Dear Editor,

Somatic hypoxia-inducible factor 2 alpha (HIF2A) mutations are responsible for a newly discovered syndrome of multiple paragangliomas (PGL) and duodenal somatostatinomas associated with polycythemia (Pacak–Zhuang syndrome) (Zhuang et al. 2012, Pacak et al. 2013, Toyoda et al. 2014). In this syndrome, found exclusively in females, somatostatinomas are located in the 2nd portion of the duodenum.

Gangliocytic PGL (GPGL), a mixed neuroectodermal–endodermal tumor, is a rare and unique type of PGL that is almost exclusively located in the 2nd portion of the duodenum, much like HIF2A-related somatostatinomas. Both GPGLs and HIF2A-related somatostatinomas produce and/or secrete somatostatin. Clinically, GPGL patients often present with gastrointestinal bleeding, iron deficiency anemia, abdominal pain, and weight loss. Despite their clinical presentation, signs and symptoms of catecholamine excess are not present, because these tumors do not produce catecholamines due to the absence of tyrosine hydroxylase, a rate-limiting enzyme in catecholamine biosynthesis. Until now, no familial cases of GPGL have been described.

Gain-of-function somatic HIF2A mutations in patients (with this syndrome or without it) are located close to the oxygen-dependent degradation domain (ODD) of HIF2α (Zhuang et al. 2012, Comino-Mendez et al. 2013, Pacak et al. 2013, Toledo et al. 2013). The mutated HIF2α protein prevents proper hydroxylation by prolyl hydroxylases, resulting in abnormally low binding to the von Hippel–Lindau (VHL) protein and enhances HIF2α stabilization and activation. Consequently, the HIF2α downstream genes, including the cancer signaling pathway genes, are upregulated, contributing to the pathogenesis of cancer. This notion was nicely presented in a study of Toledo et al., where HIF2α 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 (Toledo et al. 2013). Moreover, mice injected with mutant HIF2A tumor cells developed tumors with a shorter latency than tumors in mice injected with wildtype (WT) HIF2A tumor cells. As concluded by the authors, these results further support a direct oncogenic role for somatic gain-of-function HIF2A mutations in human neoplasia and strengthen the link between hypoxic pathways and PGL.

We tested whether duodenal GPGLs share a similar pathogenic mechanism with PGLs associated with somatic HIF2A mutations. Ten GPGL tissues (Fig. 1A–D) were screened for somatic HIF2A mutations; patient 1 was also tested for a potential germline HIF2A mutation, which included blood leukocytes and gallbladder tissue. Genomic DNA was extracted from paraffin-embedded tissue and white blood cells using a DNeasy Blood & Tissue Kit (Qiagen). Two female patients were found to have pathogenic somatic HIF2A mutations in their GPGLs. No HIF2A mutation was found in the blood or gallbladder tissue of patient 1. In patient 1 (a 54-year-old woman), a periamplullar GPGL (Fig. 1E) was found to have a CαT substitution at base 1556 in exon 12 of HIF2A. In patient 2 (a 46-year-old woman), a periamplullar GPGL (Fig. 1E) exhibited a CαT substitution at base 1630 of the same exon. The mutations resulted in amino acid substitutions in HIF2α protein to T519M and P544S, respectively. Alignment of multiple HIF2α peptide sequences indicated that both amino acids are located in the proximal region of the primary hydroxylation site of HIF2α protein and are evolutionary conserved across different species (Fig. 1F). Both mutations were closely located to the oxygen-sensing domain of HIF2α, which affects its protein ubiquitination in the presence of oxygen.

To understand the functional impact of either mutation, we obtained a pcDNA3-HA-HIF2α plasmid containing human HIF2A coding sequence (Addgene plasmid 18950) and introduced T519M and P544S mutations through Quikchange Lightning Site-directed...
Mutagenesis Kit (Agilent). The plasmids were introduced into HEK293 cells with Lipofectamine 2000 (Invitrogen) for detection of HIF2α ubiquitination and measurement of protein half-lives.

Through an immunoprecipitation assay, we found that the ubiquitination of HIF2α was reduced in either the T519M or P544S HIF2α variant compared with WT protein, with a reduction of 35.16% and 61.76%, respectively (Fig. 2A). We confirmed the changes in protein stability of the mutant HIF2α via a cycloheximide (CHX) chase assay. WT HIF2α protein exhibited a half-life of 7.60 min, suggesting rapid turnover of HIF2α protein under normoxic conditions. By contrast, T519M and P544S variants were more stable, with half-lives extended to 67.21 and 81.09 min, respectively, when compared with WT protein with a half-life of 7.6 min (Fig. 2B and C). Furthermore, the expression of canonical hypoxia-related genes in tumors was measured through quantitative real-time PCR (Fig. 2D). We identified the upregulation of genes downstream of HIF2α, including EDN1, EPO, GLUT1, GNA14, LDHA, and VEGFA measured through a real-time PCR assay on a ViiA 7 real-time PCR system (Applied Biosystems).

