Effective Therapeutic Intervention and Comprehensive Genetic Analysis of mTOR Signaling in PEComa: A Case Report

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Abstract. *Background/Aim: Perivascular epithelioid cell tumors (PEComas) are rare mesenchymal neoplasms. The exact genetic alterations underlying the pathophysiology of PEComas are largely unknown, although it has been shown that activation of the Mammalian target of rapamycin (mTOR) signaling pathway plays a pivotal role. Herein we describe the successful treatment of a patient with metastatic PEComa with the mTOR inhibitor everolimus and a comprehensive analysis to identify mechanisms for response. Materials and Methods: Immunohistochemistry, array comparative genomic hybridization (aCGH) and genetic analyses were performed. Results: Immunohistochemistry confirmed constitutive activation of mTOR. aCGH revealed a hyperdiploid karyotype affecting large regions of the genome. Next-generation sequencing did not reveal any tumor-specific mutations in mTOR-related genes. Conclusion: Our results show the complexity of determining causal genetic alterations that can predict responsiveness to mTOR inhibition, even for a tumor with a complete remission to this specific treatment.*

Perivascular epithelioid cell tumors (PEComa) are rare mesenchymal neoplasms with perivascular epithelioid cell differentiation (1, 2), characterized by a unique immunohistochemical profile of melanocytic and myogenic markers (3). The PEComa family comprises of angiomyolipoma located in kidney or liver, clear cell 'sugar' tumor located in the lung, lymphangioleiomyomatosus and an assembly of uncommon neoplasms arising in skin, viscera or soft tissue (4).

The molecular mechanism underlying PEComas became apparent from the observation that lymphangioleiomyomatosus and angiomyolipoma often occurred in patients with tuberous sclerosis, a hereditary disease associated with genetic defects in Tuberous sclerosis 1 (*TSC1*) or *TSC2*, both inhibitors of Mammalian target of rapamycin (mTOR) (5, 6). PEComas show constitutive mTOR activation, as shown by elevated phospho-mTOR and phospho-S6 immunohistochemistry (IHC), but thus far no causal mutations in mTOR-related genes have been discovered, although gross chromosomal aberrations, including loss of chromosome 16p containing the *TSC2* gene, have been observed (7, 8).

Recently, three patients with PEC tumors were successfully treated with sirolimus, a selective mTOR-complex 1 inhibitor (6). The choice of treatment was based on the predicted molecular pathophysiology of PEC tumors: an aberrant activation of the mTOR pathway. All three tumors exhibited loss of expression of tuberin, the gene product of *TSC2*. Further investigation by multiplexed ligation-dependent probe amplification and fluorescent *in situ* hybridization demonstrated a homozygous loss of *TSC1* in one of the three tumor specimens. Two out of three patients treated with sirolimus achieved a significant tumor reduction; one patient did not respond although an activated mTOR signaling pathway was confirmed by IHC. In other tumor types, phospho-S6 and phospho-mTOR IHC are also not strongly correlated with a clinical response to mTOR inhibition (9, 10). On the contrary, Dickson *et al.* reported that the mutational analysis of four patients with a response to mTOR inhibition, by conventional Sanger sequencing, revealed a loss of heterozygosity of *TSC2* in all four patients and a non-sense mutation of *TSC2* in one (11). In patients

