In vivo and in vitro oncogenic effects of HIF2A mutations in pheochromocytomas and paragangliomas

Rodrigo A Toledo1,*, Yuejuan Qin1,*, Subramanya Srikantan1, Nicole Paes Morales1, Qun Li1, Yilun Deng1, Sang-Woo Kim1,†, Maria Adelaide A Pereira3, Sergio P A Toledo3, Xiaoping Su4, Ricardo C T Aguiar1,2 and Patricia L M Dahia1,2

1Division of Hematology and Medical Oncology, Department of Medicine, Cancer Therapy and Research Center at the University of Texas Health Science Center, 7703 Floyd Curl Drive, MC 7880, San Antonio, Texas 78229-3900, USA
2Greehey Children Cancer Research Institute, San Antonio, Texas, USA
3University of São Paulo School of Medicine, São Paulo, SP, Brazil
4Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
*(R A Toledo and Y Qin contributed equally to this work)
†S-W Kim is now at Department of Biological Sciences, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea

Abstract

Pheochromocytomas and paragangliomas are highly vascular tumors of the autonomic nervous system. Germline mutations, including those in hypoxia-related genes, occur in one third of the cases, but somatic mutations are infrequent in these tumors. Using exome sequencing of six paired constitutive and tumor DNA from sporadic pheochromocytomas and paragangliomas, we identified a somatic mutation in the HIF2A (EPAS1) gene. Screening of an additional 239 pheochromocytomas/paragangliomas uncovered three other HIF2A variants in sporadic (4/167, 2.3%) but not in hereditary tumors or controls. Three of the mutations involved proline 531, one of the two residues that controls HIF2A stability by hydroxylation. The fourth mutation, on Ser71, was adjacent to the DNA binding domain. No mutations were detected in the homologous regions of the HIF1A gene in 132 tumors. Mutant HIF2A tumors had increased expression of HIF2A target genes, suggesting an activating effect of the mutations. Ectopically expressed HIF2A mutants in HEK293, renal cell carcinoma 786-0, or rat pheochromocytoma PC12 cell lines showed increased stability, resistance to VHL-mediated degradation, target induction, and reduced chromaffin cell differentiation. Furthermore, mice injected with cells expressing mutant HIF2A developed tumors, and those with Pro531Thr and Pro531Ser mutations had shorter latency than tumors from mice with wild-type HIF2A. Our results support a direct oncogenic role for HIF2A in human neoplasia and strengthen the link between hypoxic pathways and pheochromocytomas and paragangliomas.
Introduction

Pheochromocytomas and paragangliomas are sympathetic neural-derived neoplasms. Over one third of these tumors carry germline mutations in one of the ten distinct genes (Jafri & Maher 2012). Remarkably, somatic events in pheochromocytomas have been limited to mutations of some of these susceptibility genes (Burnichon et al. 2011).

A majority of hereditary pheochromocytomas and paragangliomas are related to the hypoxia pathway through mutations of the VHL gene or those encoding the succinate dehydrogenase (SDH) complex (Jafri & Maher 2012). Loss-of-function mutations in these genes lead to increased stability of main components of the hypoxic profile to pheochromocytomas and paragangliomas (Jafri & Maher 2012). Remarkably, somatic events in some of these susceptibility genes (Burnichon et al. 2011).

Materials and methods

Patients and samples

Tumor and matching normal tissue were obtained from patients with pheochromocytomas and paragangliomas according to institutionally approved protocols. Details on this cohort of 239 tumors are provided in Supplementary Materials and methods, see section on supplementary data given at the end of this article. Normal tissue was obtained from blood or adjacent normal tissue.

DNA and RNA isolation

Blood and tumor DNA and RNA were isolated using Qiagen kits or Trizol respectively following the manufacturers’ protocols.

