mTORC1 Complex is Significantly Over-Activated in SDHX-Mutated Paragangliomas

mTORC1 complex is significantly over-activated in *SDHX*-mutated paragangliomas

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Abstract

Aim. We aimed at exploring the activation pattern of mTOR pathway in sporadic and hereditary pheochromocytomas (PCC) and paragangliomas (PGL).

Methods. A total of 178 PCC and 44 PGL, already characterized for the presence of germline mutations in *VHL*, *RET*, *NF-1*, *MAX*, *SDHA*, *SDHB*, *SDHC*, *SDHD* and somatic mutations in *VHL*, *RET*, *H-RAS* and *MAX*, were included into five TMAs and tested using immunohistochemistry for mTOR and Rictor and the phosphorylated forms of mTOR, p70S6K, AMPK, AKT, 4E-BP1, S6 and Raptor.

Results. The positive correlation among most of the molecules investigated proved the functional activation of the mTOR pathway in PCC/PGL. Total mTOR, p-S6K and p-S6 and mTORC1-associated molecules p-Raptor and p-AMPK were all significantly over-expressed in PGLs rather than in PCCs, and in head and neck rather than in abdominal locations. None of the markers, except the low expression of p-mTOR, was associated to malignancy. Cluster 1 had higher total mTOR, p-Raptor and p-S6 expression than Cluster 2 PCC/PGL. In contrast, p-mTOR and mTORC2-associated molecule Rictor were significantly over-expressed in Cluster 2 tumors. Within Cluster 1, molecules active in the mTORC1 complex were significantly over-expressed in *SDHX*- as compared to *VHL*-mutated tumors.

Conclusions. In summary, the mTOR pathway is activated in a high proportion of PCC/PGLs, with a preferential over-activation of mTORC1 complex in PGLs of the head and neck and/or harbouring *SDHX* mutations.

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neuroendocrine tumors arising from chromaffin cells of the adrenal medulla or of paraganglia in the head and neck region or along the sympathetic trunk. PCC and PGL can be either familial or sporadic. Germline mutations in the *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2* (together *SDHX*), *VHL*, *RET*, *NF1*, *TMEM127*, *MAX*, *KIF1B*, *PHD2*, *FH* or the most recently identified *HIF2A* are identified in about 40% of PCC/PGL patients [1]. Somatic mutations in *RET*, *VHL*, *MAX* and *HIF2A* genes are also reported in 17% of sporadic tumors. Moreover, recent reports identified somatic *NF1* and *H-RAS* mutations in 22-26% and 5-7% of sporadic PCCs/PGLs, respectively [2-5]. Although the disease is the perfect example of genetic heterogeneity, two main transcriptomic signatures have been evidences. The first one, named cluster 1, is enriched with *VHL*- , *SDHX*- and *FH*-mutated tumors, and shares a pseudohypoxic profile. The second one, named cluster 2, groups tumours related to mutations in *RET*, *NF1*, *TMEM127* and *MAX*, and involves a kinase pathway [1]. The first integrative genomic study, recently published, demonstrated the crucial role of predisposing mutations as being the main drivers of PCC/PGLs [6].

The mTOR pathway is of great interest since it functionally interacts with genes whose alterations characterize both PCC/PGL clusters. In fact, several cancer models demonstrated that the components of the mTOR pathway have signalling interactions with *RET*, *TMEM127*, *MAX*, *NF1* and *VHL* gene products as well as with the succinate dehydrogenase complex. The mTOR protein is a kinase acting downstream in the phosphoinositide-3-kinase (PI3K)/AKT signalling pathway and forms two multiprotein complexes, named mTORC1 (sensitive to rapamycin) and mTORC2 (resistant to rapamycin). The mTORC1 complex is activated by diverse stimuli, such as growth factors, nutrients, oxygen availability, energy and stress

signals in order to control cell growth, proliferation and survival, whereas mTORC2 regulates the cytoskeleton function and is generally insensitive to nutrients and energy signals [7]. Hence, the mTOR pathway has been reported to be de-regulated in several human tumors, including - among others - neuroendocrine ones [11]. In PCCs, altered expression of mTOR-pathway molecules (phosphorylated forms of AKT and the mTOR downstream effector S6) has been documented in small series [12,13]. Moreover, total mTOR protein was investigated in a larger series of PCC and PGL apparently with a very low proportion of tumours (5 out of 100 cases) showing mTOR expression [14]. However, despite incomplete evidence of mTOR activation in PCC/PGL tumor tissues, therapeutic strategies selectively inhibiting mTOR have been tested both *in vitro* and *in vivo*. In fact, everolimus, a clinically used mTOR inhibitor, proved to be effective, although partially, in patients with progressive malignant PCCs/PGLs [15], whereas the dual inhibition of both mTORC1 and mTORC2 complexes has shown to be highly effective in PCC primary cell cultures and the MTT cell line [16].