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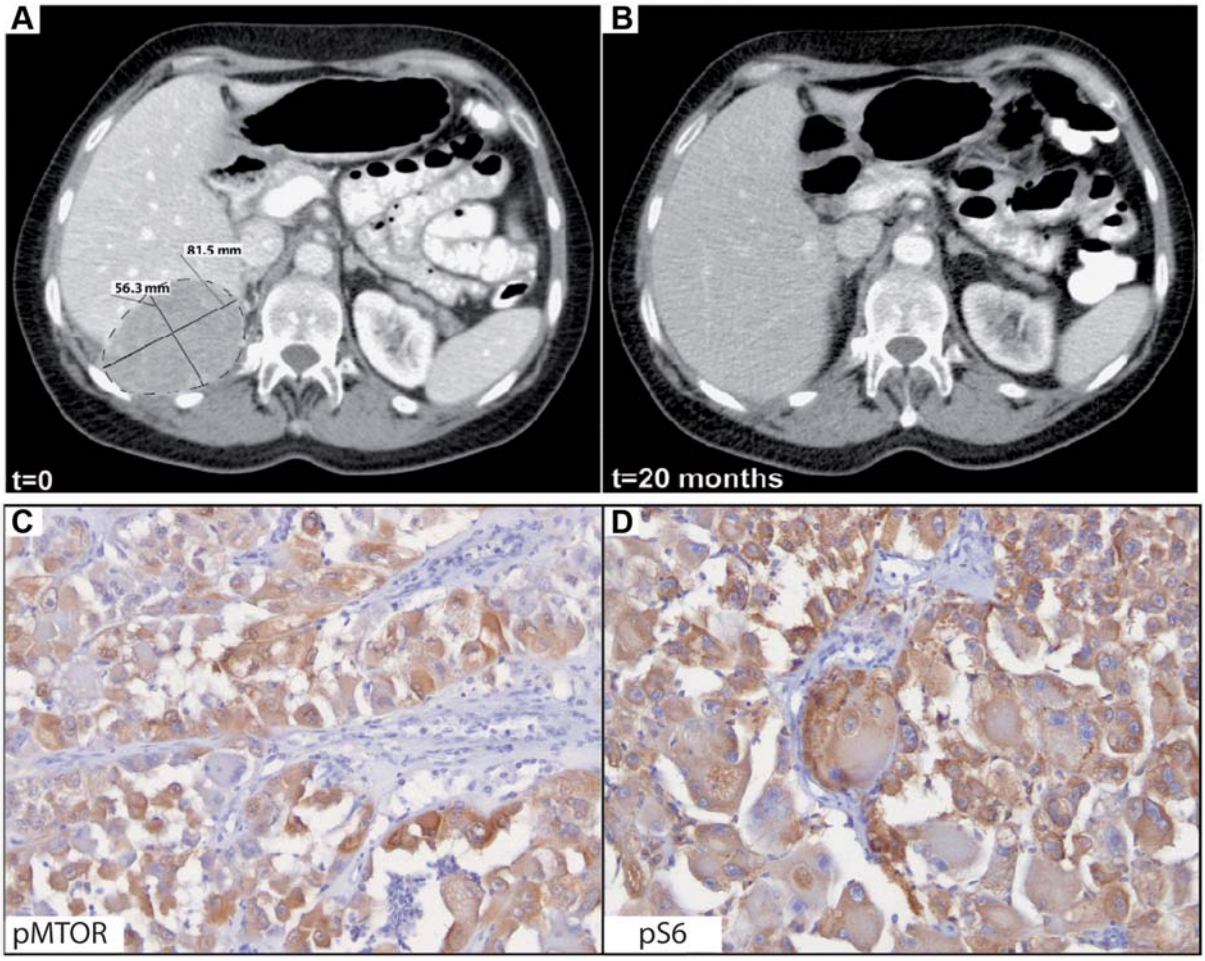

Figure 1. *Computed tomographic (CT) imaging and immunohistochemistry of metastatic perivascular epithelioid cell tumor. A: The baseline CT scan showing a large lesion dorsal of the liver. B: The CT scan taken after 20 months of treatment with everolimus and demonstrates a complete remission. Immunohistochemical analysis of phospho-S6 (C) and phospho-mammalian target of rapamycin (mTOR) (D). Both portray diffuse cytoplasmic staining indicating ectopic activation of the mTOR signaling pathway. Magnification, ×200.*

with metastatic bladder cancer, mutations in *TSC1* have also shown a strong correlation with response to everolimus (12). These findings suggest that genetic profiling of key signaling components of the mTOR pathway allows for improved stratification of patients suitable for mTOR inhibition compared to IHC, thereby increasing treatment efficiency.

