Immunohistochemical Evidence of Dysregulation of the Mammalian Target of Rapamycin Pathway in Primary and Metastatic Pheochromocytomas

Alcides Chaux, Fadi Brimo, Nilda Gonzalez-Roibon, Smit Shah, Luciana Schultz, Jean-Marc Rizk, Pedram Argani, Jessica Hicks, and George J. Netto

OBJECTIVE To characterize the status of the mammalian target of rapamycin pathway using formalin-fixed, paraffin-embedded specimens from patients with primary and metastatic pheochromocytoma.

METHODS Tissue microarrays were built from 19 normal adrenal medullas, 39 primary pheochromocytomas, and 8 unrelated metastatic pheochromocytomas. In 2 of the 8 cases of metastatic pheochromocytoma tissues, samples from the primary tumor were available. The expression levels of phosphatase and tensin homolog, phosphorylated Akt, phosphorylated S6, p27, and c-myc were evaluated by immunohistochemistry.

RESULTS The levels of phosphatase and tensin homolog and p27 were greater in the nontumor tissue than in the primary and metastatic pheochromocytomas. Increasing levels of phosphorylated Akt were noted in the nontumor adrenal medulla, primary pheochromocytomas, and metastatic pheochromocytomas. Finally, the levels of phosphorylated S6 were greater in the metastatic pheochromocytomas than in the nontumor adrenal medulla and primary pheochromocytomas.

CONCLUSION We found evidence of dysregulation of the mammalian target of rapamycin pathway in primary and metastatic pheochromocytomas, with increased phosphorylated S6 and phosphorylated Akt, and decreased phosphatase and tensin homolog and p27 expression levels. Because the currently available treatment modalities are less than optimal, our findings lend additional support to continuing to explore the utility of mammalian target of rapamycin pathway-targeted therapy for pheochromocytomas. UROLOGY 80: 736.e7–736.e12, 2012. © 2012 Elsevier Inc.

Adrenal pheochromocytoma is a catecholamine-secreting tumor arising from the chromaffin cells of the adrenal medulla. Although most pheochromocytomas have a benign clinical course, about 10% of cases are malignant. Only the presence of metastatic disease reliably establishes the malignancy of these tumors. Therefore, close clinical and biochemical follow-up is recommended in all cases, even in tumors with bland histologic findings.[1,2] For malignant pheochromocytomas, the reported 5-year survival rate from the point of diagnosis is <50%.[3-5] The main therapeutic option is in the form of surgical cytoreduction, which is not curative and rarely feasible in the event of disseminated metastatic disease.[3,6,7] In the past, palliative cytoreductive therapies have been explored, including iodine-131 metaiodobenzylguanidine, streptozotocin, cyclophosphamide, vincristine, and decarbazine, mitotane, and tyrosine kinase inhibitors, all with limited success.[7-11]

Dysregulation of the mammalian target of rapamycin (mTOR) pathway has been reported in several types of malignancies, including lung carcinomas, clear cell renal cell carcinomas, urothelial carcinomas, and neuroendocrine tumors.[7,12-15] Additionally, in vitro studies have suggested that dysregulation of the mTOR pathway is implicated in the pathogenesis of pheochromocytoma.[16,17] First-generation mTOR inhibitors, such as everolimus and temsirolimus, are already approved for the treatment of renal cell carcinoma, and second-gen-

Alcides Chaux and Fadi Brimo contributed equally to this work.

Financial Disclosure: *The authors declare that they have no relevant financial interests.*

Financial Support: *Supported in part by the Johns Hopkins Medicine—Patana Fund for Research. Dr. Alcides Chaux was partially supported by an award granted by the Consejo Nacional de Ciencia y Tecnologia, CONACYT (National Council of Science and Technology), dependent of the Presidency of the Republic of Paraguay, as an Active Researcher of Level 1 of the Programa Nacional de Incentivo a los Investigadores, PRONII (National Incentive Program for Researchers).*

Presented in part at the 2011 United States and Canadian Academy of Pathology Meeting, San Antonio, Texas.

