *et al*. 2009) using an anti-human rabbit polyclonal primary antibody (Sigma–Aldrich, HPA002868, 1:200).

Tumor tissue processing

Representative snap-frozen tumor samples were cut in approximately five sections of 20 μm in a cryostat at −20 °C, put in lysis buffer (RLT, Qiagen), and stored at −80 °C until RNA isolation. For histological confirmation, additional slices were H&E stained for histological re-evaluation by a pathologist.

RNA extraction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) with on-column DNase-I treatment. RNA concentrations were determined by measuring the spectrophotometric absorption at 260 nm using the GeneQuant (Amersham).

RT reaction

cDNA synthesis was performed using 10 μl sample volume, containing ∼1 μg total RNA and 10 μl RT-mix, with a total volume of 20 μl. cDNA synthesis was performed using a PTC-200 PCR apparatus (MJ Research, Waltham, MA, USA). Samples were denatured for 10 min at 70 °C and immediately cooled on ice. After random hexamers were annealed for 10 min at 21 °C, cDNA synthesis was performed for 45 min at 42 °C, followed by an enzyme inactivation step for 5 min at 99 °C.

Quantitative PCR

For the quantitative PCR (qPCR), SYBR Green assays were used for *aHIF* and *VEGF*, and Taqman for *aquaporin 3* (Gill blood group), *cytochrome b561*, *p57Kip2*, *slit homolog 3* (*Drosophila melanogaster*), and *SDHC*. The latter five genes were selected because in a previous study by Dahia *et al*. (2005), their RNA expression was shown to mark either cluster 1 or 2

Table 2 Characteristics of the patients with metastatic paraganglioma

Patient	Age at diagnosis	Gender	Primary tumor location	Tumor size (cm)	Genotype	Total follow-up (years)	Time until metastases (years)	Deceased	Survival (years)
1	53	F	Extra-adrenal	7	SDHB	9.99	2.27	Yes	9.99
2	20	M	Extra-adrenal	7	SDHB	20.96	19.38	No	20.96
3	58	M	Adrenal	17	SDH negative[a]	16.32	15.10	Yes	16.32
4	44	F	Adrenal	7	SDH negative[a]	0.61	0.61	Yes	0.61
5	40	M	Adrenal	13	SDH negative[a]	1.19	0.00	Yes	1.26
6	61	M	Adrenal	12	SDH negative[a]	2.34	0.00	No	2.34
7	42	M	Adrenal	8.5	Sporadic	4.26	1.13	Yes	4.26

[a]As assessed by positive SDHB immunohistochemistry.

PGL. Overexpression of VEGF and aquaporin 3 mark cluster 1 tumors, whereas cytochrome b561, P57Kip2, slit homolog 3, and SDHC mark cluster 2 tumors. qPCR was performed using the Taqman Universal PCR Master Mix or SYBR. Green Universal Master Mix (PE Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) in a total volume of 25 µl. The forward and reverse primers, as well as the optimal concentrations (for each target gene), were optimized in previous studies at the Department of Laboratory Medicine of the RUNMC for the SYBR Green assays, whereas Assays on Demand (PE Applied Biosystems) was used for the Taqman-based assays. Hypoxanthine–guanine phosphoribosyltransferase (*HPRT1*) was used as a housekeeping gene for normalization of all samples (de Kok *et al*. 2005). qPCR was performed using the ABI Prism 7700 Sequence Detector (PE Applied Biosystems). The amplification reactions were carried out with denaturation at 95 °C for 10 min, 40 cycles of 15 s at 95 °C (melting), and 60 s at 60 °C (annealing and elongation).

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (for Windows 16.0; SPSS 16.0, Chicago, IL, USA). Aquaporin-3 mRNA levels were normally distributed (one-sample Kolmogorov–Smirnov), whereas those of other genes were not. After log transformation, a normal distribution of mRNA expression was found for the other genes. Thus, parametric tests were used for all the log-transformed mRNA levels, with the exception of aquaporin-3, which was already normally distributed before log transformation. ANOVA with *post hoc* Tukey HSD test was used to test for the differences in mRNA expression of the target genes between clusters 1 and 2 PGLs, sporadic PGLs, and apparently sporadic PGLs with unknown genotype. Differences in the mRNA expression between metastatic and non-metastatic tumors were tested using an independent samples *t*-test. In addition, the relation between expression of individual genes and metastasis-free survival was investigated. Normalized mRNA levels of an individual target gene were correlated with metastasis-free survival after dichotomizing on the basis of the median mRNA expression of individual genes using univariate Cox regression survival analysis. Metastasis-free survival of patients with an above versus below median expression was compared using the Kaplan–Meier method and a log-rank test for equality of survival distributions. A similar analysis was performed for overall survival.

