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Phospho-mTOR is not upregulated in metastatic *SDHB* paragangliomas

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Abstract

Background—Pheochromocytomas (PCCs)/paragangliomas (PGLs) are neuroendocrine tumours that may cause arrhythmia and death if untreated. Treatment for patients with metastatic tumours is lacking. As new PCC/PGL susceptibility genes are discovered that are associated with the mTOR pathway, treatment targets focusing on this pathway are being intensively explored.

Design—Twenty-one human PCC/PGLs were analysed from two tertiary care centres. Immunohistochemistry (IHC) analysis was performed for phospho-mTOR (pmTOR), phospho-S6K (pS6K), phosphoinositide 3-kinase (PI3K), phospho-4EBP1 (p4EBP1), HIF1 α and MIB-1 in 6 metastatic *SDHB* PCC/PGLs, 15 nonmetastatic PCC/ PGLs, (including 1 *TMEM127* PCC and 1

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Contributions

HKG designed the experiment, analysed data and wrote the manuscript; AG designed the experiment and provided necessary reagents for experiments; AC performed the experiment and analysed data; PK performed the experiment and analysed data; AC and X-JX performed the statistical analysis; VM analysed data and wrote the manuscript; JWS designed the experiment and provided necessary reagents for experiments; RFS designed the experiment and provided necessary reagents for experiments; KP designed the experiment, provided necessary reagents for experiments and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interests.

nonmetastatic *SDHB* PGL) and 6 normal adrenal medullas. The product of the intensity of stain and percentage of cells stained was calculated as an H score.

Results—Using a two-sample *t*-test and paired *t*-test, pmTOR and pS6K had significantly higher H scores in nonmetastatic PCC/PGLs than in metastatic *SDHB* PCC/PGLs. HIF1 α had significantly higher H scores in metastatic *SDHB* PCC/PGLs compared with nonmetastatic PCC/PGLs and normal adrenal medulla. No difference in H scores was seen with p4EBP1, PI3K and MIB-1 when comparing metastatic *SDHB* PCC/PGLs and nonmetastatic PCC/PGLs. Significantly higher difference in pS6K was seen in normal adrenal medullas compared to nonmetastatic PCC/PGLs and metastatic *SDHB* PCC/PGLs.

Conclusion—The present results suggest that the use of mTOR inhibitors alone for metastatic *SDHB* PCC/PGLs may not achieve good therapeutic efficacy in patients.

Keywords

Paranglioma; pmTOR; *SDHB*

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neuroendocrine tumours that arise from the adrenal medulla and produce catecholamines [1]. Patients with excessive catecholamine secretion from these tumours may suffer from severe hypertension, myocardial infarction, arrhythmia and stroke, which are commonly associated with death [2,3]. In addition, patients with PCC/PGL can develop metastatic disease, for which there is currently no cure [4]. Furthermore, patients who are not surgical candidates or those with metastatic disease have very limited treatment options.

Currently, 30–35% of patients with PCC/PGLs have an underlying germline mutation in one of several well-described genes, including neurofibromin 1 (*NF1*), von Hippel-Lindau (*VHL*), transmembrane protein 127 (*TMEM127*), MYC-associated factor X (*MAX*), ret proto-oncogene (*RET*), hypoxia-inducible factor 2A (*HIF2A*) [5–7] and succinate dehydrogenase genes (*SDHA*, *SDHB*, *SDHC* and *SDHD*). These succinate dehydrogenase genes encode subunits of the mitochondrial enzyme succinate dehydrogenase, which is responsible for the conversion of succinate to fumarate in the Krebs cycle and oxidative phosphorylation [8,9]. *SDHB* mutations are found to be associated with aggressive and often metastatic behaviour [10]. Mutations in these mitochondrial genes cause pseudo-hypoxic conditions with an increase in hypoxia-inducible factor alpha (HIF α) [10]. As a result, levels of angiogenic growth factors, like vascular endothelial growth factor (VEGF), and glucose transporter 1 increase to allow sufficient blood and nutrient supply for tumour growth [10]. In addition, tumour cell mitogenicity may increase through the phosphatidylinositol 3-kinase (PI3K) pathway, which is also involved in the activation of HIF [11] and the mammalian target of rapamycin (mTOR) pathway [12].