The present study was therefore designed to explore the activation pattern of mTOR signalling pathway in a large series of sporadic and hereditary PCC/PGL, in order to check its relation to clinical, pathological and genetic features.

Materials and Methods

Case series. A total of 222 genetically well-characterized PCCs and PGLs were included in the study from the databases of the following centres: Department of Pathology, Erasmus MC, Rotterdam, The Netherlands (7 cases); the Spanish National Cancer Research Centre (CNIO) and ISCIII Center for Biomedical Research on Rare Diseases (CIBERER), Madrid, Spain (41 cases); INSERM, UMR970, Paris-Cardiovascular Research Center (PARCC)) and Biological Resources Center and Tumor Bank Platform, Hôpital européen Georges Pompidou (BB-0033-

00063, 75015 Paris, France (78 cases); the Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy (51 cases) and the Division of Pathology, Department of Oncology, University of Turin at San Luigi Hospital, Orbassano, Turin, Italy (45 cases). Institutional review board approval was obtained for the study by each of the centers, and informed consent was obtained from all patients. The overall series included 178 PCC and 44 PGL. Fourteen cases were metastatic. The genetic characterization in all cases for the presence of germline mutations in the *VHL*, *RET*, *MAX*, *TMEM127*, *SDHA*, *SDHB*, *SDHC*, *SDHD* and *FH* and of somatic mutations in *VHL*, *RET* and *MAX* was performed in the enrolling centres as clinical routine work. The presence of *NF1* mutations was determined in cases with clinically suspected neurofibromatosis type 1 (i.e. presence of neurofibromata and skin spots). Methodological conditions are available from the authors upon request. Moreover, *H-RAS* mutations were investigated in this series in a recent study by some of the present authors [5]. The baseline characteristics of the included samples are summarized in **Table 1**.

Immunohistochemistry. Five tissue micro arrays were prepared at the Erasmus University Medical Centre and at the University of Turin for immunohistochemical analysis using the ATA-27 Automated Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI, USA) or the semi-automated Quick-RAY^{TM} tissue arrayer (Bio-Optica, Milan, Italy). For each case, two samples of tumor tissue were selected from a representative hematoxylin and eosin-stained slide, and tissue cylinders with a diameter of 1 mm were punched from the representative areas of the 'donor' block and brought into the 'recipient' paraffin block.

All cases included on the five TMAs were analysed by means of immunohistochemistry using the following antibodies: mTOR (rabbit monoclonal, 7C10, diluted 1:50, Cell Signaling), phospho-mTOR (rabbit monoclonal, 49F9, Ser2448, diluted 1:100; Cell Signaling Tech,

Beverly, MA), phospho-p70S6K (mouse monoclonal, 1A5, Thr389, diluted 1:400; Cell Signaling), phospho-AMPK (rabbit monoclonal, 40H9, Thr172, diluted 1:100; Cell Signaling), phospho-AKT (rabbit monoclonal, 736E11, Ser473, diluted 1:40; Cell Signaling), phospho-4E-BP1 (rabbit monoclonal, diluted 1:300; Cell Signaling), p-S6 (rabbit polyclonal, 2211, Ser 235/236, diluted 1:400; Cell Signaling), Rictor (rabbit monoclonal, diluted 1:100, Cell Signaling) and phospho-Raptor (rabbit polyclonal, diluted 1:100; Cell Signaling). Immunoreactions were revealed by means of a biotin-free, dextran-chain detection system (Envision, Dako, Glostrup, Denmark) and developed using diaminobenzidine (DAB) as the chromogen. For all antibodies, immunohistochemical staining was scored in each core by multiplying the most prevalent staining intensity (0=negative, 1=weak, 2=moderate, 3=strong) and the quantity of staining (0-100%) giving a final IHC score (IHS) from 0 to 300. The mean score of the two cores for each tumor was recorded for subsequent statistical correlations. All TMAs were evaluated by one of us (LO); moreover, random slides or cases with equivocal staining were assessed at a multihead microscope by two observers (LO and MV) to uniform the staining interpretation or reach a consensus. These data were used for statistical purposes. Moreover, a second blind round of evaluation of all TMA was performed by an independent investigator (EK) to test inter-observer agreement.