Exome sequencing

We performed whole exome sequencing of matched germline and tumor DNA from six pheochromocytomas/paragangliomas without an identifiable PSG (PSGS) mutation. Libraries were prepared and exomes enriched using Agilent Sure Select 44Mbp at the Beijing Genomics Institute, as described (Gui et al. 2011). Paired-end reads of enriched exomes were generated in illumina HiSeq2000 (average depth of 50×) and aligned using the Mosaic algorithm (Hillier et al. 2008). Variants detected at the somatic level were selected using a modified version of the GigaBayes/FreeBayes pipeline for somatic variants (Marth et al. 1999). Synonymous sequence variants and those present in the dbsNP135 database were excluded.

Sanger sequencing

Primers flanking the 16 exons of the HIF2A gene and exons 9, 12, and 16 of HIF1A (sites of three regulatory hydroxylation domains) were used for PCRs and
Sanger sequencing as described in Supplementary Materials and methods.

Microarray-based expression profiling

We used normalized transcription data from 126 pheochromocytomas that were generated using Affymetrix U133 microarrays (GEO accession numbers GSE28416 and GSE199877) (Dahia et al. 2005, Qin et al. 2010). Two HIF2A mutant tumors (37 and 208) were available in this cohort and were used for comparisons with tumors from other well-established hereditary pheochromocytomas. Details are described in Supplementary Materials and methods.

Quantitative Real-time PCR (qRT-PCR)

This was performed in cDNA isolated from tumors and cell lines (Qin et al. 2010). Additional details are provided in Supplementary Materials and methods.

Clones and constructs

A HA-HIF2alpha-pBabe-puro construct containing the wild-type HIF2A coding sequence, originated from Dr Willam Kaelin’s laboratory (Kondo et al. 2003), was obtained from Addgene, Cambridge, MA, USA. Site-direct mutagenesis was used to generate each of the HIF2A mutants as described, all maintaining the wild-type Pro405, to mimic the tumor-related mutations (Qin et al. 2010). An HA-tagged full-length VHL construct was cloned into the MSCV-GFP retroviral vector.

Cell culture, transductions, and transfections

HEK293, 786-0, and PC12 cell lines were cultured as described in Supplementary Materials and methods. For hypoxia experiments, cells were maintained in a hypoxia chamber at 1% O₂ (InVivo200, Ruskinn, Bridgend, UK) for 16 h. Retroviral transduction was performed as described (Qin et al. 2010). MSCV-HA-VHL constructs were sorted by flow cytometry based on GFP expression (Qin et al. 2010). Puromycin-resistant pools or clones were obtained.

Cycloheximide assay

HEK293 cells stably expressing wild-type or various HIF2A mutants were treated with cycloheximide 100 μg/ml for the indicated times.

Nerve growth factor differentiation and withdrawal

PC12 cells were exposed to nerve growth factor (NGF) 50 ng/ml (Harlan, Houston, TX, USA) for 5–7 days followed by its withdrawal for 24 h, as described previously (Lee et al. 2005).

Nude mouse xenografts

Nude mouse xenograft assays were performed as described (Rai et al. 2010). Cells stably expressing empty vector, wild-type HIF2α, and 531 mutants were injected subcutaneously in nude mice (six or seven mice per group). Animals were killed after 28 days and tumor weights were recorded. Two additional cohorts were performed and in these, animals that developed tumors (n=5–10 per group) were killed when tumor reached maximum size, following UTHSCSA-IACUC-approved protocols.

Statistical analysis

The statistical significance of in vitro assays was determined with a two-tailed Student’s t-test and one-way ANOVA or Kruskal–Wallis for the xenograft assay. In all instances, P<0.05 was considered significant. Data analyses were performed with the Prism software (GraphPad, La Jolla, CA, USA) and Excel (Microsoft).