The mTOR pathway is involved in protein synthesis and cellular proliferation [13]. Interestingly, the mTOR pathway components have signalling interactions with the succinate dehydrogenase complex (*SDHx*) as well as with the *TMEM127*, *VHL*, *MAX* and

NF1 gene products, reinforcing the rationale to use drugs targeting the mTOR pathway in PCC/PGLs [5]. However, when the mTOR 1 inhibitor everolimus (Afinitor) was used for patients with unresectable, metastatic PCC/PGLs, the results were disappointing [14].

Thus, in this study our aim was to explore protein expression of components of the mTOR pathway, such as pmTOR and its downstream targets, including pS6K and p4EBP1, in metastatic *SDHB*-related PCC/PGLs compared to nonmetastatic PCC/PGLs and normal adrenal medulla. Other signalling components affecting mTOR, including PI3K and HIF1 α , were also evaluated in order to determine the relevance of future therapeutic options using mTOR inhibitors in these tumours.

Materials and methods

Patients and tumours

PCC/PGLs included 15 nonmetastatic PCC/PGLs (1 with a *TMEM127* mutation, 1 with *SDHB* mutation), 6 metastatic *SDHB* PCC/PGLs and 6 normal adrenal medullas collected at the National Institutes of Health (NIH) and the University of Texas Southwestern Medical Center. This study was carried out in accordance with the institutional review board (IRB) protocol from both institutions.

Immunohistochemistry

Standard immunohistochemistry analysis was performed for the following mTOR and related pathway members: pS6K (Ser 235/236), p4EBP1 (Thr37/46), pmTOR, PI3K, HIF1 α and MIB-1. Immunostaining was performed using the Benchmark XT automated stainer (Ventana) for all antibodies. Briefly, formalin-fixed, paraffin-embedded tissue microarray sections were cut at 3–4 micron and air-dried overnight. The sections were deparaffinized, rehydrated and subjected to heat-induced epitope retrieval. Sections were then incubated with the appropriate primary antibody. For signal detection, the ultraView universal detection system (Ventana) was used. The slides were developed using 3-3'-diaminobenzidine chromogen and counter-stained with haematoxylin–eosin. The immunohistochemical stains were standardized and validated in a CLIA laboratory using appropriate positive and negative tissue controls. These tissue controls were carefully selected using the information provided in package inserts, tissue with known antibody expression status (e.g. pS6K expression by Western blot on metastatic lung carcinoma to brain) and antibody expression of various benign and neoplastic tissue available on the internet (<http://www.proteinatlas.org>). Once the protocol was standardized and validated, appropriate positive tissue and negative antibody controls were utilized for each run of immunostains and checked for validation of the assay [15,16].

Interpretation

Immunohistochemistry (IHC) stains were performed on sections of tumour and benign tissue for each marker. The staining pattern (nuclear vs. cytoplasmic), extent (percentage of positive cells: 10/high power field) and intensity (0 for negative, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive) were evaluated by a clinical pathologist (P.K.). p4EBP1 positivity and HIF1 α positivity were interpreted as nuclear and/or

cytoplasmic expression; all other antibodies were interpreted as exclusively cytoplasmic patterns of expression. An H score was assigned to each section as the product of intensity of staining and the extent of immunoexpression (percentage of cells staining). The final H scores for each were used during statistical analyses for all markers. As noted in Table 2A–C, there were some slides that could not have an H score assigned because enough tissue may not have been present to verify the type of tissue and therefore could not be further evaluated for the appropriate stains.

Statistics

A Student's *t*-test was used to compare nonmetastatic PCC/ PGLs with metastatic *SDHB* PCC/PGLs, as well as metastatic *SDHB* PCC/PGLs with normal adrenal medullas. A paired *t*-test was used to compare nonmetastatic PCC/PGLs with normal adrenal medullas. A *P*-value of <0.05 was considered statistically significant. Bonferroni correction was also used for multiple comparison adjustment. All analyses were performed using *SAS* Version 9.3 for Windows (Cary, NC, USA).