Statistical analysis. The association between immunohistochemical findings, known clinical and pathological parameters and genotype was assessed by non-paired Student's *t* test. The Spearman test was used to analyse the correlation index among the expression of markers and between two independent observers. The level of significance was set at $p<0.05$. Statistical analysis was performed using the GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

Results

The mTOR pathway is activated in PCCs/PGLs. The functional activation of the mTOR pathway in the series analyzed was demonstrated by the positive correlation among most of the molecules investigated (**Table 2**). Total mTOR protein expression was positively associated to p-S6K, p-S6, p-AKT, p-Raptor and p-AMPK expression (Spearman's correlation coefficient R: $\geq$0.3). The specific functional activation of the mTORC1 complex was strengthened by the reciprocal correlation of p-Raptor (which couples with mTOR in the mTORC1 complex) and both p-S6K and p-S6, and by the positive correlation of p-AMPK (which specifically interacts with the mTORC1 complex) with p-S6K, p-S6, p-AKT and p-Raptor. By contrast, RICTOR (which couples with mTOR in the mTORC2 complex) was correlated with p-AKT, only. Phospho-mTOR protein, which represents the activated form of mTOR and interacts with both mTORC1 and mTORC2 complexes, was not significantly associated to a specific molecule, except for p-AKT. Robustness of immunohistochemical data was proved by the strong correlation between two observers evaluating independently all TMA (**Table 3**)

The mTORC1 complex is over-expressed in PGLs. Molecules active in the mTOR pathway were differentially expressed in PCCs as compared to PGLs. Total mTOR, p-S6K and p-S6 were all significantly over-expressed in PGLs than in PCCs. The mTORC1-associated molecules (p-Raptor and p-AMPK) showed the same profile. By contrast, p-mTOR and the mTORC2-associated molecule Rictor were over-expressed in PCCs (**Table 4**). When comparing tumor location, head and neck PGLs displayed a significantly higher expression of mTOR, p-S6K, p-S6, p-AMPK and p-Raptor as compared with abdominal PCC/PGLs (p<0.0001 for all markers). This association retained statistical significance restricting the

analysis to extra-adrenal and head and neck PGLs, only. Phospho-4EBP1 expression did not show significant differences between tumor type (PCC/PGL) and tumor location (abdominal/head and neck). None of the markers was significantly associated to the presence of malignant behaviour, except for p-mTOR which showed a higher mean IHS in benign cases. When comparing mean age at diagnosis, the expression of p-mTOR and Rictor was higher in older patients (using the median age of 45 years as the cut off), while the expression of p-AMPK and p-4EBP1 was higher in younger patients. Finally, any of the investigated markers was significantly associated to patients' gender.

mTOR pathway activation is associated with specific genotypes. The expression of the mTOR markers tested across the diverse PCC/PGL susceptibility genes and in cases with no germline or somatic mutations detected (n=109) was heterogeneous (**Table 5**). Highest levels of expression of mTOR, pS6K, pS6, p-Raptor and p-AMPK were detected in *SDHX*-mutated tumors. By contrast, *TMEM127*-mutated cases had very low protein expression levels of all markers. Cases with no known mutations showed expression levels for each marker generally close to the mean levels of the overall series. P-4EBP1 and p-AKT were the only markers that lacked any significant association with tumor genotype. For statistical comparison, all tumors from patients with known mutations in one of the PCC/PGL susceptibility genes were arbitrarily grouped into clusters, as proposed in the literature [17]: Cluster 1 included *SDHX*-, *FH*- and *VHL*-mutated tumors (n=53), whereas Cluster 2 included *NF1*, *RET*, *TMEM127*, *MAX* and *H-RAS* PCC/PGL (n=57). Cluster 1 tumors had significantly higher total mTOR, p-Raptor and p-S6 expression than Cluster 2 PCC/PGL. In contrast, p-mTOR and Rictor expression were significantly higher in Cluster 2 tumors as compared to Cluster 1 tumors. Among genes in Cluster 2, a significant difference was observed between *MAX* and *H-RAS* for p-mTOR expression (p=0.0170), and between *RET* and *H-RAS* for p-S6K expression

(p=0.0128). More interestingly, within Cluster 1, *VHL*- and *SDHX*-mutated cases showed significantly different mTOR pathway profiles. Molecules active in the mTORC1 complex (p-AMPK and p-Raptor) and mTOR itself were significant over-expressed whereas p-mTOR expression was reduced in *SDHX*- as compared to *VHL*-mutated tumors (**Figure 1**). Finally, restricting the analysis to PGL cases only, mTORC1 complex was over-expressed in *SDHX* mutated (28 cases) *vs* not mutated (14 cases) tumors, whereas p-mTOR expression was reduced (**Figure 2**).