Here we describe a 56-year-old female patient with malignant PEComa who achieved a prolonged complete remission on everolimus treatment (3 years).

Case Report

The patient was diagnosed in 2006 with a tumor originating from the omentum. After complete resection (omentectomy and bilateral adnectomy), pathology classified it as a PEComa due to expression of CD99, myogenic and melanocytic markers. The patient was disease-free until December 2008, when multiple recurrences localized in the liver, adrenal gland and peritoneal cavity were discovered. In March 2009, palliative chemotherapy was started with six cycles single-agent adriamycin (doxorubicin; 75 mg/m^2/*q3* weeks) with partial remission as best response. The tumor progressed six months after the start of treatment. Based on the reported successful treatment of PEComa by sirolimus, the patient was treated with the mTOR inhibitor everolimus (10 mg *qd*) and metastatic lesions were evaluated after three months (6). All target lesions showed partial remission. Six months after start of treatment, a virtually complete remission was observed that was sustained for another two and a half years (Figure 1A and B). Three years after the start of everolimus the patient developed hepatic and peritoneal metastases. A resection of liver segments 6/7 and the peritoneal lesions was performed

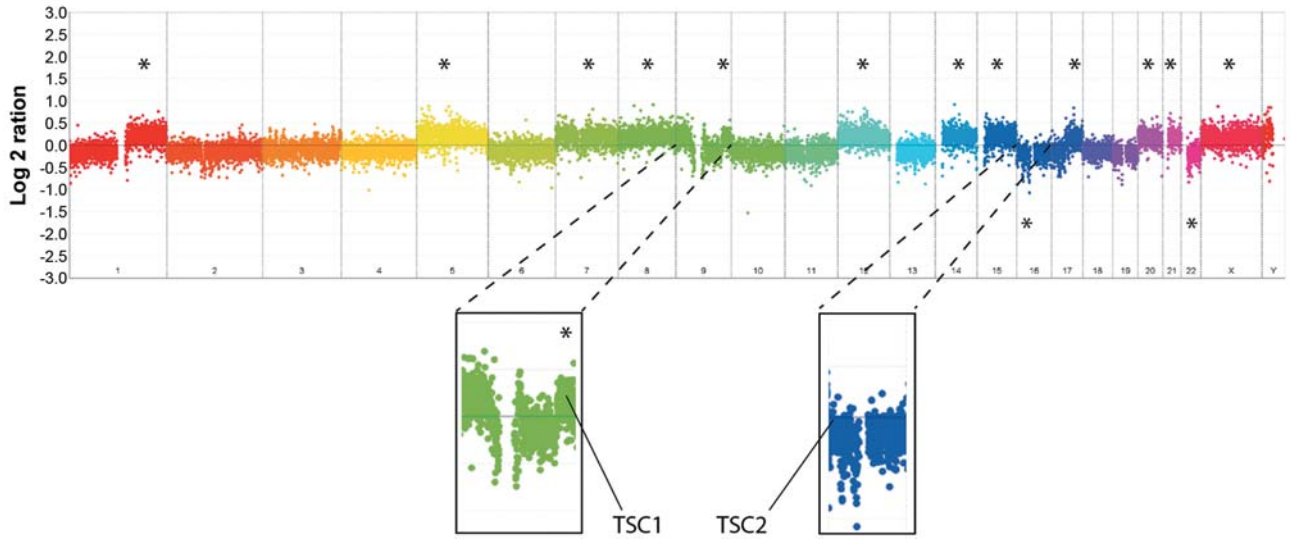

Figure 2. *Array comparative genomic hybridization analysis showing amplification of a significant part of the genome and only a small number of deletions (indicated by an asterisk). No loss of 9q (Tuberous sclerosis 1 (TSC1)) or 16p (TSC2) was observed, contrary to previously reported cases (insets).*