Results

SDHB expression in apparently sporadic tumor tissues

The presence of SDHB immunoreactivity and a granular staining pattern typical of mitochondrial proteins was detected in all the tumor tissues of apparently sporadic cases. This includes the four metastatic cases with incomplete germline mutation testing (Supplementary figure, see section on supplementary data given at the end of this article).

Genotype-specific mRNA expression

Figure 1 illustrates the differences in mRNA expression of the individual target genes between sporadic PGLs ($n=18$), apparently sporadic PGLs ($n=44$), and

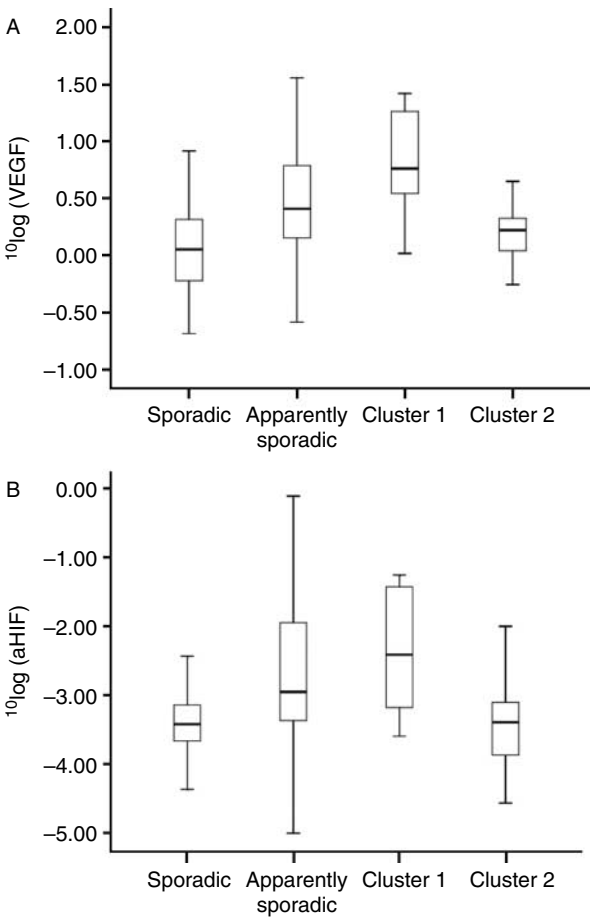

Figure 1 Box and whiskers plots (box = median with 25th and 75th percentile, and whiskers are minimum and maximum values) of log-normalized expression levels of VEGF (A) and aHIF (B) in sporadic, apparently sporadic, and cluster 1 or 2 type of PGLs. Significant differences, as assessed by ANOVA and *post hoc* Tukey HSD testing, were found only between clusters 1 and 2 tumors.

cluster 1 PGLs (*SDHB* ($n=3$), *SDHD* ($n=1$), and *VHL* ($n=3$)) and cluster 2 PGLs (*MEN2A* ($n=10$), *MEN2B* ($n=1$), and *NF1* ($n=7$)). After performing an ANOVA with *post hoc* Tukey HSD test to compare mRNA expression of individual genes, both VEGF (Fig. 1A) and aHIF (Fig. 1B) expression was found to be significantly higher in cluster 1 compared with cluster 2 PGLs ($P=0.018$ and $P=0.021$ respectively). Sporadic and apparently sporadic tumors exhibited expression levels that were not significantly different from clusters 1 and 2 tumors. No differences were found in aquaporin 3, cytochrome b561, p57Kip2, slit homolog 3, or SDHC expression (not shown).