Results

Sporadic pheochromocytomas and paragangliomas carry somatic mutations of the HIF2A but not HIF1A gene

To discover novel somatic mutations involved in pheochromocytomas and paragangliomas, we generated exome sequences of six paired constitutive and tumor DNA samples without a mutation in any of the known pheochromocytoma susceptibility genes. We identified 269 somatic variants and herein focus on a heterozygous HIF2A gene mutation affecting the hydroxyl-acceptor residue, proline 531 (Pro531Thr), identified in one of these patients (Table 1).

This mutation was confirmed by Sanger sequencing in the tumor and was absent from the patient’s germline DNA (Fig. 1A). We next extended the screening of the entire HIF2A coding sequence to 239 pheochromocytomas/paragangliomas, including 166 sporadic and 72 with a germline PSG mutation. We identified 32 variants (Supplementary Table 1, see section on supplementary data given at the end of this article), of which four, including the change originally detected in the exome,
fulfilled our criteria for pathogenic significance: involved a conserved residue, were absent in controls, and were predicted to change protein function in silico (Table 1). Similar to the exome finding, two other variants also targeted the 531 codon (Pro531Ser and Pro531Leu). The remaining one was detected immediately distal to the DNA binding domain of HIF2α (Ser71Tyr). All the four variants were found in sporadic, but not hereditary, pheochromocytomas and paragangliomas (four of 167 tumors, 2.3%) and were absent in an ethnically matched control group (n=214 alleles) or publicly available sequences from the SNP database (http://www.ncbi.nlm.nih.gov/snp/) and 1000 Genomes Project (http://www.1000genomes.org/). The mutations were exclusively detected in tumor DNA of two cases in which germline material was available (Fig. 1A). In two other samples, the somatic status of the mutations could be established due to unavailability of constitutive DNA. The homologous region of the HIF1A gene was also sequenced in 132 of these tumors, including the tumors with HIF2A mutations, but no pathogenic variants were identified (Supplementary Table 1).

Clinically, two of the mutations occurred in paragangliomas, one metastatic and the other recurrent, while the other two were in pheochromocytomas (Table 1). All four tumor DNA sequence traces had a heterozygous appearance, indicating retention of both alleles (Fig. 1A). In one case, sequence of tumor cDNA showed that both alleles were transcribed (Supplementary Figure 1).