Results

Table 1 shows patient characteristics including age, tumour location, catecholamine production and treatments that the patients have received.

Tumour tissue staining was performed for pmTOR, PI3K, p4EBP1, pS6K, HIF1 α and MIB-1. Six metastatic *SDHB* PCC/ PGLs, fifteen nonmetastatic PCC/PGLs (13 sporadic, 1 with a *TMEM127* mutation, 1 with *SDHB* mutation) and 6 normal adrenal medullas were stained. pmTOR as well as pS6K were found to have higher H scores, whereas HIF1 α had lower H scores in nonmetastatic PCC/PGLs compared to metastatic *SDHB* PCC/PGLs. No differences in H scores were observed between nonmetastatic PCC/PGLs and metastatic *SDHB* PCC/ PGLs for MIB-1, PI3K and p4EBP1. When comparing metastatic *SDHB* PCC/ PGLs with normal adrenal medulla, HIF1 α had higher H scores in metastatic *SDHB* PCC/ PGLs, while pS6K had statistically higher H scores in normal adrenal medullas. Comparing nonmetastatic PCC/PGLs with normal adrenal medulla, pS6K had significantly higher H scores in normal adrenal medulla. pmTOR was lower in normal adrenal medulla compared with nonmetastatic PCC/PGLs; however, this did not achieve statistical significance using a paired two-tailed *t*-test due to increased variation. Table 2A–C show results comparing the different tissue types. Figures 1 and 2 show a pictorial summary of the H score results. Figure 3a,b shows representative IHC staining. Nonmetastatic PCC/PGLs had positive staining for pmTOR and pS6K and negative staining for HIF1 α . On the other hand, metastatic *SDHB* PCC/ PGLs stained positively for HIF1 α , but negatively for pmTOR and pS6K. All these significant associations stay statistically significant after Bonferroni corrections.

Discussion

Our results show that pmTOR expression in nonmetastatic PCC/PGLs is elevated compared to metastatic *SDHB* PCC/ PGLs and that metastatic *SDHB* PCC/PGLs have elevated expression of HIF1 α compared to nonmetastatic PCC/PGLs. The statistically significant

differences in pmTOR expression in nonmetastatic PCC/PGLs compared to metastatic *SDHB* PCC/ PGLs suggest that mTOR inhibition alone may not reach good therapeutic efficacy for patients with metastatic *SDHB* PCC/PGLs.

With the successful use of mTOR inhibitors in neuroendocrine tumours such as carcinoid [17] and pancreatic neuroendocrine tumours [18], much hope has been placed in their application towards treating other (neuro) endocrine tumours, including metastatic PCC/ PGLs. Further enthusiasm for studying the mTOR pathway in these tumours occurred when the *TMEM127* gene was linked to their pathogenesis [5]. *TMEM127* is a tumour suppressor gene whose product modulates mTOR activity [5]. As a result, research interest in studying the mTOR pathway in PCC/PGL patients is on the rise. In one study, which enrolled four patients with metastatic PCC/PGLs, a trial of everolimus (mTOR inhibitor) was given for 3 months in two patients, 5 months in the third patient and 6 months in the fourth patient. Two of these patients received chemotherapy in addition to everolimus. Unfortunately, none of the patients treated with everolimus had any survival benefit, as tumour progression occurred in all cases [14]. Perhaps the reason is that the *TMEM127* gene mutation is found in nonmetastatic tumours as opposed to metastatic ones; however, the results of this treatment in a small sample population would need confirmation in larger clinical trials.