To further explore the significantly higher expression of VEGF and aHIF in cluster 1 compared with cluster 2 PGLs, genotype-specific mRNA expression levels are shown in Fig. 2. SDHB, SDHD, and VHL (cluster 1) tumors on an average exhibit higher expression levels for both aHIF and VEGF than MEN2A-, MEN2B-, and NF1 (cluster 2)-related tumors. The size of the subgroups precluded relevant statistical analyses.

mRNA expression and metastasis-free survival

The mRNA expression of each gene in non-metastatic PGLs ($n=80$) versus the mRNA expression in the primary tumors of metastatic PGLs ($n=7$) is visualized in Fig. 3. The RNA expression of both VEGF ($P=0.001$) and aHIF ($P=0.001$) was significantly higher in the primary tumors of PGLs that metastasized during follow-up than in the non-metastatic PGLs (Fig. 3A and B). Aquaporin-3 expression was not significantly different between groups (Fig. 3C), whereas the expression of slit homolog 3 ($P=0.019$), p57KIP2 ($P=0.035$), cytochrome b561 ($P=0.003$), and SDHC ($P=0.039$) was significantly higher in the non-metastatic group than in the metastatic group (Fig. 3D–G).

Subsequently, we assessed whether metastasis-free survival differed between the group of patients after dichotomization at the median expression level using a Kaplan–Meier analysis with log-rank testing. This analysis indicates that high expression of VEGF is associated with decreased metastasis-free survival (Fig. 4A; $P=0.004$). This is also true for high aHIF expression (Fig. 4B; $P=0.011$). Among the seven metastatic cases, high VEGF and aHIF expression did not seem to correlate with (metastasis free) survival (data not shown). An opposite effect was observed for SDHC expression (Fig. 4G; $P=0.008$). For the other genes, metastasis-free survival was not significantly different between the high- versus low-expression groups, although low levels of expression were without exception associated with poor prognosis. Overall survival was not significantly different between the high- versus low-expression groups, although there was trend toward a higher mortality in patients with high VEGF expression ($P=0.067$, data not shown).

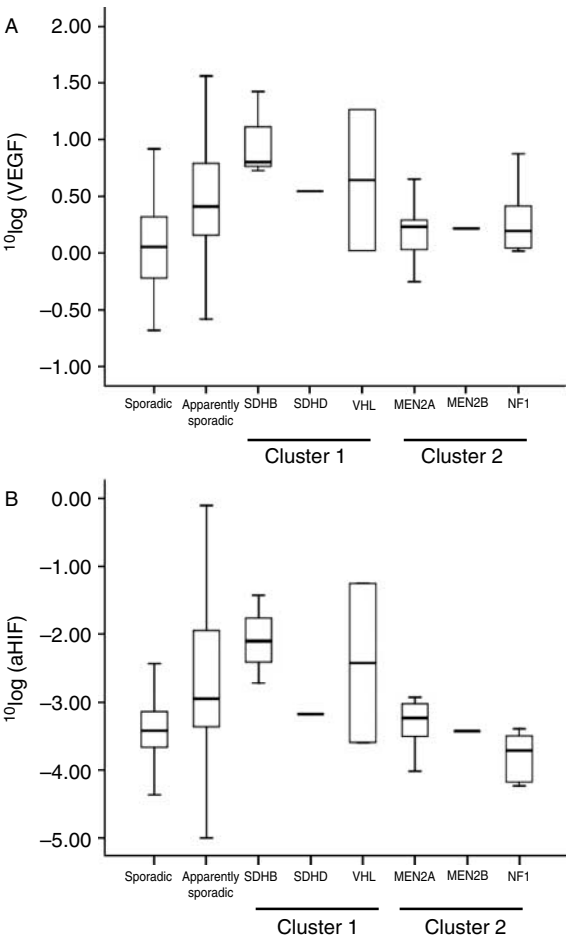

Figure 2 Box and whiskers plots (box = median with 25th and 75th percentile and whiskers are minimum and maximum values) of l og-normalized expression levels of VEGF (A) and aHIF (B) in sporadic, apparently sporadic, and PGLs defined by specific genotype.