Discussion

The mTOR signalling pathway in PCC/PGLs has attracted research interest because cluster 2 PCC/PGLs are associated with a de-regulation of this pathway and components of the mTOR pathway have signalling interactions with *SDHX* and *VHL* gene products (i.e. cluster 1 PCC/PGLs) as well. Therefore, the use of drugs targeting the mTOR pathway has been considered suitable in PCC/PGL patients.

In this study, we investigated the immunohistochemical expression of mTOR-signalling components in a very large series of PCC/PGLs. We correlated the expression of a variety of markers acting in the mTOR pathway with major clinical data and genotype of the tumors. Although a few studies have investigated the protein expression of single or various components of the mTOR pathway [12-14] in this setting, a comprehensive assessment of all key members of this intracellular signalling cascade in a genetically well-characterized set of PCC/PGLs has not been performed. Examining the entire population, a substantial activation of the mTOR pathway emerged by the positive correlation between mTOR protein expression and its down and upstream regulators, with special reference to those acting in the mTORC1 complex. Our data are partly in contrast with the findings by Pinato [14], who found a very

low expression of mTOR and AKT in a series of PCC and PGL. However, in the present study the protein expression data were supported by the integrated analysis of several molecules active in the same pathway which were all consistent and significantly correlated with each other. Phosphorylated-mTOR was not directly correlated with mTOR and other proteins active in the mTORC1 complex except for p-AKT. These findings are probably related to the fact that the phosphorylated form of mTOR is also active in the mTORC2 complex, thus its detection at the tissue level is the consequence of more complex stimuli.

When trying to compare the patterns of expression of all molecules investigated with major clinical and pathological parameters, it was clearly evident that PCC and PGL have opposite profiles of activation. In fact, mTORC1-active molecules and mTOR itself were over-expressed in PGLs as compared to PCCs. This same profile was observed in head and neck PGLs when compared to extra-abdominal tumors. By contrast, mTORC2 protein Rictor and p-mTOR were over-expressed in PCCs as compared to PGLs, but failed to be significantly different when comparing head and neck versus extra-abdominal PGLs. With regard to other clinical or pathological parameters, all markers failed to show relevant associations. Phospho-mTOR was significantly over-expressed in non-metastatic cases, a finding which is in agreement with what observed by Ghayee and coworkers [13] in a smaller series, but this association is more probably the result of the higher expression in PCC cases observed in our entire population. Hence, we might argue that the mTOR pathway is expressed in both benign and malignant PCC/PGLs and mTOR inhibition might be a successful therapy target in malignant PCC/PGL cases which need medical treatment for disease control.

A further aim of this study was to extensively explore the association between mTOR activation status and genotype of PCC/PGL cases. The hypothesis of specific genetic-driven activation profiles of the mTOR pathway in PCC/PGLs partly stemmed from previous observations by some of the present authors on the association between mTOR activation and

Ret mutational status in medullary thyroid carcinoma [10]. This hypothesis was also partly sustained by Pinato and co-workers who found, although at very low levels - as commented above - a preferential expression of mTOR and AKT in *SDHX*-mutated tumors [14].

Comparing genes grouped into the two major molecular clusters, namely *SDHX* and *VHL* in cluster 1 and *NF1*, *RET*, *TMEM127*, *MAX* and *H-RAS* in cluster 2 PCC/PGL, it was strongly evident that mTORC1 complex molecules (including p-S6, p-Raptor and mTOR itself) were over-expressed in Cluster 1 tumors, whereas phospho-mTOR and Rictor were over-expressed in Cluster 2 tumors. It is worth to notice that restricting the analysis to Cluster 1 a significant over-expression of some of the above mentioned molecules (p-Raptor and mTOR itself) together with p-AMPK (all belonging to the mTORC1 complex) was observed in *SDHX* as compared to *VHL*- mutated tumors. Moreover, mTORC1 complex over-activation in *SDHX* mutated tumors was significant also in the PGL group examined separately. Interestingly, our data are in agreement with a previous study describing the increased expression of phospho-p70S6K in a subgroup of Cluster 1 cases enriched for SDHx mutations as compared to Cluster 1 cases enriched for VHL mutations [18].

These findings overall suggest two major issues: first, that although grouped into major molecular clusters, PCC and PGL with different genetic profiles are characterized by specific and more heterogeneous intracellular signalling activation patterns; second, that if the genetic landscape of tumors is a major responsible for mTOR activation in PCC/PGL, the hypothetical strategy of mTOR-targeting therapies in PCC/PGL should take into consideration not only the biological behaviour of tumors but also their genetic characteristic to be of clinical meaningfulness. However, our data are descriptive in nature and validation of immunohistochemical biomarkers on tissue microarrays is methodologically limited in part, therefore their potential utility need to be validated in the clinical practice and corroborated by functional pharmacogenomic studies.