We therefore hypothesized that mTOR pathway activity would be much more prevalent in nonmetastatic PCC/PGLs than in metastatic ones, often linked to *SDHB* mutations. To test this hypothesis, tissue staining for components of the mTOR pathway on *SDHB* mutated metastatic tumours was compared to nonmetastatic PCC/PGL tumours. The results showed that HIF1 α has intense staining in metastatic *SDHB* tumours, whereas the nonmetastatic PCC/PGL tumours have intense staining for pmTOR and pS6K. Both nonmetastatic PCC/ PGLs and metastatic *SDHB* tumours have similar staining for p4EBP1. Our data suggest that the mTOR pathway is prevalent in nonmetastatic tumours as in tumours with *TMEM127* gene mutations, but not in metastatic *SDHB* PCC/PGLs, which may explain why using everolimus in the treatment of metastatic tumours has been unsuccessful.

How can the mTOR pathway play a role in targeted therapy for PCC/PGLs? It has been found that the binding of growth factors to cell surface receptors activates PI3K, which increases cellular glucose uptake, glycolysis, lactate production and perhaps later the Warburg effect. Activation of PI3K suppresses macromolecule breakdown in cells, whereas the mTOR protein complex itself plays a key role in protein translation. All of these various components share the common goal of cellular survival and/or proliferation. When the mTOR pathway is activated as a result of growth factor stimulation, increased cellular transcription associated with cellular proliferation occurs due to pS6K and 4EBP1. However, inhibiting the mTOR pathway to affect tumorigenesis is not a simple task. The mTOR complex is composed of two components, mTORC1 and mTORC2. mTORC1 is sensitive to the antibiotic rapamycin and is associated with controlling protein synthesis. mTORC2, on the other hand, is not sensitive to rapamycin and is associated with regulating the cell cytoskeleton as well as affecting the activation of mTORC1 [19]. Therefore, treating metastatic tumours with mTORC1 inhibitors alone will not be able to control aggressive disease, such as those seen with metastatic *SDHB* PCC/PGLs. An effort is underway to further study the successful role of dual mTORC1 and mTORC2 inhibitors in mouse models

of PCC [20]. One potential area of bias of comparing nonmetastatic PCC/PGLs of different hereditary backgrounds exclusively to *SDHB* metastatic PCC/PGLs is that other hereditary causes of nonmetastatic PCC/PGLs may utilize pathways other than mTOR. Nevertheless, in our study, we conclude that mTOR inhibition alone may not be the favourable treatment target for metastatic *SDHB*-related PCC/PGLs.

We speculate that perhaps other targets in the mTOR pathway should be investigated in metastatic PCC/PGLs. Although HIF is known to regulate mTOR by activating a tumour suppressor complex (TSC) which results in mTOR inhibition [21,22], further studies are warranted to examine these new possibilities, including HIF inhibitors.

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Figure 1. mTOR pathway staining of nonmetastatic PCC/PGL tumours. The H score was calculated by multiplying the staining intensity by the cell percentage. Darker colours correlate to higher H scores.

Figure 2. mTOR pathway staining in metastatic *SDHB* tumours. The H score was calculated by multiplying the staining intensity by the cell percentage. Darker colours correlate to higher H scores.

Legend: Immunohistochemistry (IHC) stains for normal adrenal medulla (NAM), metastatic *SDHB* tumors, and non-metastatic pheochromocytoma (PCC) and paraganglioma (PGL). Magnification is 200x with scale of 100μm.

Legend: Immunohistochemistry (IHC) stains for normal adrenal medulla (NAM), metastatic *SDHB* tumors, and non-metastatic pheochromocytoma (PCC) and paraganglioma (PGL). Magnification is 200x with scale of 100μm. Examples of positive and negative controls are shown.

Figure 3. Immunohistochemistry (IHC) stains for normal adrenal medulla (NAM), *SDHB* metastatic tumours and nonmetastatic PCC/PGL. Nonmetastatic PCC/PGLs have intense staining for (a) pmTOR and (b) pS6K. *SDHB* metastatic tumours have intense IHC staining for (a) HIF1α but no staining for pmTOR and (b) pS6K. Examples of positive and negative controls are included.