From the Departments of Pathology, Urology, and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD; Departments of Urology and Pathology, McGill University Health Centre, Montreal, Quebec, Canada; and Universidad del Norte School of Medicine, Asuncion, Paraguay

Reprint requests: George J. Netto, M.D., Department of Pathology, Johns Hopkins University, 401 North Broadway, Weinberg 2242, Baltimore, MD 21231. E-mail: gnetto1@jhmi.edu

Submitted: February 28, 2012, accepted (with revisions): April 17, 2012

http://dx.doi.org/10.1016/j.urology.2012.04.032

eration mTOR inhibitors have also entered the clinical trials arena.[18] This approval has recently been extended to pancreatic neuroendocrine tumors.[15] However, experience with mTOR inhibitors in pheochromocytomas is limited.[19] In the present study, we evaluated the immunohistochemical expression of members of the mTOR pathway in primary and metastatic pheochromocytomas. We also evaluated the expression levels of these biomarkers in nontumor tissue of the adrenal medulla.

MATERIAL AND METHODS

The present study included tissue samples from 47 patients with pheochromocytoma who were diagnosed and treated by surgery at the Johns Hopkins Medical Institutions (Baltimore, MD) from 1996 to 2009. Of the 47 patients, 39 had primary pheochromocytomas and 8 unrelated patients had metastatic pheochromocytomas. Tissue from the primary tumor was available in 2 of these 8 metastatic pheochromocytomas. All tissue samples were fixed in buffered formalin and embedded in paraffin. The histologic sections were retrieved and reviewed for confirmation of the original diagnosis. A first tissue microarray (TMA) was built using triplicate tissue samples from the unmatched primary and metastatic pheochromocytomas, as previously described.[20] A second TMA was built using adrenal glands removed during nephrectomy for renal cancer. This second TMA included 19 triplicate samples of microscopically normal adrenal medulla. Finally, the 2 primary tumors that were matched to their corresponding metastatic pheochromocytomas were evaluated using whole sections.

The immunohistochemical expression of the following mTOR pathway markers was evaluated: phosphatase and tensin homolog (PTEN) (clone D4.3; dilution 1:50; Cell Signaling Technology, Danvers, MA), phosphorylated (phos)-Akt (clone 736E11; dilution 1:50; Cell Signaling Technology), phos-S6 (polyclonal antibody; dilution 1:200; Cell Signaling Technology), p27 (clone 57; dilution 1:4000; BD Transduction Laboratories, San Jose, CA), and c-myc (clone Y69; dilution 1:300; Epitomics, Burlingame, CA). For each marker, the percentage of positive cells (0%-100%) and the intensity of staining (0, 1+, 2+, and 3+) per each extent were recorded. At each TMA, the spot intensity and extent were multiplied, and their values summed to form an H score (range 0-300). For the whole slide sections, PTEN was evaluated in 3 selected spots (20×) showing the lowest immunohistochemical expression ("cold spots"). The remaining biomarkers were evaluated in 3 selected spots (20×) showing the greatest immunohistochemical expression ("hot spots"). For each case, the average H score of the spots was used for analysis.

Statistical Analysis

The H scores were compared using the paired Student *t* test for matched cases and 1-way analysis of variance test with the Šidák test for post hoc pair wise comparisons for unmatched cases. The significance level was set at $P = .05$ for the Student *t* test and analysis of variance test and, after Bonferroni's correction, at $P = .01$ for post hoc comparisons. All *P* values provided are 2-tailed. Data were analyzed using Stata, release 11 (StataCorp, College Station, TX).

RESULTS

Primary Pheochromocytomas

The mean patient age was 44 years (range 10-71). Of the 39 patients with primary pheochromocytomas, 21 (54%) were men and 18 (46%) were women (male/female ratio 1.16:1). The tumor size ranged from 0.9 to 12.5 cm (mean 5.3). Of the 39 cases, 31 were sporadic and 8 were associated with genetic syndromes (5 with multiple endocrine neoplasia type 2A, 2 with neurofibromatosis, and 1 with Von Hippel-Lindau disease). The patients with syndromic cases presented at an earlier age than those with sporadic cases (age 38 vs 46 years), but the difference was not significant ($P = .48$). The syndromic cases also presented with smaller tumors than the sporadic cases (3.2 vs 5.9 cm, $P = .037$).