In summary, our data show that the mTOR pathway is activated in a relevant proportion of PCC/PGLs, with a preferential over-expression of mTORC1 complex proteins in PGLs of the head and neck and/or harbouring *SDHX* mutations.

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Figure Legends

Figure 1. Representative immunohistochemical pictures (above) and boxplots (below) showing the differential expression of mTOR-pathway molecules in *VHL* as compared to *SDHX* mutated tumors.

Figure 2. Boxplots showing the differential expression of mTOR-pathway molecules reaching statistical significance in non-mutated as compared to *SDHX*-mutated PGLs.

Table 1. Baseline characteristics of patients.

Baseline characteristic	**n = 222**
Gender	
Male	98
Female	124
Age	
< mean 45	103
≥ mean 45	119
PCC genotype	177
VHL germline/somatic	14/3
RET germline/somatic	30/3
NF1 germline/somatic	6/4
MAX germline/somatic	5/1
TMEM127 germline	2
SDHB germline	2
SDHD germline	3
H-RAS somatic	6
No mutation found	98
EA PGL genotype	21
VHL germline/somatic	1/1
NF1 germline	1
SDHB germline	6
SDHC germline	2
SDHD germline	2
No mutation found	8
HNPGL genotype	22
SDHB germline	5
SDHD germline	13
SDHX * germline	1
No mutation found	3
META genotype	2
SDHB germline	1
FH germline	1
Behaviour	
Non-metastatic	208
Metastatic	14

PCC = pheochromocytoma; EA PGL = extra-adrenal paraganglioma; HNPGL = head&neck paraganglioma; meta = metastasis; **SDHB* immunonegative, but no *SDHB*/*SDHC*/*SDHD*/*SDHAF2* mutation identified with Sanger sequencing.

Table 2. Reciprocal correlations among markers investigated.

	p-mTOR	**p-S6K**	**p-S6**	**p-AKT**	**p-RAPTOR**	**RICTOR**	**p-AMPK**	**p-4EBP-1**
mTOR	R: 0.1019 p: 0.1347	**R: 0.39** **p: <0.0001**	**R: 0.45** **p: <0.0001**	**R: 0.34** **p: <0.0001**	**R: 0.37** **p: <0.0001**	R: 0.11 p: 0.09	**R: 0.51** **p: <0.0001**	R: 0.23 p: 0.001
p-mTOR	-	R: -0.02 p: 0.82	R: 0.02 P: 0.72	**R: 0.34** **p: <0.0001**	R: 0.04 p: 0.59	R: 0.21 p: 0.002	R: 0.15 p: 0.02	R: 0.07 p: 0.32
p-S6K	-	-	**R: 0.42** **p: <0.0001**	R: 0.18 p: 0.01	**R: 0.40** **p: <0.0001**	R: -0.01 p: 0.89	**R: 0.50** **p: <0.0001**	R: 0.29 p: <0.0001
p-S6	-	-	-	R: 0.19 p: 0.006	**R: 0.47** **p: <0.0001**	R: 0.03 P: 0.66	**R: 0.41** **p: <0.0001**	R: 0.22 p: 0.001
p-AKT	-	-	-	-	R: 0.16 p: 0.02	**R: 0.38** **p: <0.0001**	**R: 0.38** **p: <0.0001**	R: 0.18 p: 0.008
p-RAPTOR	-	-	-	-	-	R: 0.00 p: 0.94	**R: 0.32** **p: <0.0001**	R: 0.13 p: 0.06
RICTOR	-	-	-	-	-	-	R: 0.16 p: 0.02	R: 0.16 p: 0.02
p-AMPK	-	-	-		-	-	-	R: 0.28 p: <0.0001

Table 3. Interobserver agreement**.**

IHC marker	**Observer LO *vs* Observer EK - Spearman's correlation**
mTOR	R: 0.85 p: <0.0001
p-mTOR	R: 0.92 p: <0.0001
p-S6K	R: 0.84 p: <0.0001
p-S6	R: 0.72 p: <0.0001
p-AKT	R: 0.86 p: <0.0001
p-RAPTOR	R: 0.88 p: <0.0001
RICTOR	R: 0.87 p: <0.0001
p-AMPK	R: 0.95 p: <0.0001
p-4EBP-1	R: 0.91 p: <0.0001

Table 4. Correlation of mTOR pathway molecules with clinical and pathological characteristics