Discussion

This is the first study in which the role of the natural antisense transcript aHIF is investigated in PGL. We found that aHIF, along with VEGF, is overexpressed in PGLs with a pseudohypoxic signature due to *SDH* and *VHL* mutations. Overexpression of both factors is also associated with a decreased metastasis-free survival, marking an increased malignant potential of apparently benign primary PGLs. Conversely, slit homolog 3, p57Kip2, cytochrome b561, and SDHC showed over-expression in non-metastatic tumors, whereas no

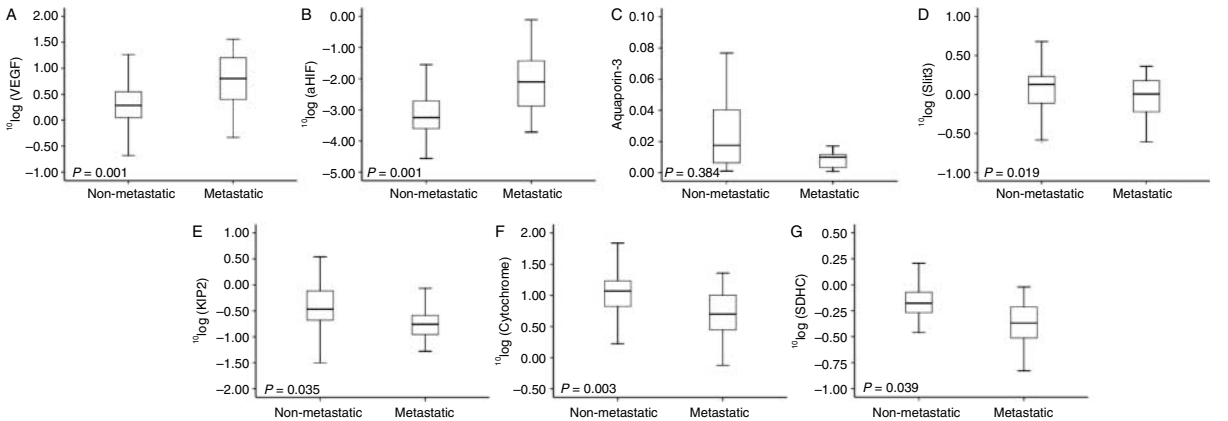

Figure 3 Box and whiskers plots (box = median with 25th and 75th percentile and whiskers are minimum and maximum values) of expression levels of VEGF (A), aHIF (B), aquaporin-3 (C), SLIT3 (D), KIP2 (E), cytochrome b561 (F), and SDHC (G) between non-metastatic and metastatic PGL tumors. Significant differences were found using student's *t*-test as marked by the *P*-values.

difference was observed for aquaporin 3. Higher expression levels of SDHC are correlated with an increased metastasis-free survival.

HIF1a regulates the transcription of a number of genes that are known to be involved in tumorigenesis and angiogenesis (Selak *et al*. 2005), including VEGF (Forsythe *et al*. 1996). In VHL disease, stabilization of HIF1a in PGL tumor cells is the result of decreased pVHL-dependent proteasomal degradation of HIF1a by the E3 ubiquitin ligase complex, after being hydroxylated by prolyl hydroxylase (Kaelin 2007). In case of underlying mutations of the mitochondrial enzyme *SDH*, succinate accumulation inhibits prolyl hydroxylase activity and consequently HIFa stabilization (Selak *et al*. 2005). As shown in *SDHA*-, *B*-, and *D*-associated PGL, abolishment of SDH activity results in overexpression of *VEGF*, a target gene of HIF1a (Gimenez-Roqueplo *et al*. 2001, 2002, Burnichon *et al*. 2010). Mitochondrial dysfunction due to *SDH* mutations and activation of the hypoxic pathway is probably interlinked with other mechanisms of tumorigenesis involving oxidative stress and apoptosis resistance (Gottlieb & Tomlinson 2005). Reactive oxygen species were shown to inhibit the action of prolyl hydroxylase, thereby increasing HIF-1α and VEGF availability (Pouyssegur & Mechta-Grigoriou 2006). Another important theory interlinking the pathogenesis of different hereditary forms of PGL pertains to the developmental culling of sympathetic neuronal precursor cells by affecting c-Jun-dependent