Metastatic Pheochromocytomas

For the 8 patients with metastatic pheochromocytomas, the mean patient age at presentation was 52.5 years (range 36-71). A marked male predominance was observed, with 7 men and 1 woman. The site of the metastatic disease was retroperitoneal lymph nodes in 6 patients and bone in 2 patients. At 1.5 and 2 years postoperatively, 4 patients were alive with disease and 2 had died of their disease, respectively.

Analysis of Immunohistochemical Expression

The patterns of immunohistochemical expression for the mTOR pathway-related biomarkers are depicted in Figure 1. The expression levels in nontumor adrenal medulla, primary pheochromocytomas, and metastatic pheochromocytomas are listed in Table 1 and Figure 2A. The PTEN and p27 levels were greater in the nontumor tissue than in the primary and metastatic pheochromocytomas. Increasing levels of phos-Akt were noted in the nontumor adrenal medulla, primary pheochromocytomas, and metastatic pheochromocytomas. Finally, the phos-S6 levels were greater in the metastatic pheochromocytomas than in the nontumor adrenal medulla and primary pheochromocytomas and were similar in the latter 2.

The biomarker levels between the matched primary and metastatic cases are listed in Table 2 and Figure 2B. The PTEN levels were markedly greater in the primary pheochromocytomas than in the metastatic pheochromocytomas. The opposite trend was noted for phos-Akt, with markedly greater levels in metastatic pheochromocytomas compared to primary pheochromocytomas. In metastatic tumors, the phos-S6 levels were slightly greater and p27 levels were slightly greater than the primary tumors. Expression of c-myc was not observed in these cases.

COMMENT

In the present study, we analyzed the expression of mTOR pathway-related members in a large series of pheochromocytomas. Our findings of decreased PTEN