Table I. *Sequencing statistics.*

	PEComa	Reference
Total number of reads	35,630,848	28,694,234
Percentage of reads mapped	76%	79%
Mapped on target (%)	23,045,280 (65%)	18,675,848 (65%)
Target coverage	94.8%	93.3%
Target coverage >20	80.8%	79.8%

Table II. *Sequence statistics for genes directly involved in mTOR signaling.*

	All reads		Uniquely mapped reads	
Gene	%cov	%cov ≥20	%cov	%cov ≥20
MTOR	96,55%	92,45%	96,55%	92,45%
PTEN	100,00%	99,92%	81,67%	79,50%
RHEB	97,15%	91,93%	96,99%	90,66%
TSC2	91,20%	57,13%	91,20%	57,13%
PIK3CA	100,00%	100,00%	93,35%	93,60%
RICTOR	99,69%	98,15%	99,69%	98,15%
RPTOR	98,50%	79,80%	98,50%	79,80%
AKT1	98,81%	60,69%	98,81%	60,69%
TSC1	100,00%	95,17%	100,00%	95,17%

(tumor-free margins <1 mm) and everolimus was re-introduced one month later. The patient was treated with everolimus for another year before progressive disease occurred.

Tissue samples. The tumor specimen was obtained from the resected primary tumor (Formalin-fixed, paraffin-embedded). A blood sample served as reference for germline variations. DNA was isolated from the primary tumor as described previously (13). Tumor tissue and blood were collected through secondary use and therefore did not require ethical review. All samples were coded and anonymized.

Immunohistochemistry. Immunohistochemistry was performed using rabbit monoclonal antibodies for phospho-S6 (#4857, 1:50, ARS pH 6, 1 h incubation; Cell Signaling, Danvers, MA, USA) and Phospho-mTOR (#2976, 1:200, ARS pH 6, overnight incubation at 4˚C; Cell Signaling, Danvers, MA, USA). The BrightVision+poly-HRP detection system (ImmunoLogic DPVB110HRP, Duiven, the Netherlands) and DAB (Sigma-Aldrich D5637, St. Louis, MO, USA) were used for staining. Positive control: ductal carcinoma (human epidermal growth factor receptor "HER2neu^{+}"), negative control: healthy breast tissue.

Array comparative genomic hybridization. Genomic DNA (600 ng) of both tumor and blood were labeled with Cy3 and Cy5 respectively (#ENZ-42670; Enzo Life Sciences, Farmingdale, NY, USA). Samples were pooled equimolarly and subsequently hybridized to a NimbleGen (Basel, Switzerland) 12X135K CGH array (20 kb resolution) according to manufacturers' protocol. Segment analysis was performed by determining the Cy3/Cy5 ratio at 532/635 nm. Due to a hyperdiploid karyotype

resulting in a downward shift of the baseline, a log2ratio of ≥0.15 is classified as a gain (≤–0.45 as a loss).

Sequencing of mTOR-related genes. Library preparation was performed according to Nijman *et al*. (14). Briefly, 500 ng per sample (primary tumor and healthy blood) was fragmented and blunt-ended, followed by barcoded-adaptor ligation. After amplification and size selection, equimolar amounts of samples were pooled, followed by multiplexed sequence enrichment using a custom designed SureSelect sequence enrichment kit (Agilent, Santa Clara, CA, USA) according to methods established by Harakalova *et al*. (15). Samples were subsequently processed using the EZbead system and sequenced on the SOLiD4 sequencing platform according to standard protocols (Life Technologies, Carlsbad, CA, USA).

Sequence reads were mapped against the reference genome build hg19 using BWA (16). Custom scripts were used to detect and annotate variants with different stringency criteria. All calls with less than 20× or more than 2,000× coverage were removed, as well as all calls with a base-quality below 10. Only reads mapped to a unique location in the genome were allowed. A maximum of 5 identical reads were allowed to filter out the effects of clonality. The variant was required to be found at least three times in the seed of the read (first 25 bases), to have at least three independent start sites, and the percentage of forward calls compared to the reverse calls or vice versa was at least 10%. The minimum variant frequency was set at 20%. To identify somatic mutations, all variants identified in both tumor and blood were excluded from further analysis.