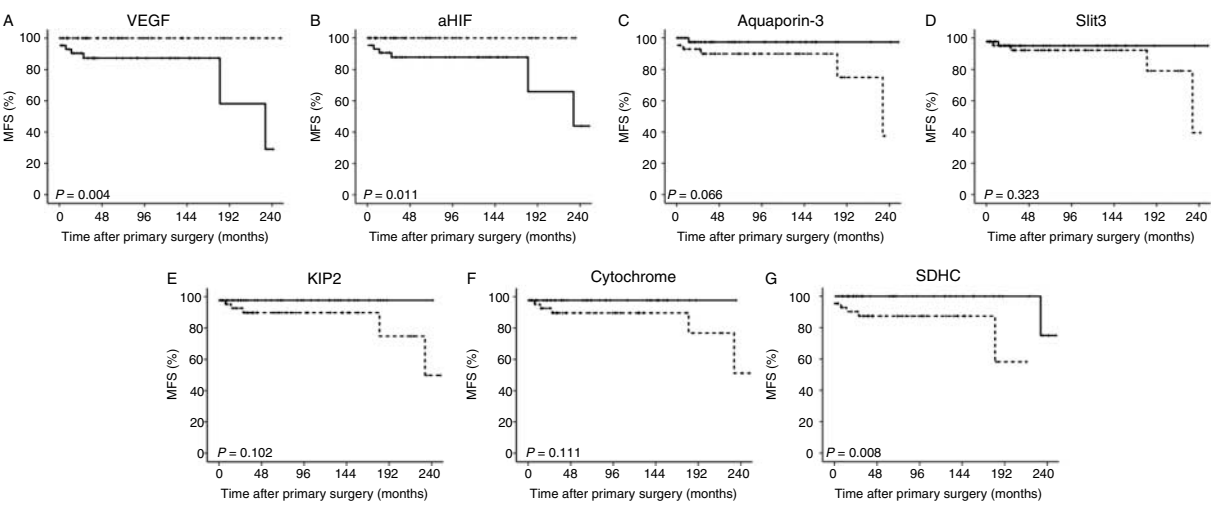

Figure 4 Kaplan–Meier plots of metastasis-free survival in PGL patients dichotomized by the mean tumor expression level of VEGF (A), aHIF (B), aquaporin-3 (C), SLIT3 (D), KIP2 (E), cytochrome b561 (F), or SDHC (G). Straight lines > median, dashed lines < median. Significant differences were found using the log-rank test.

neuronal apoptosis following withdrawal of neuronal growth factor (Lee *et al*. 2005).

The role of hypoxia and angiogenesis as a pathophysiological mechanism in a subset of PGLs was confirmed by gene expression profiling studies by Dahia *et al*. (2005). PGLs caused by germline *VHL* and *SDH* mutations exhibit a clear signature of (pseudo)hypoxia and angiogenesis, assigned as 'cluster 1' tumors. On the other hand, *RET*- and *NF1*-related PGLs, so-called cluster 2 tumors, are characterized by a deregulated RAS/RAF/MAP kinase signaling cascade that is associated with metabolism, translation initiation, and RNA/protein synthesis. Subsets of sporadic tumors were shown to fit either the cluster 1 or 2 profile. Our current findings of overexpression of VEGF in cluster 1 tumors and corroborate these previous findings. The expression of other genes that were suggested to be cluster related, i.e. p57Kip2, slit homolog 3, aquaporin 3, cytochrome b561, and SDHC, was not significantly different between the groups in our study.

Besides by factors mentioned earlier, the expression of HIF1a is also regulated by aHIF, a naturally occurring antisense transcript RNA that forms a double-stranded RNA molecule with the antisense transcript of HIF1a (Thrash-Bingham & Tartof 1999, Uchida *et al*. 2004). Naturally occurring antisense transcript expression can lead to the downregulation of the sense transcript by RNA interference (Yelin *et al*. 2003, Chen *et al*. 2004). Naturally occurring antisense transcript RNAs also exhibit important biological functions in DNA methylation (Tufarelli *et al*. 2003), genomic imprinting (Rougeulle & Heard 2002), X-chromosome inactivation (Ogawa & Lee 2002), genomic recombination (Bolland *et al*. 2004), and RNA splicing, polyadenylation, editing, stability, transport, and translation (Mello & Conte 2004, Makalowska *et al*. 2005). *In vitro* experiments in renal cell carcinoma cells have yielded insight in the oxygen-dependent regulation of HIF-1α (Thrash-Bingham & Tartof 1999, Uchida *et al*. 2004). Hypoxic conditions initially induce overexpression of HIF-1α. During sustained hypoxia, however, *HIF-1α* mRNA decreases in parallel with increased expression of aHIF, suggesting inhibition of HIF-1α by aHIF through a negative feedback loop. Our current finding of increased aHIF expression in parallel with VEGF in *SDH*- and *VHL*-related PGL fits a pattern of a chronic (pseudo)hypoxic state. This suggests that aHIF can serve as a surrogate marker of chronic HIF-1α activation, taking into account that measurement of the HIF-1α protein itself is cumbersome.