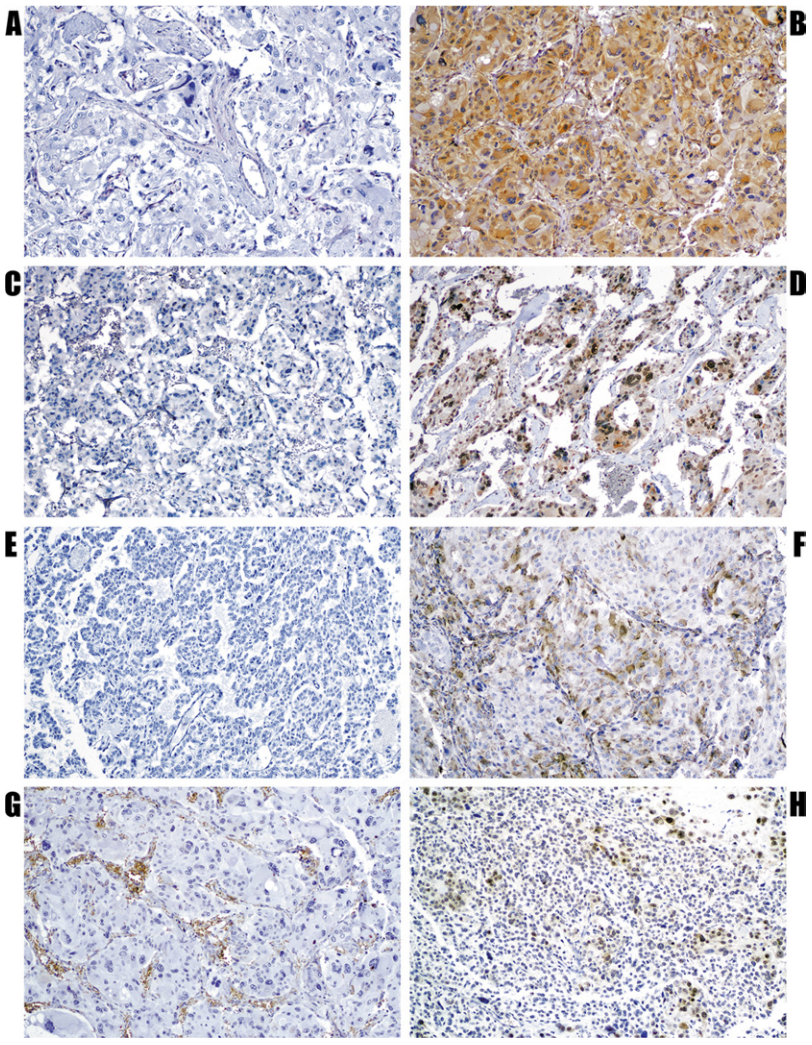

Figure 1. Patterns of immunohistochemical expression of mTOR-related members in pheochromocytomas. **(A)** Immunohistochemical loss of PTEN expression. **(B)** Positive cytoplasmic PTEN expression. **(C)** Negative phos-Akt expression. **(D)** Nuclear expression of phos-Akt. **(E)** Negativity for phos-S6 immunohistochemical expression. **(F)** Cytoplasmic positivity for phos-S6. **(G)** Loss of p27 immunohistochemical expression. **(H)** Nuclear staining for p27. Immunohistochemical expression of c-myc not depicted, because it was negative in all cases.

Table 1. Immunohistochemical expression of biomarkers in unmatched cases of adrenal medulla, primary pheochromocytoma, and metastatic pheochromocytomas*

Variable	Adrenal Medulla (n = 19)	Primary Pheochromocytoma (n = 39)	Metastatic Pheochromocytoma (n = 8)	*P* Value†
PTEN	230 ± 52	15 ± 13	16 ± 16	< .0001
Phos-Akt	68 ± 37	108 ± 92	139 ± 135	.12
Phos-S6	6 ± 7	6 ± 12	45 ± 66	.0003
p27	91 ± 37	2 ± 5	23 ± 57	< .0001
c-myc	0 ± 0	0 ± 0	0 ± 0	NA

PTEN, phosphatase and tensin homolog; NA, not applicable.
* Data presented as mean ± standard deviation of H scores.
† One-way analysis of variance test.

and p27 levels and increased phos-S6 levels compared to normal tissues suggest a dysregulation of the mTOR pathway in pheochromocytomas. In addition, the greater levels of phos-Akt and phos-S6 and lower levels of PTEN and p27 in metastatic pheochromocytomas compared to primary tumors might also indicate a role for mTOR pathway dysregulation in tumor progression. We have seen the same staining patterns of immunohistochemical expression in urothelial carcinomas of the urinary bladder and in primary and metastatic clear cell and papillary renal cell carcinomas.[12,21,22] Considering these previous studies, our present observations might provide a frame-