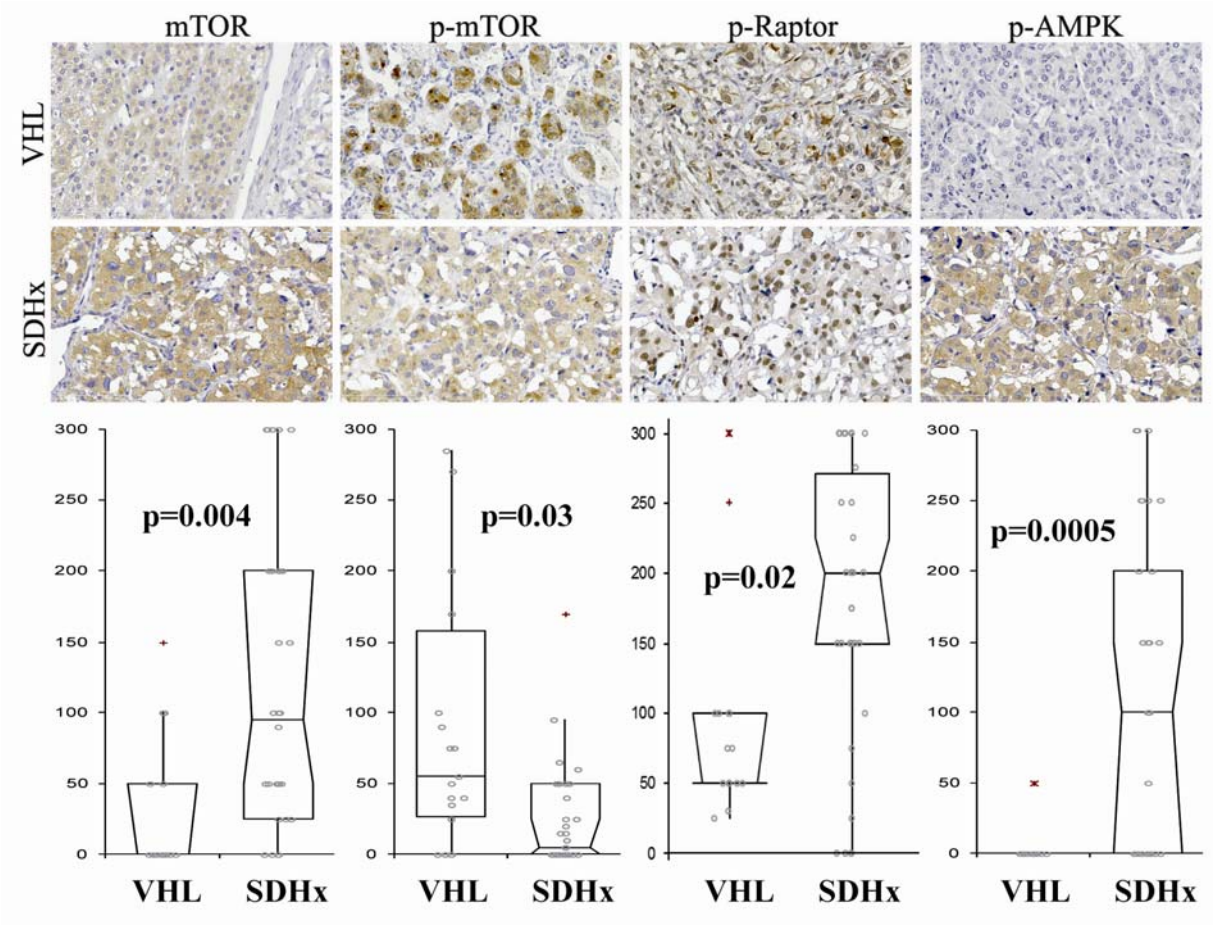
	mTOR		**p-mTOR**		**p-S6K**		**p-S6**		**p-AKT**		**p-Raptor**		**Rictor**		**p-AMPK**		**p-4EBP1**	
	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p
PCC	42.0 ± 4.7		91.1 ± 5.9		30.7 ± 3.8		14.0 ± 2.6		144.7 ± 6.1		82.8 ± 6.1		155.0 ± 6.3		45.9 ± 6.0		38.8 ± 5.1	
PGL	101.9 ± 16.5	**<0.001**	42.0 ± 9.1	**<0.001**	53.3 ± 6.9	**0.001**	42.3 ± 8.8	**<0.001**	130 ± 10.9	0.25	144.2 ± 16.2	**<0.001**	110.1 ± 12.3	**<0.001**	89.5 ± 15.6	**0.004**	57.3 ± 12.9	0.17
EAPGL	36.9 ± 12.0		44.3 ± 13.7		31.7 ± 9.7		13.5 ± 5.3		114.0 ± 15.8		81.0 ± 17.9		122.1 ± 20.2		52.4 ± 18.4		74.3 ± 20.1	
HNPGL	163.9 ± 23.5	**<0.001**	39.8 ± 12.3	0.72	75.0 ± 7.3	**0.001**	69.7 ± 14.2	**<0.001**	145.2 ± 14.6	0.19	204.5 ± 19.4	**<0.001**	98.1 ± 14.0	0.55	125.0 ± 22.7	**0.015**	41.1 ± 16.0	0.16
non-mets	54.7 ± 5.5		84.2 ± 5.4		34.8 ± 3.3		18.8 ± 2.8		143.7 ± 5.5		93.0 ± 6.1		148.0 ± 5.9		55.0 ± 6.0		40.4 ± 4.8	
mets	35.8 ± 11.6	0.86	35 ± 11.4	**0.02**	37.3 ± 11.9	0.60	28.6 ± 18.6	0.82	114.6 ± 16.8	0.16	130.4 ± 29.4	0.24	144.6 ± 28.0	0.75	42.3 ± 19.5	0.66	78.6 ± 27.9	0.33
Female	51.7 ± 7.0		88.1 ± 7.3		34.2 ± 4.3		13.7 ± 2.8		136.9 ± 7.1		88.2 ± 7.8		150.2 ± 7.9		54.1 ± 8.0		39.3 ± 6.2	
Male	56.0 ± 7.9	0.65	72.7 ± 7.4	0.16	35.9 ± 4.6	0.87	26.7 ± 5.2	0.13	148.0 ± 7.9	0.38	104.4 ± 9.3	0.19	144.6 ± 8.4	0.64	54.3 ± 8.4	0.80	47.3 ± 7.7	0.31
age<mean	54.9 ± 7.8		67.4 ± 7.2		39.7 ± 5.0		26.0 ± 4.9		134.0 ± 7.8		105.8 ± 9.5		134.4 ± 8.6		68.6 ± 9.5		51.9 ± 7.8	
age>mean	52.5 ± 7.0	0.98	93.1 ± 7.3	**0.01**	30.8 ± 3.9	0.24	13.7 ± 3.0	0.35	148.8 ± 7.2	0.11	86.2 ± 7.5	0.19	159.5 ± 7.5	**0.02**	41.7 ± 6.8	**0.04**	34.9 ± 5.9	**0.04**