Until now, most GPGLs were considered sporadic, although some of them are found rarely in association with neurofibromatosis type 1. This is the first report that shows that somatic gain-of-function HIF2A mutations are present in 20% of GPGLs in the present series. The mutations appear to be located in the hot spot of the oxygen-sensing domain of HIF2α, resulting in increased HIF2α stabilization and impaired ubiquitination and degradation, as also described in studies by Dahia et al. (2005), Toledo et al. (2013), and Comino-Mendez et al. (2013). Increased half-life of HIF2α and enhanced activity trigger its downstream regulated genes and thus, the upregulation of the HIF signaling pathway, classifying these tumors as cluster 1. The evidence of HIF2A oncogenicity of PGLs was previously demonstrated by Dahia et al. (2005). The authors showed that HIF2A mutations conferred growth advantage (e.g., by resistance to VHL-mediated degradation, increased tumor cell stability, and reduced PC12 cell differentiation) in a mouse subcutaneously injected with various mutant HIF2A tumor cells.

The developmental origin of GPGL is currently unclear, but it is believed that this tumor derives from a defective sheet of intraembryonic endoderm progenitor cells (primordial gut; epithelial cells in GPGL), rests that have recruited progenitor cells of neuroectoderm, nerves/Schwann and ganglion cells (spindle cells and ganglion-like cells, respectively in GPGL), and smooth muscle (Perrone et al. 1985, Witzigmann et al.)

Figure 1
Histopathological features of GPGLs from patient 1 (A) and patient 2 (B). In both tumors, the neoplastic cells were arranged in solid nests, trabeculae, and fascicles with areas of epithelioid (arrow A), ganglion-like (arrow B), and spindle (arrow C) cells. Hematoxylin & eosin stain, magnification ×20. Positive immunohistochemical stains for chromogranin A (C) and somatostatin (D) in the tumor from patient 1, magnification ×20. Both GPGLs were negative for tyrosine hydroxylase (Immunostar) immunohistochemistry (data not shown). (E) Genomic DNA sequencing of HIF2A exon 12 nucleotides 1551–1561. Heterozygous T519M (patient 1) and P544S (patient 2) somatic mutations were identified in both GPGLs. No HIF2A mutations were found in the blood or gallbladder tissue of patient 1. (F) Alignment of amino acid sequence of HIF2α residues 1625–1635 in humans, mice, rats, ox, chickens, and frogs.
GPGLs are often positive for somatostatin, and both tumors are initially derived from endoderm, which is capable of recruiting neuroendocrine cells. Since somatic \textit{HIF2A} mutations are found in both tumor types, we hypothesize that these mutations with HIF signaling pathway upregulation may affect common precursor cells and their differentiation to somatostatin-secreting endocrine cells. Therefore, some PGLs, somatostatinomas, and GPGLs share similarities in their pathogenic mechanisms.

Amino acid substitutions in the ODD domain have been linked to abnormalities in oxygen-dependent hydroxylation of HIF2α, as well as abnormal accumulation of HIF2α protein under a normal oxygen level. Indeed, similar to previous findings related to HIF2α stabilization and degradation, both mutations resulted in significant increased stability (half-lives extended to 67.21 and 81.09 min for T519M or P544S, respectively) and decreased protein ubiquitination (reduction of 35.16 and 61.76% for T519M or P544S, respectively). The results confirm that HIF2α mutant protein found in both patients escaped from the degradation mechanism and likely induced a pseudohypoxic phenotype, as well as oncogenic gene transcription, through the canonical hypoxia pathway.

Moreover, mutated \textit{HIF2A} overexpression could contribute to a more immature phenotype of GPGL since \textit{HIF2A} knockdown in neuroblastomas, a tumor with similar developmental, biochemical, and localization characteristics as a PGL, promotes a sympathetic neuronal differentiation (Pietras \textit{et al.} 2009). By contrast, high normoxic and hypoxic levels of HIF2α protein in neuroblastoma cells are associated with immature recruitment of endothelial cells as well as a neural crest-like phenotype, all which resemble GPGL as an immature tumor (Pietras \textit{et al.} 2009).

Whether \textit{HIF2A} mutations may be associated with the presence of other (neuro)endocrine tumors or health-related abnormalities, especially in tumors that are found in the 2nd portion of the duodenum apart from those associated with MEN1, NF1, and \textit{HIF2A}-related somatostatinomas, is currently unclear. If a \textit{HIF2A} mutation is found, patients may be considered to have personalized clinical and therapeutic management with a HIF2α-targeted drug as for other hereditary neuroendocrine tumors undergoing various genetic screening, including next-generation sequencing (Neumann & Eng 2009).
Gangliocytic paraganglioma

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Development and the National Institute of Neurological Disorders and Stroke, NIH.

Acknowledgements
The authors would like to acknowledge the technical assistance of Katherine Wolf in the production and editing of this manuscript.

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Received in final form 3 April 2016
Accepted 18 April 2016
Accepted Preprint published online 29 April 2016