**HIF2A mutations induce target genes in primary tumors and cell lines and increase HIF2α stability**

The clustering of the mutations (3/4) at the primary site of HIF2α hydroxylation suggested that these variants are functionally relevant (Fig. 1B). To explore the consequences of these HIF2A mutations, we measured expression of HIF target genes in primary tumor samples. Two of the tumors, carrying a mutation on Pro531Ser and Pro531Leu, had been previously included in our global transcriptional study of pheochromocytomas (Dahia et al. 2005). Reassuringly, both HIF2A mutant tumors aligned with the pseudohypoxic group, which was comprised of samples carrying germline VHL and SDHB or SDHD gene mutations (known as cluster 1), by supervised clustering analysis (Dahia et al. 2005). Normalized data from probes encoding HIF target genes were extracted from this dataset and plotted against expression data from other pheochromocytomas carrying either cluster 1 or cluster 2 (RET, NF1, or TMEM127) mutations (Fig. 1C). RNA was also obtained.
Figure 1
Somatic mutations of HIF2A in pheochromocytomas and paragangliomas. (A) Sequence traces of tumor DNA showing the four mutations identified (Ser71Tyr, Pro531Thr, Pro531Leu, and Pro531Ser) and either their respective normal sequences from germline DNA or a reference control (#). Mutations are indicated with an asterisk. Border coloring matches the mutated amino acid on HIF2A sequence shown in B. (B) Amino acid sequence alignment of HIF2A showing high conservation at the DNA binding domain (basic helix–loop–helix = bHLH), PAS1 (PER-ARNT-SIM) domain, and the N-terminal transactivation domain (NTAD) oxygen-dependent degradation domain (ODD) across various species. The residues mutated in pheochromocytomas and paragangliomas are shown in color, matching the respective DNA sequences displayed in (A). (C) Expression levels of VEGFA, CCND1, IGF2, and c-MYC transcripts obtained from microarray-based profiling of combined HIF2A mutant tumors (37 and 208) in comparison with pheochromocytomas mutated for NF1 and TMEM17 genes (C2 = cluster 2) and VHL, SDHB, and SDHD genes (C1 = cluster 1), as detailed in the Materials and methods section. Values of the C2 cluster samples have been set to 1 (statistically significant difference between HIF2A mutant and C2 tumors; **statistically significant difference between HIF2A mutant samples and both C1 and C2 tumors, at P < 0.05). (D) Expression levels of VEGFA, CCND1, IGF2, and c-MYC transcripts in one HIF2A-mutated primary tumor (tumor from sample 9) along with other pheochromocytomas carrying mutations of VHL, SDHB, or NF1 genes assayed by quantitative real-time PCR (qRT-PCR). Samples were run in triplicate and the experiment was repeated at least twice. Results are shown as the average and bars represent S.E.M. Values of NF1 samples were set to 1. (*statistically significant difference between HIF2A mutant samples and NF1 tumors, **statistically significant difference between HIF2A mutant between HIF2A mutant samples and NF1-, VHL-, or SDHB-related tumors, at P < 0.05).
from another HIF2A mutant tumor and compared to genetically defined pheochromocytomas or paragangliomas. Quantitative real-time PCR (qRT-PCR) of HIF-responsive genes encoding VEGFA, cyclin D1 (CCND1), IGF2, and c-MYC (MYC) was performed in these tumors (Fig. 1D). The HIF2A mutant tumors, both by microarray and by qRT-PCR analyses, displayed increased target gene expression (Fig. 1C and D). Moreover, cyclin D1 (also known as CCND1) and c-MYC, considered HIF2α-specific growth-promoting determinants in renal cell carcinomas (Raval et al. 2005, Gordan et al. 2007), and IGF2, associated with hypoxic neuroblastomas (Pietras et al. 2009), were also induced, suggesting that these effectors may contribute to the protumorigenic effects of HIF2A mutations in pheochromocytomas/paragangliomas.

The site of the mutations on the critical 531 prolyl residue suggested that these variants affected HIF2A stability. We examined these effects by generating HEK293 cell lines stably expressing each mutation, created by site-directed mutagenesis in a retroviral backbone. Constructs containing wild-type 531 sequence or a HIF2A mutation (Pro531Ala), previously shown to be both resistant to PHD action and defective in VHL binding (Kondo et al. 2002), were also used. All constructs, analogous to the primary tumors, were wild type for proline 405, the secondary HIF2α hydroxylation site. Stable expression of the HIF2A 531 mutations led to increased stability and prolonged half-life of HIF2A in vitro under normoxia to levels similar to those seen with the Pro531Ala mutant, as shown by a time course assay with cycloheximide (Fig. 2A and Supplementary Figure 2, see section on supplementary data given at the end of this article). These findings are consistent with structural studies of HIF1α indicating that replacement of the equivalent proline (Pro564) with other residues prevents hydroxylation at this site (Min et al. 2002). The expression levels of the juxta-DNA binding domain mutation, Ser71Tyr, in contrast, did not differ from the levels of the wild-type construct, suggesting that this mutation does not affect HIF2α stability (Fig. 2A).

**Mutant HIF2α is stabilized by hypoxia and is resistant to VHL-mediated degradation**

Despite already high normoxic levels of mutant HIF2A in HEK293 cells, these constructs could be further stabilized and activated by hypoxia, as measured by an increase in HA-HIF2A and GLUT1 protein abundance respectively (Fig. 2B). This response likely results from the effect of hypoxia on the intact proline 405, which leads to HA-HIF2A product escape from VHL-mediated proteasome degradation. Similar to the primary tumors, HEK293 cell lines expressing HIF2A mutations showed increased expression of VEGFA, OCT4/POU5F1, and GLUT1/SLC2A1 transcripts (Fig. 2C). These results suggest that the 531 mutations are associated with constitutive HIF2α activation.