Note: PCC= pheochromocytoma; PGL= paraganglioma; EAPGL= extra adrenal paraganglioma; HNPGL= head and neck paraganglioma; mets: metastatic.

Table 5. Correlation of mTOR pathway components with genotype

	mTOR		**p-mTOR**		**p-S6K**		**p-S6**		**p-AKT**		**p-Raptor**		**Rictor**		**p-AMPK**		**p-4EBP1**	
	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p	mean ± SE	p
H-RAS (#6)	58.33 ± 23.86		166.67 ± 28.07		5 ± 3.16		10 ± 6.32		183.3 ± 30.73		73.3 ± 29.20		153.33 ± 18.65		33.33 ± 24.72		40.83 ± 20.02	
MAX (#6)	30 ± 30		37 ± 13.38		18 ± 7.84		0		130.0 ± 48.99		43 ± 28.27		100 ± 25.0		10.0 ± 10		108.33 ± 49.20	
NF1 (#10)	45.91 ± 20.64		73.18 ± 16.79		21,36 ± 5.80		20 ± 11.06		159.1 ± 25.95		102.3 ± 23.94		137.27 ± 28.99		31.82 ± 13.94		45.45 ± 31.23	
RET (#33)	41.29 ± 12.64		110.61 ± 13.90		61,56 ± 11.28		16,6 ± 7.23		142.6 ± 15.62		90 ± 16.58		167.58 ± 14.55		103.03 ± 19.09		48.28 ± 12.76	
TMEM127 (#2)	50 ± 50.0		12.5 ± 12.5		10 ± 0		0		25.0 ± 25.0		7.5 ± 7.5		50 ± 50		25.0 ± 25		0.0	
SDHX (#35)	118,09 ± 18.60		24.24 ± 6.36		62,58 ± 7.74		49.47 ± 10.80		128.9 ± 10.64		180.0 ± 16.71		110.29 ± 13.23		108.82 ± 18.58		54.57 ± 12.58	
FH (#1)	0		0		0		0		100		100		300		0.0		0.0	
VHL (#17)	26.32 ± 10.38		90 ± 20.88		29,11 ± 8.90		28.16 ± 11.24		114.2 ± 16.30		87.1 ± 16.32		128.16 ± 17.98		5.26 ± 3.62		60.26 ± 22.51	
NO MUT*(#109)	36.09 ± 7.05	**0.002**	85.43 ± 10.55	**<0.001**	18.48 ± 4.05	**<0.001**	13,80 ± 5.92	**0.003**	149.81 ± 7.51	0.12	75.65 ± 7.14	< **0.001**	137.5 ± 10.04	**0.031**	26.09 ± 8.48	**<0.001**	26.91 ± 8.32	0.33
CLUSTER1 (#53)	86.83 ± 14.01		45.29 ± 9.3		51.24 ± 6.32		42.16 ± 8.16		124.15 ± 9.09		149.15 ± 13.74		116.06 ± 10.81		73.08 ± 13.97		40.83 ± 20.02 ±	
CLUSTER2 (#57)	43.36 ± 8.95	**0.012**	99.39 ± 10.26	**<0.001**	41.88 ± 7.24	0.21	14.45 ± 4.69	**<0.001**	144.82 ± 11.88	0.18	83.60 ± 11.48	**<0.001**	150.18 ± 11.03	**0.018**	71.05 ± 12.64	0.9	108.33 ± 49.20	0.65