Sanger sequencing was used to validate identified mutations by SOLiD sequencing and resequence *TSC1* and *TSC2* to confirm the absence of mutations.

Results of analyses. In search of a predictive genetic profile for mTOR inhibition, we first confirmed constitutive activation of mTOR by IHC staining of phospho-mTOR and phospho-S6. (Figure 1C and D). Subsequently, aCGH analysis identified gross chromosomal aberrations (Figure 2), showing that a substantial part of the genome was duplicated (3n), with only a very limited number of focal deletions (none of which involved mTOR-related genes). Previous CGH analysis on PEComa showed a recurrent loss of 16p13 in the majority of patients (7, 8), which was not observed in this patient. Due to this severe aneuploidy, we were unable to identify a single genomic region explaining activation of the mTOR pathway.

To determine whether single nucleotide variations could activate mTOR, we sequenced all genes functioning in the mTOR signaling pathway using next-generation sequencing for 2,000 cancer-related genes (Gene list is provided in Vermaat *et al*. (13); sequence statistics in Table I; sequence statistics specific for genes directly involved in mTOR signaling in Table II). Data analysis did not identify any somatic mutations in mTOR-related genes, potentially activating the pathway (see Table III). Due to the low percentage of uniquely mapped genes for *TSC2* and the overall importance of *TSC1* and *TSC2* for mTOR signaling, both genes were re-sequenced using the Sanger method, confirming the absence of somatic mutations.

Table III. *Somatic mutations identified by SOLiD4 sequencing*.

Gene	Gene name	Mutation	Type
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	P713L	Known (rs3748022)
SMC1B	Structural maintenance of chromosomes 1B	F1055L	Known (rs61735519)
SREBF1	Sterol regulatory element binding transcription factor 1	Non-essential splice site	Known (rs11868035)
TCF4	Transcription factor 4	H398Y	Novel
CASP9	Caspase 9, apoptosis-related cysteine peptidase	Q221R	Known (rs1052576)
COL4A4	Collagen, type IV, alpha 4	P482S	Known (rs2229814)
MAP2K7	Mitogen-activated protein kinase kinase 7	Intronic-undefined	Known (rs10412007)

Discussion

This case report shows the effectiveness of inhibiting mTOR activity by everolimus in treating PEComa. Although a very strong therapeutic response of tumor load was observed in this patient, we still face major challenges in understanding, and ultimately predicting, therapy response. Even with knowledge on the driving signaling pathway, we did not find one single genetic alteration explaining this observation. There are multiple scenarios possibly explaining this: i) our knowledge of the complexity of genes regulating the mTOR signaling pathway is incomplete; ii) our knowledge of interactions between different signaling pathways is incomplete; iii) large-scale genomic instability (copy number variants) affects the expression level of (various) genes; iv) structural variations, such as translocations, are driving tumorigenesis; v) disturbed epigenetic regulation of chromosomal organization might be affected; vi) everolimus does not exclusively inhibit the mTOR pathway; vii) mutations in mTOR-related genes were not identified on account of sequencing low-quality DNA due to formalin fixation; or viii) there are genetic differences between

the primary tumor and its metastases. In summary, our data illustrate the complexity of determining genetic events underlying treatment response. To fully exploit the potential of next-generation sequencing technology in order to establish personalized cancer treatment, we require high-quality (fresh frozen) pre-treatment sampling of metastatic lesions, allowing for comprehensive analysis of genetic alterations in combination with treatment response data of the patient. Ultimately, combining various datasets, will enable us to identify more biomarkers for response or resistance, tailoring cancer treatment based on the individual genomic profile.

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Conflicts of Interest

The Authors declare there are no conflicts of interest.

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