We next stably expressed the HIF2A mutants in the VHL-null 786-0 renal carcinoma cells reconstituted with VHL and measured transcription of target genes. Again, mutants showed increased expression of VEGFA, OCT4/POU5F1, and GLUT1/SLC2A1 (Fig. 2D). We also compared GLUT1 protein levels in 786-0 cells expressing various HIF2A mutations without VHL or after its reconstitution. VHL expression normalized GLUT1 in cells expressing endogenous HIF2α and an empty vector control, as expected, but it was unable to overcome the effect of the new mutations or the positive control HIF2A Pro531Ala on this target (Fig. 2E). These results suggest that mutations of this site abrogate VHL binding to the HIF2α ODD (Kondo et al. 2002). Unlike the 531 mutants, HIF2A-Ser71Tyr mutant expression was undetectable in the presence of VHL similar to wild-type HIF2A (Supplementary Figure 3, see section on supplementary data given at the end of this article), suggesting that binding to VHL was not altered by this variant.

**HIF2A mutations change the differentiation pattern of chromaffin cells**

To explore the effect of HIF2A mutations in a more tissue-specific context, we used rat pheochromocytoma PC12 cells. Expression of the four HIF2A mutants also led to increased target transcription in comparison with empty vector-expressing cells (Fig. 3A). PC12 cells are capable of differentiating into neurons after exposure to NGF (Lee et al. 2005). Under these conditions, cells extended neurites and increased expression of chromaffin cell markers chromogranin A (CHGA) and neuropeptide Y (NPY), while transcription of HIF2A, its target, VEGFA, and a marker of cell dedifferentiation, Nanog, were decreased (Supplementary Figure 4A, see section on supplementary data given at the end of this article). In contrast, exposure of PC12 cells expressing various HIF2A mutations to NGF for 7 days led to reduced transcription of CHGA and NPY in comparison with cells expressing empty vector (Fig. 3B). Likewise, chromaffin markers NPY and dHAND were downregulated in a HIF2A mutant tumor (Fig. 3C), suggesting that the PC12 model recapitulates in vivo effects of these mutations. Furthermore, markers of cell dedifferentiation or stemness, NANOG, OCT4, JAGGED2
JAG2, KRT19, SOX9, and CHD2, were increased in HIF2A mutant pheochromocytomas/paragangliomas (Fig. 3C and D). This differential transcription pattern is reminiscent of the effects of hypoxia, and specifically, HIF2α, in promoting an immature, stem cell-like phenotype of the sympathetic-derived tumors neuroblastomas (Pietras et al. 2009). Thus, increased HIF2α activation caused by these mutations may lead to a shift in the NGF-induced transcriptional program. Parenthetically, we did not detect differences in apoptosis of these cells following...
NGF removal (Supplementary Figure 4B), in agreement with earlier findings of a HIF-independent, inhibitory effect of pheochromocytoma mutations in sympathetic precursor cell death (Lee et al. 2005). The downregulation of chromaffin markers in both mutant HIF2α cell lines and tumors accompanied by the increased expression of dedifferentiation markers are consistent with the notion that mutant HIF2α may contribute to a less differentiated phenotype of pheochromocytomas and paragangliomas.