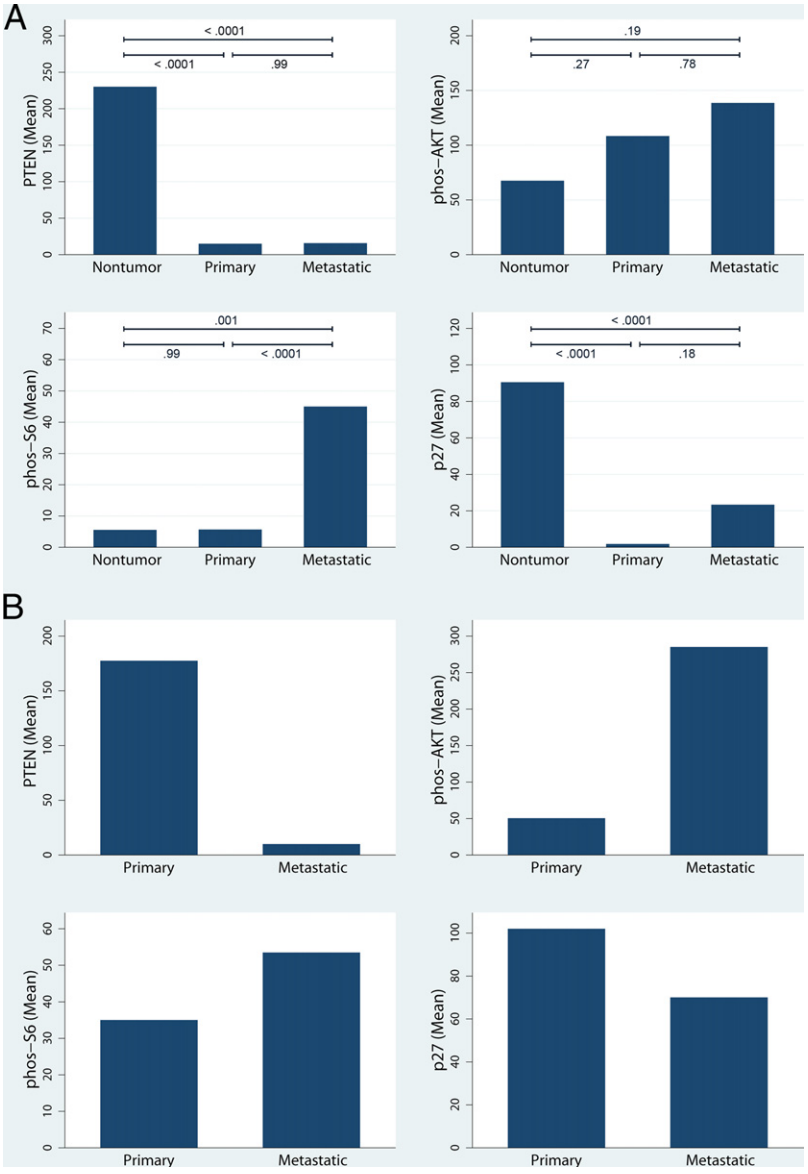

Figure 2. mTOR pathway in normal adrenal gland and pheochromocytomas. **(A)** Expression levels of mTOR pathway-related biomarkers in nontumor adrenal medulla and unrelated primary and metastatic pheochromocytomas. Bars represent mean values of biomarker using H score. Lines with capped ends show *P* values between categories. *P* values estimated using Šidák test. **(B)** Expression levels of mTOR pathway-related biomarkers in matched primary and metastatic pheochromocytomas (n = 2). Bars represent mean values of biomarker using H score.

Table 2. Immunohistochemical expression of biomarkers in 2 matched cases of primary and metastatic pheochromocytomas*

	Primary Pheochromocytoma			Metastatic Pheochromocytoma		
Variable	Case 1	Case 2	Mean ± SD	Case 1	Case 2	Mean ± SD
PTEN	220	135	178 ± 60	15	5	10 ± 7
Phos-Akt	73	28	50 ± 32	270	300	285 ± 21
Phos-S6	60	10	35 ± 35	27	80	54 ± 38
p27	200	4	102 ± 139	140	0	70 ± 99
c-myc	0	0	0	0	0	0

SD, standard deviation.
* Statistical tests were not run for matched primary and metastatic cases owing to the small sample size.

work for exploring the potential role of the currently available mTOR inhibitors in malignant pheochromocytomas, because the existing treatment modalities for this disease are suboptimal. The activation of the mTOR pathway in pheochromocytomas has been previously reported in both tumor cells and tumor samples.[17,23] Our results have confirmed these initial findings, further supporting a possible link between activation of the mTOR

pathway and oncogenesis in pheochromocytomas. In addition, a new susceptibility gene (*TMEM127*) has recently been identified that was associated to the mTOR pathway, mainly in the sporadic forms of pheochromocytoma.[24]