Table 1

(A) Clinical characteristics of patients with nonmetastatic PCC/PGL. Fifteen patients with nonrecurrent PCC/PGL are listed, including one with a *TMEM127* gene mutation and one with *SDHB* gene mutation. (B) Clinical characteristics of patients with metastatic *SDHB* PCC/PGL

Gender	Genetics	Tumor location	Type	Biochemistry before operation	Later disease	Other treatments	Survival
(A)							
F	<i>TMEM127</i>	R adrenal	Primary	EPI, NE, MN	No known recurrence	None	8 years since surgery
F		Paraspinal	Primary	NE, NMN	No known recurrence	None	4 years since surgery
F		R adrenal	Primary	NE, MN, NMN	No known recurrence	None	4–5 years since surgery
M		R adrenal	Primary	EPI, MN, NMN	No known recurrence	None	3 years since surgery
F		L renal vein	Primary	None	No known recurrence	None	2–5 years since surgery
F		R carotid	Primary	None	No known recurrence	None	1–5 years since surgery
F		R carotid	Primary	DA	No known recurrence	None	1–5 years since surgery
F		R adrenal	Primary	NE, NMN, DA	No known recurrence	None	1 yr since surgery
F		R adrenal	Primary	MN, NMN	No known recurrence	None	11 months since surgery
M		L adrenal	Primary	MN	No known recurrence	None	11 months since surgery
M		R adrenal	Primary	MN, NMN	No known recurrence	None	5 years since surgery
F		L adrenal	Primary	MN, NMN	No known recurrence	None	5 years since surgery
F		L adrenal	Primary	MN, NMN	No known recurrence	None	4 years since surgery
F		R adrenal	Primary	EPI, MN, NMN	No known recurrence	None	4–5 years since surgery
F	<i>SDHB</i>	Iliac bifurcation	Multiple	NE, NMN, DA	No known recurrence	None	3 years since surgery
(B)							
M	<i>SDHB</i>	Retroperitoneal	Metastatic	None	Metastatic	CVD; radiation therapy; RFA	2–5 years since surgery

Gender	Genetics	Tumor location	Type	Biochemistry before operation	Later disease	Other treatments of liver lesions	Survival
M	<i>SDHB</i>	Retroperitoneal	Metastatic	NE, NMN, DA	Metastatic	CVD	Deceased (4 years after surgery)
M	<i>SDHB</i>	Nasopharyngeal	Metastatic	NE, NMN, DA	Metastatic	None	4 years since surgery
M	<i>SDHB</i>	Intraaortic, right renal hilar	Metastatic	NE, NMN	Metastatic	None	4-5 years since surgery
F	<i>SDHB</i>	Liver	Metastatic	NE, NMN	Metastatic	MIBG; CVD; radiation therapy	Deceased (3 years after surgery)
F	<i>SDHB</i>	Lung	Metastatic	NE	Metastatic	CVD; RFA to metastatic lesions	3 years since surgery

M, male; F, female; R, right; L, left; EPI, epinephrine; NE, norepinephrine; MN, metanephrine; NMN, normetanephrine; DA, dopamine; *TMEM127*, transmembrane 127; *SDHB*, succinate dehydrogenase B; RFA, radiofrequency ablation; CVD, cyclophosphamide, vincristine, dacarbazine; MIBG, metaiodobenzylguan.

Table 2

Comparison of nonmetastatic, metastatic PCC/PGL and normal adrenal medulla. (A) Two-tailed *t*-test was performed comparing nonmetastatic PCC/PGL and metastatic *SDHB* tumours*. (B) Two-tailed *t*-test was performed comparing normal adrenal medulla and metastatic *SDHB* tumours†. (C) Paired *t*-test for the difference in H score by nonmetastatic PCC/PGL vs. normal adrenal medulla‡.