Note: *: p value as compared to cases with any type of mutation.


mTOR
p-mTOR
p-Raptor
p-AMPK
VHL
SDHx
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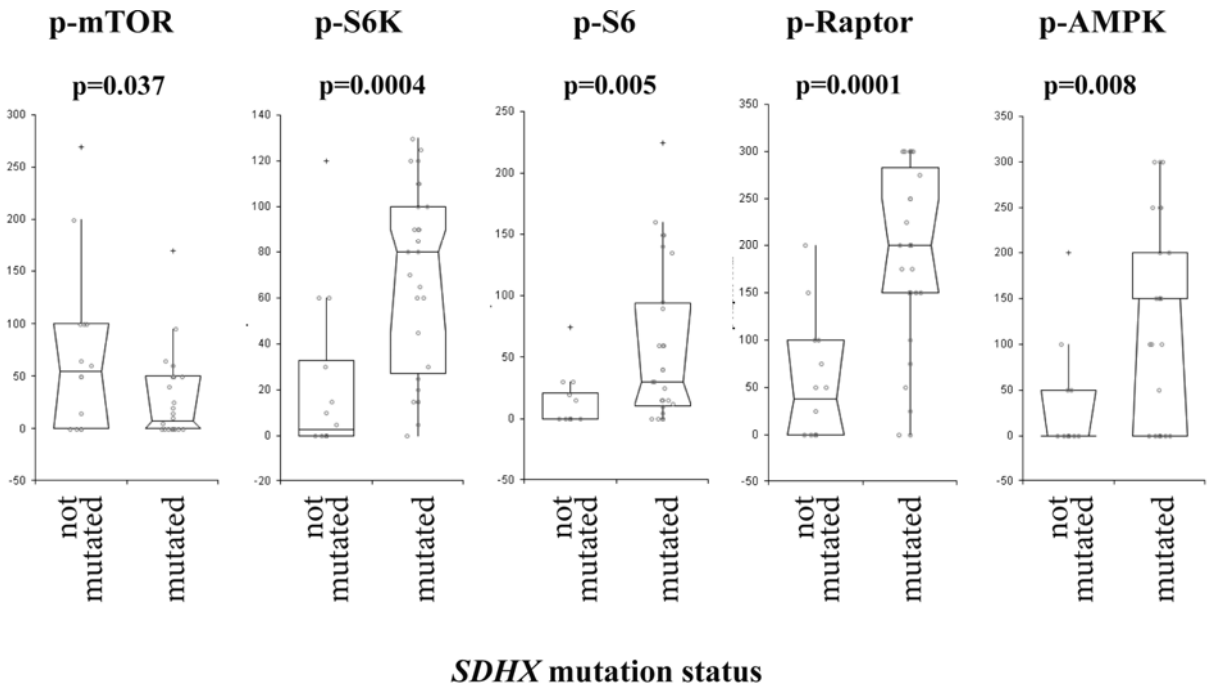
p=0.03
p=0.02
p=0.0005
300
250
200
150
100
50
0
VHL
SDHx

16

17

18


p-mTOR
p=0.037
p-S6K
p=0.0004
p-S6
p=0.005
p-Raptor
p=0.0001
p-AMPK
p=0.008
not mutated
mutated
SDHX mutation status

19