Evidence that mTOR inhibitors could be beneficial for patients with neuroendocrine tumors has been provided by several recent studies. Missiaglia et al[14] found decreased immunohistochemical expression of PTEN in 61% of 72 pancreatic neuroendocrine tumors. They also noted an association between PTEN, tumor progression, and disease-free survival. Jiao et al[25] found mutations in genes of the mTOR pathway in 14% of pancreatic neuroendocrine tumors, including mutations in *PTEN*, *TSC2*, and *PIK3CA*. Moreover, in a Phase III clinical trial, everolimus significantly prolonged progression-free survival in patients with advanced pancreatic neuroendocrine tumors.[15] However, to date, only 4 patients with malignant pheochromocytomas have been treated with everolimus, with disappointing results.[19] Nonetheless, we believe that our current data should lend further support for continuing to explore the utility of these drugs, alone or combined with other chemotherapeutic agents. Thus, the antagonists of the vascular endothelial growth factor, such as sunitinib, were reported to inhibit the mTOR pathway in pheochromocytomas tumor cells.[17]

Given the difficulty in determining their biologic behavior on pathologic grounds alone, pheochromocytomas pose a clinical challenge, even in their primary setting. Histologically, the most useful scoring system to predict the behavior of primary tumors is the Pheochromocytoma Adrenal Gland Scaled Score. The Pheochromocytoma Adrenal Gland Scaled Score includes factors, such as capsular invasion, vascular invasion, necrosis, and increased mitotic activity, with scores of ≥ 4 correlating with malignant behavior.[2] However, some of the Pheochromocytoma Adrenal Gland Scaled Score histologic parameters, such as diffuse cellular growth, hyperchromasia, severe atypia, and increased cellularity, are, to some extent, subjective. This unavoidable subjectivity, combined with the lack of long-term follow-up in most studies conducted on the subject, makes this histologic scoring system far from perfect.[1,26,27] Therefore, the need for additional prognosticators in pheochromocytomas continues to fuel the search for biomarkers. However, most of the cell cycle and apoptosis-related gene products tested, including p53, p21, p27, BCL2, cyclin-D1, and MDM2, proved to be of little significance.[1] The usefulness of prognostic models combining both the Pheochromocytoma Adrenal Gland Scaled Score system and the expression status of mTOR pathway members could be explored in future studies. Finally, the mechanisms underlying decreased PTEN expression should be further characterized. The potential mechanisms include loss of heterozygosity and/or PTEN mutation or gene promoter hypermethylation, among others.

One limitation of the present study was the small sample size of matched tissues from primary and metastatic pheochromocytomas. Even with this limitation, our results consistently suggested a role for mTOR dysregulation in the oncogenesis of pheochromocytomas.

Conclusions

We found immunohistochemical evidence of dysregulation of the mTOR pathway in pheochromocytomas, with decreased PTEN and p27 expression levels compared with the normal adrenal medulla. The expression levels of phos-Akt and phos-S6 were greater in metastatic pheochromocytomas compared with primary pheochromocytomas. Because the current available treatment modalities are less than optimal, our findings might lend additional support for continuing to explore the utility of mTOR pathway-targeted therapy in these tumors.