Besides its involvement in hypoxia-related tumorigenesis, HIF-1α stabilizes the tumor suppressor gene *p53* (An *et al*. 1998). Inhibition of HIF-1α by aHIF during sustained hypoxia results in the loss of p53 and subsequent tumor cell proliferation (Uchida *et al*. 2004). This aHIF-mediated escape from p53-mediated apoptosis theoretically translates into more aggressive tumor behavior and increased metastatic potential. Overexpression of aHIF has clearly been established in malignant non-papillary clear cell renal carcinoma (Thrash-Bingham & Tart 1999). In breast cancer, overexpression of aHIF was shown to be a marker of poor prognosis (Cayre *et al*. 2003). In this study, aHIF expression in primary tumors turned out to be a predictor of decreased metastasis-free survival. There are currently no reliable histological criteria for malignant PGL besides the presence of metastatic lesions in locations where chromaffin tissue is normally absent (Linnoila *et al*. 1990). The 5-year survival of patients with metastatic PGL is ∼50% (Eisenhofer *et al*. 2004, Timmers *et al*. 2008), and no cure is available. It is therefore critical to identify biomarkers of malignancy such as aHIF. Besides aHIF, VEGF was also negatively correlated with metastasis-free survival. High expression of cytochrome b561, p57Kip2, slit homolog 3, and SDHC on the other hand was associated with a benign course. These expression profiles of malignant PGL fit those of the cluster 1 tumors. Among cluster 1 tumors, specifically, *SDHB* mutations are strongly associated with a malignant course, whereas VHL- and SDHD-related PGLs are rarely malignant. Our findings, however, probably do not specifically reflect the *SDHB* genotype, as only two out of seven patients who developed metastases were known mutation carriers. However, considering the low frequency of malignancy in VHL/SDHD-related tumors, a cluster 1 signature of hypoxia/angiogenesis along with increased aHIF and VEGF expression as observed in our VHL and SDHD samples does not automatically translate into a high malignant potential. The discrepancy in metastatic potential between cluster 1 tumors of different genotypes suggests that other pathophysiological mechanisms besides hypoxia/angiogenesis are involved. Also, we emphasize that the expression of aHIF is highly variable, so it cannot be used as a marker of malignancy in individual patients. It is likely that individual risks of developing metastases can only be estimated by the use of a combination of clinical, biochemical, genetic, and molecular markers (Eisenhofer *et al*. 2004, Suh *et al*. 2009). In this context, protein expression of SNAIL, a zinc-finger transcription factor, has been identified as a useful marker of metastatic potential of PGL (Häyry *et al*. 2009).

Our current study has several limitations. The study includes a limited number of patients with metastatic

disease. The role of aHIF as a predictor of malignancy needs to be further evaluated in a larger cohort with a longer follow-up. Also, the role of aHIF as a marker of the upregulation of hypoxic and angiogenic pathways in the tumorigenesis of PGL needs further exploration across tumors of different genotypes, specifically *SDHB* versus other cluster 1 genotypes. Since the samples that we investigated spanned several decades, the integrity of mRNA could theoretically have decreased over time in the older samples. We did, however, not find such a correlation for aHIF expression. Formal genetic testing was omitted in several patients with apparently sporadic PGL, i.e. in those with a negative family history and lack of syndromal features. In these cases, SDHB immunostaining was used as 'surrogate' genotyping. The sensitivity and specificity of this approach awaits prospective evaluation. In conclusion, along with VEGF, the expression of aHIF, a naturally occurring antisense transcript of HIF-1α, is increased in cluster 1 PGL, fitting a profile of pseudohypoxia and angiogenesis. Moreover, overexpression of aHIF and VEGF marks a higher metastatic potential in PGL.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-10-0184.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV & Neckers LM 1998 Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature* **392** 405–408. (doi:10.1038/32925)

Baysal BE 2008 Clinical and molecular progress in hereditary paraganglioma. *Journal of Medical Genetics* **45** 689–694. (doi:10.1136/jmg.2008.058560)

Bolland DJ, Wood AL, Johnston CM, Bunting SF, Morgan G, Chakalova L, Fraser PJ & Corcoran AE 2004 Antisense intergenic transcription in V(D)J recombination. *Nature Immunology* **5** 630–637. (doi:10.1038/ni1068)