HIF2α mutations promote tumor growth in mice

No changes were seen in HIF2α mutant cell proliferation in vitro, similar to reports of HIF2α overexpression in renal carcinomas (Supplementary Figure 5, see section on
Thus, we explored the transforming properties of the 531 mutations with a murine xenograft model. Six to seven nude mice per group were injected subcutaneously with HEK293 cells expressing empty vector, wild-type or mutant HIF2A constructs. While mice carrying vector-expressing cells did not form tumors after 28 days, most HIF2A-injected mice developed macroscopic tumors (Fig. 4A). In particular, animals injected with Pro531Thr-expressing cells produced significantly larger tumors than any other cell group (Fig. 4A and B, P < 0.05). These results were validated in two other cohorts, in which five to ten mice per group developed tumors. In these groups, the tumor latency in mice carrying HIF2A Pro531Thr and Pro531Ser-expressing cells was shorter than in those injected with HIF2A without these mutations (P < 0.05, Fig. 4C). In our model, the remaining mutants, Pro531Leu and the positive control Pro531Ala, did not yield significantly more aggressive tumors (Fig. 4A and B). The mild phenotype of the Pro531Ala-injected cells might suggest a cell type-dependent effect. It is also possible that the transcriptional transactivation properties of the amino acid sequence surrounding the Pro531 region may contribute to mutation-specific differences (Kondo et al. 2003, Yan et al. 2007). Additional in vitro and in vivo models may elucidate the reasons for this genotype-phenotype variability.
Discussion

Our data support an oncogenic role for HIF2A in cancer and confirm recent reports of somatic mutations of this gene in pheochromocytomas and paragangliomas, which became available as we were completing this study (Burnichon et al. 2012, Favier et al. 2012, Lorenzo et al. 2012, Zhuang et al. 2012, Comino-Mendez et al. 2013). Furthermore, our work expands on these observations by showing the tumorigenic effect of mutations of the 531 residue in vivo. Moreover, our finding of the inhibitory effect of these mutations on chromaffin marker expression suggest that activated HIF2α may contribute to a more aggressive phenotype of target cells, similar to poorly oxygenated areas of various cancers (Semenza 2012). Despite its effect on increased transcription of target genes, expression of the Ser71Tyr mutant did not enhance HIF2α protein stability or resistance to VHL degradation, so a potential ‘passenger’ effect of this mutation cannot be ruled out.

Despite notable recent advances, the genetic basis of most pheochromocytomas, including those with a pseudohypoxic profile, still remains unresolved. Future investigations may uncover other genetic variations that can strengthen the link between the hypoxia pathways and cancer.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0101.

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.

Funding
This work was supported by a Voelcker Fund Award and a CTSA-IIIMS pilot project as part of the NIH-BUL1TR000149 to P L M Dahia; a Voelcker Fund; and NCI-SR01CA138747 to R C T Aguiar. The Flow Cytometry and DNA Nucleic Acid Core are supported by UTHSCSA, NIH-NCI P30-CAS4174 to the Cancer Therapy and Research Center (CTRC at UTHSCSA).

Author contribution statement
R A Toledo designed experiments; processed and extracted nucleic acids from tissues and cell lines; validated mutations; and performed quantitative gene expression, statistical analyses, and preliminary exome analysis. Y Qin was responsible for generating most stable cell line pools and clones and for performing cellular and biochemical studies. S Srikanth generated cell lines and performed biochemical analyses. N P Morales assisted with sample processing and sequencing and generated mutagenized clones. Q Li and S-W Kim performed the mouse xenograft studies, Y Deng assisted with tumor and cell line expression analysis. M A A Pereira provided clinical information. S P A Toledo provided clinical samples and reviewed patients’ records. X Su performed bioinformatic and somatic analysis of exomes. R C T Aguiar provided reagents, designed and performed experiments, managed xenograft studies, and analyzed the data. P L M Dahia conceived the study, designed and performed experiments, analyzed the data, and wrote the manuscript, with input from R A Toledo, R C T Aguiar, and other authors.

Acknowledgements
This work is dedicated to the memory of Alberto António Dahia. The authors thank the International Familial Consortium members for their continuing collaboration and the patients and their families for their participation.

References


Received in final form 15 March 2013
Accepted 20 March 2013
Made available online as an Accepted Preprint 26 March 2013