References

1. Strong VE, Kennedy T, Al-Ahmadie H, et al. Prognostic indicators of malignancy in adrenal pheochromocytomas: clinical, histopathologic, and cell cycle/apoptosis gene expression analysis. *Surgery*. 2008;143:759-768.
2. Thompson LD. Pheochromocytoma of the adrenal gland scaled score (PASS) to separate benign from malignant neoplasms: a clinicopathologic and immunophenotypic study of 100 cases. *Am J Surg Pathol*. 2002;26:551-566.
3. Ahlman H. Malignant pheochromocytoma: state of the field with future projections. *Ann N Y Acad Sci*. 2006;1073:449-464.
4. Eisenhofer G, Bornstein SR, Brouwers FM, et al. Malignant pheochromocytoma: current status and initiatives for future progress. *Endocr Relat Cancer*. 2004;11:423-436.
5. Adler JT, Meyer-Rochow GY, Chen H, et al. Pheochromocytoma: current approaches and future directions. *Oncologist*. 2008;13:779-793.
6. Szalat A, Fraenkel M, Doviner V, et al. Malignant pheochromocytoma: predictive factors of malignancy and clinical course in 16 patients at a single tertiary medical center. *Endocrine*. 2011;39:160-166.
7. Adjallé R, Plouin PF, Pacak K, et al. Treatment of malignant pheochromocytoma. *Horm Metab Res*. 2009;41:687-696.
8. Gross DJ, Schlank E, Ipp E. Streptozotocin therapy for malignant pheochromocytoma. *Arch Intern Med*. 1985;145:367-368.
9. Scholz T, Eisenhofer G, Pacak K, et al. Clinical review: current treatment of malignant pheochromocytoma. *J Clin Endocrinol Metab*. 2007;92:1217-1225.
10. Bates SE, Shieh CY, Mickley LA, et al. Mitotane enhances cytotoxicity of chemotherapy in cell lines expressing a multidrug resistance gene (mdr-1/P-glycoprotein) which is also expressed by adrenocortical carcinomas. *J Clin Endocrinol Metab*. 1991;73:18-29.
11. Gross DJ, Munter G, Bitan M, et al. The role of imatinib mesylate (Glivec) for treatment of patients with malignant endocrine tumors positive for c-kit or PDGF-R. *Endocr Relat Cancer*. 2006;13:535-540.
12. Schultz L, Albadine R, Hicks J, et al. Expression status and prognostic significance of mammalian target of rapamycin pathway members in urothelial carcinoma of urinary bladder after cystectomy. *Cancer*. 2010;116:5517-5526.
13. Seufferlein T, Rozengurt E. Rapamycin inhibits constitutive p70s6k phosphorylation, cell proliferation, and colony formation in small cell lung cancer cells. *Cancer Res*. 1996;56:3895-3897.
14. Missiaglia E, Dalai I, Barbi S, et al. Pancreatic endocrine tumors: expression profiling evidences a role for AKT-mTOR pathway. *J Clin Oncol*. 2010;28:245-255.
15. Yao JC, Shah MH, Ito T, et al. Everolimus for advanced pancreatic neuroendocrine tumors. *N Engl J Med*. 2011;364:514-523.
16. Harthill JE, Pozuelo Rubio M, Milne FC, et al. Regulation of the 14-3-3-binding protein p39 by growth factors and nutrients in

rat PC12 pheochromocytoma cells. *Biochem J*. 2002;368: 565-572.
17. Saito Y, Tanaka Y, Aita Y, et al. Sunitinib induces apoptosis in pheochromocytoma tumor cells by inhibiting VEGFR2/AKT/mTOR/S6K1 pathways through modulation of Bcl-2 and BAD. *Am J Physiol Endocrinol Metab*. 2012;302:E615-E625.
18. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
19. Druce MR, Kaltsas GA, Fraenkel M, et al. Novel and evolving therapies in the treatment of malignant phaeochromocytoma: experience with the mTOR inhibitor everolimus (RAD001). *Horm Metab Res*. 2009;41:697-702.
20. Fedor HL, De Marzo AM. Practical methods for tissue microarray construction. *Methods Mol Med*. 2005;103:89-101.
21. Schultz L, Chaux A, Albadine R, et al. Immunoexpression status and prognostic value of mTOR and hypoxia-induced pathway members in primary and metastatic clear cell renal cell carcinomas. *Am J Surg Pathol*. 2011;35:1549-1556.
22. Chaux A, Schultz L, Albadine R, et al. Immunoexpression status and prognostic value of mTOR and hypoxia-induced pathway members in papillary cell renal cell carcinomas. *Hum Pathol*. (In press).
23. Favier J, Igaz P, Burnichon N, et al. Rationale for anti-angiogenic therapy in pheochromocytoma and paraganglioma. *Endocr Pathol*. 2012;23:34-42.
24. Jiang S, Dahia PL. Minireview: the busy road to pheochromocytomas and paragangliomas has a new member, TMEM127. *Endocrinology*. 2011;152:2133-2140.
25. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science*. 2011;331:1199-1203.
26. Clarke MR, Weyant RJ, Watson CG, et al. Prognostic markers in pheochromocytoma. *Hum Pathol*. 1998;29:522-526.
27. Gao B, Meng F, Bian W, et al. Development and validation of pheochromocytoma of the adrenal gland scaled score for predicting malignant pheochromocytomas. *Urology*. 2006;68:282-286.