Burnichon N, Brière JJ, Libé R, Vescovo L, Rivière J, Tissier F, Jouanno E, Jeunemaitre X, Bénit P, Tzagoloff A *et al.* 2010 SDHA is a tumor suppressor gene causing paraganglioma. *Human Molecular Genetics* **19** 3011–3020. (doi:10.1093/hmg/ddq206)

Cayre A, Rossignol F, Clottes E & Penault-Llorca F 2003 aHIF but not HIF-1alpha transcript is a poor prognostic marker in human breast cancer. *Breast Cancer Research* **5** R223–R230. (doi:10.1186/bcr652)

Chen J, Sun M, Kent WJ, Huang X, Xie H, Wang W, Zhou G, Shi RZ & Rowley JD 2004 Over 20% of human transcripts might form sense–antisense pairs. *Nucleic Acids Research* **32** 4812–4820. (doi:10.1093/nar/gkh818)

Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, Barontini M, Kung AL, Sanso G, Powers JF, Tischler AS *et al.* 2005 A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. *PLoS Genetics* **1** 72–80. (doi:10.1371/journal.pgen.0010008)

DeLellis RA, Lloyd RV, Heitz PU & Eng C 2004 Pathology and Genetics. In *World Health Organization Classification of Tumours of Endocrine Organs*, ch 3, pp 151–166. Lyon, France: IARC Press.

Eisenhofer G, Bornstein SR, Brouwers FM, Cheung NK, Dahia PL, de Krijger RR, Giordano TJ, Greene LA, Goldstein DS, Lehnert H *et al.* 2004 Malignant pheochromocytoma: current status and initiatives for future progress. *Endocrine-Related Cancer* **11** 423–436. (doi:10.1677/erc.1.00829)

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD & Semenza GL 1996 Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Molecular and Cellular Biology* **16** 4604–4613.

Gimenez-Roqueplo AP, Favier J, Rustin P, Mourad JJ, Plouin PF, Corvol P, Rötig A & Jeunemaitre X 2001 The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activity of complex II in the mitochondrial respiratory chain and activates the hypoxia pathway. *American Journal of Human Genetics* **69** 1186–1197. (doi:10.1086/324413)

Gimenez-Roqueplo AP, Favier J, Rustin P, Rieubland C, Kerlan V, Plouin PF, Rötig A & Jeunemaitre X 2002 Functional consequences of a SDHB gene mutation in an apparently sporadic pheochromocytoma. *Journal of Clinical Endocrinology and Metabolism* **87** 4771–4774. (doi:10.1210/jc.2002-020525)

Gimenez-Roqueplo AP, Burnichon N, Amar L, Favier J, Jeunemaitre X & Plouin PF 2008 Recent advances in the genetics of phaeochromocytoma and functional paraganglioma. *Clinical and Experimental Pharmacology & Physiology* **35** 376–379. (doi:10.1111/j.1440-1681.2008.04881.x)

Gottlieb E & Tomlinson IP 2005 Mitochondrial tumour suppressors: a genetic and biochemical update. *Nature Reviews. Cancer* **5** 857–866. (doi:10.1038/nrc1737)

Häyry V, Salmenkivi K, Arola J, Heikkilä P, Haglund C & Sariola H 2009 High frequency of SNAIL-expressing cells confirms and predicts metastatic potential of phaeochromocytoma. *Endocrine-Related Cancer* **16** 1211–1218. (doi:10.1677/ERC-09-0049)

Hoogsteen IJ, Marres HA, Bussink J, van der Kogel AJ & Kaanders JH 2007 Tumor microenvironment in head and neck squamous cell carcinomas: predictive value and clinical relevance of hypoxic markers. A review. *Head & Neck* **29** 591–604. (doi:10.1002/hed.20543)

Kaelin WG 2007 Von Hippel–Lindau disease. *Annual Review of Pathology* **2** 145–173. (doi:10.1146/annurev.pathol.2.010506.092049)

Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J *et al.* 2005 Antisense transcription in the mammalian transcriptome. *Science* **309** 1564–1566. (doi:10.1126/science.1112009)

de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW & Span PN 2005 Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Laboratory Investigation* **85** 154–159.