	Non-metastatic N	Mean ± SE	Metastatic N	Mean ± SE	P-value
(A)					
HIF1α	15	173.7 ± 17.2	6	273.3 ± 15.8	0.0029
MIB-1	15	6.2 ± 1.0	6	16.8 ± 7.1	0.1953
PI3K	15	44.7 ± 11.8	6	82.9 ± 20.9	0.1105
p4EBP1					
All Scores	15	8.0 ± 4.2	6	7.1 ± 6.6	0.9090
Certain Scores	14	4.3 ± 2.2	5	0.5 ± 0.5	0.1173
pmtOR					
All Scores	15	22.4 ± 6.2	6	5.6 ± 4.1	0.1181
Certain Scores	15	22.4 ± 6.2	5	1.7 ± 1.5	0.0053
pS6K					
All Scores	15	17.8 ± 5.9	6	1.3 ± 1.3	0.0144
Certain Scores	15	17.8 ± 5.9	5	0.0 ± 0.0	0.0089
	Metastatic N	Mean ± SE	Normal N	Mean ± SE	P-value
(B)					
HIF1α	6	273.3 ± 15.8	8	160.0 ± 0.0	0.0008
MIB-1					

	Non-metastatic N	Mean $\pm$ SE	Metastatic N	Mean $\pm$ SE	Mean $\pm$ SE	P-value
All Scores	6	16.8 $\pm$ 7.1	6	16.8 $\pm$ 7.1	5.8 $\pm$ 2.9	0.1818
Certain Scores	6	16.8 $\pm$ 7.1	5	16.8 $\pm$ 7.1	3.0 $\pm$ 0.5	0.1094
PI3K	6	82.9 $\pm$ 20.9	8	82.9 $\pm$ 20.9	38.8 $\pm$ 6.9	0.0911
p4EBP1						
All Scores	6	7.1 $\pm$ 6.6	5	7.1 $\pm$ 6.6	0.0 $\pm$ 0.0	0.3319
Certain Scores	5	0.5 $\pm$ 0.5	5	0.5 $\pm$ 0.5	0.0 $\pm$ 0.0	0.3739
pmTOR						
All Scores	6	5.6 $\pm$ 4.1	6	5.6 $\pm$ 4.1	1.7 $\pm$ 1.1	0.3888
Certain Scores	5	1.7 $\pm$ 1.5	6	1.7 $\pm$ 1.5	1.7 $\pm$ 1.1	0.9853
pS6K						
All Scores	6	1.3 $\pm$ 1.3	8	1.3 $\pm$ 1.3	56.3 $\pm$ 5.3	< 0.0001
Certain Scores	5	0.0 $\pm$ 0.0	8	0.0 $\pm$ 0.0	56.3 $\pm$ 5.3	< 0.0001
	N	Mean $\pm$ SE Non-metastatic	Normal	Mean Difference (Non-metastatic – Normal)		P-value
(C)						
HIF1 α	8	161.3 $\pm$ 20.7	160.0 $\pm$ 0.0	1.3 $\pm$ 20.7		0.9536
MIB-1						
All Scores	6	5.3 $\pm$ 1.5	5.8 $\pm$ 2.9	-0.5 $\pm$ 3.5		0.8929
Certain Scores	5	5.8 $\pm$ 1.7	3.0 $\pm$ 0.5	2.8 $\pm$ 1.5		0.1428
PI3K	8	24.4 $\pm$ 7.5	38.8 $\pm$ 6.9	-14.4 $\pm$ 10.2		0.2014
p4EBP1	5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0		–

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	Non-metastatic N	Mean ± SE	Metastatic N	Mean ± SE	P-value
pmTOR	6	28.3 ± 11.4	1.7 ± 1.1	26.7 ± 11.0	0.599
pS6K	8	19.7 ± 8.2	56.3 ± 5.3	-36.6 ± 7.5	0.0017

Bold values are considered to be statistically significant, as *P*-value is < 0.05.

* HIF1 α has higher H Scores in metastatic *SDHB* PCC/PGL compared to non-metastatic PCC/PGL. On the other hand, pmTOR and pS6K have higher H Scores in non-metastatic PCC/PGL compared to metastatic *SDHB* PCC/PGL.

[†] HIF1 α has higher H Scores in metastatic *SDHB* PCC/PGL compared to normal adrenal medulla. On the other hand, pS6K has higher H Scores in normal adrenal medulla compared to metastatic *SDHB* PCC/PGL.

[‡] pS6K has higher H Scores in normal adrenal medulla compared to non-metastatic PCC/PGL.