Lee S, Nakamura E, Yang H, Wei W, Linggi MS, Sajan MP, Farese RV, Freeman RS, Carter BD, Kaelin WG Jr *et al.* 2005 Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. *Cancer Cell* **8** 155–167. (doi:10.1016/j.ccr.2005.06.015)

Lenders JW, Eisenhofer G, Mannelli M & Pacak K 2005 Phaeochromocytoma. *Lancet* **366** 665–675. (doi:10.1016/S0140-6736(05)67139-5)

Linnoila RI, Keiser HR, Steinberg SM & Lack EE 1990 Histopathology of benign versus malignant sympathoadrenal paragangliomas: clinicopathologic study of 120 cases including unusual histologic features. *Human Pathology* **21** 1168–1180. (doi:10.1016/0046-8177(90)90155-X)

Makalowska I, Lin CF & Makalowski W 2005 Overlapping genes in vertebrate genomes. *Computational Biology and Chemistry* **29** 1–12. (doi:10.1016/j.compbiolchem.2004.12.006)

Manders P, Sweep FC, Tjan-Heijnen VC, Geurts-Moespot A, van Tienoven DT, Foekens JA, Span PN, Bussink J & Beex LV 2003 Vascular endothelial growth factor independently predicts the efficacy of postoperative radiotherapy in node-negative breast cancer patients. *Clinical Cancer Research* **9** 6363–6370.

Mello CC & Conte D Jr 2004 Revealing the world of RNA interference. *Nature* **431** 338–342. (doi:10.1038/nature02872)

Ogawa Y & Lee JT 2002 Antisense regulation in X inactivation and autosomal imprinting. *Cytogenetic and Genome Research* **99** 59–65. (doi:10.1159/000071575)

Pouyssegur J & Mechta-Grigoriou F 2006 Redox regulation of the hypoxia-inducible factor. *Biological Chemistry* **387** 1337–1346. (doi:10.1515/BC.2006.167)

Rougeulle C & Heard E 2002 Antisense RNA in imprinting: spreading silence through air. *Trends in Genetics* **18** 434–437. (doi:10.1016/S0168-9525(02)02749-X)

Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB & Gottlieb E 2005 Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell* **7** 77–85. (doi:10.1016/j.ccr.2004.11.022)

Suh I, Shibru D, Eisenhofer G, Pacak K, Duh QY, Clark OH & Kebebew E 2009 Candidate genes associated with malignant pheochromocytomas by genome-wide expression profiling. *Annals of Surgery* **250** 983–990. (doi:10.1097/SLA.0b013e3181b248bb)

Thrash-Bingham CA & Tartof KD 1999 aHIF: a natural antisense transcript overexpressed in human renal cancer and during hypoxia. *Journal of the National Cancer Institute* **91** 143–151. (doi:10.1093/jnci/91.2.143)

Timmers HJ, Brouwers FM, Hermus AR, Sweep FC, Verhofstad AA, Verbeek AL, Pacak K & Lenders JW 2008 Metastases but not cardiovascular mortality reduces life expectancy following surgical resection of apparently benign pheochromocytoma. *Endocrine-Related Cancer* **15** 1127–1133. (doi:10.1677/ERC-08-0049)

Tufarelli C, Stanley JA, Garrick D, Sharpe JA, Ayyub H, Wood WG & Higgs DR 2003 Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. *Nature Genetics* **34** 157–165. (doi:10.1038/ng1157)

Uchida T, Rossignol F, Matthay MA, Mounier R, Couette S, Clottes E & Clerici C 2004 Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. *Journal of Biological Chemistry* **279** 14871–14878. (doi:10.1074/jbc.M400461200)

Van Nederveen FH, Gaal J, Favier J, Korpershoek E, Oldenburg RA, de Bruyn EM, Sleddens HF, Derkx P, Rivière J, Dannenberg H *et al.* 2009 An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. *Lancet Oncology* **10** 764–771 . (doi:10.1016/S1470-2045(09)70164-0)

Wenger RH, Rolfs A, Spielmann P, Zimmermann DR & Gassmann M 1998 Mouse hypoxia-inducible factor-1alpha is encoded by two different mRNA isoforms: expression from a tissue-specific and a housekeeping-type promoter. *Blood* **91** 3471–3480.

Yelin R, Dahary D, Sorek R, Levanon EY, Goldstein O, Shoshan A, Diber A, Biton S, Tamir Y, Khosravi R *et al.* 2003 Widespread occurrence of antisense transcription in the human genome. *Nature Biotechnology* **21** 379–386. (doi:10.1038/nbt808)

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