Potential Targets' Analysis Reveals Dual PI3K/mTOR Pathway Inhibition as a Promising Therapeutic Strategy for Uterine Leiomyosarcomas-an ENITEC Group Initiative

Clinical Cancer Research

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Tine Cuppens[1], Daniela Annibali[1], An Coosemans[2,3], Jone Trovik[4,5], Natalja ter Haar[6], Eva Colas[7,8], Angel Garcia-Jimenez[9], Koen Van de Vijver[10], Roy P.M. Kruitwagen[11], Mariël Brinkhuis[12], Michal Zikan[13], Pavel Dundr[14], Jutta Huvila[15], Olli Carpén[15,16], Johannes Haybaeck[17], Farid Moinfar[18], Helga B. Salvesen[4,5], Maciej Stukan[19], Carole Mestdagh[20], Ronald P. Zweemer[21], Leonardus F. Massuger[22], Michael R. Mallmann[23], Eva Wardelmann[24], Miriam Mints[25], Godelieve Verbist[26], Debby Thomas[27], Ellen Gommé[27], Els Hermans[27], Philippe Moerman[28], Tjalling Bosse[6], and Frédéric Amant[1,29]

Abstract

Purpose: Uterine sarcomas are rare and heterogeneous tumors characterized by an aggressive clinical behavior. Their high rates of recurrence and mortality point to the urgent need for novel targeted therapies and alternative treatment strategies. However, no molecular prognostic or predictive biomarkers are available so far to guide choice and modality of treatment.

Experimental Design: We investigated the expression of several druggable targets (phospho-S6^{S240} ribosomal protein, PTEN, PDGFR-α, ERBB2, and EGFR) in a large cohort of human uterine sarcoma samples (288), including leiomyosarcomas, low-grade and high-grade endometrial stromal sarcomas, undifferentiated uterine sarcomas, and adenosarcomas, together with 15 smooth muscle tumors of uncertain malignant potential (STUMP), 52 benign uterine stromal tumors, and 41 normal uterine tissues. The potential therapeutic value of the most promising target, p-S6^{S240}, was tested in patient-derived xenograft (PDX) leiomyosarcoma models.

Results: In uterine sarcomas and STUMPs, S6^{S240} phosphorylation (reflecting mTOR pathway activation) was associated with higher grade ($P = 0.001$) and recurrence ($P = 0.019$), as shown by logistic regression. In addition, p-S6^{S240} correlated with shorter progression-free survival ($P = 0.034$). Treatment with a dual PI3K/mTOR inhibitor significantly reduced tumor growth in 4 of 5 leiomyosarcoma PDX models (with tumor shrinkage in 2 models). Remarkably, the 4 responding models showed basal p-S6^{S240} expression, whereas the nonresponding model was scored as negative, suggesting a role for p-S6^{S240} in response prediction to PI3K/mTOR inhibition.

Conclusions: Dual PI3K/mTOR inhibition represents an effective therapeutic strategy in uterine leiomyosarcoma, and p-S6^{S240} expression is a potential predictive biomarker for response to treatment. *Clin Cancer Res; 23(5); 1274–85. ©2017 AACR.*

[1]Department of Oncology, Gynecologic Oncology, KU Leuven (University of Leuven), Leuven, Belgium. [2]Department of Oncology, Laboratory of Tumor Immunology and Immunotherapy, ImmunOvar Research Group, KU Leuven (University of Leuven), Leuven, Belgium. [3]Department of Gynaecology and Obstetrics, Leuven Cancer Institute, UZ Leuven, Leuven, Belgium. [4]Department of Gynecology and Obstetrics, Haukeland University Hospital, Bergen, Norway. [5]Department of Clinical Science, Centre for Cancer Biomarkers, University of Bergen, Bergen, Norway. [6]Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands. [7]Biomedical Research Group in Gynecology, Vall d'Hebron Institute of Research (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain. [8]Department of Pathology and Molecular Genetics/Oncologic Pathology Group, Arnau de Vilanova University Hospital, University of Lleida, IRB Lleida, Lleida, Spain. [9]Department of Pathology, Vall d'Hebron University Hospital, Barcelona, Spain. [10]Department of Pathology, GROW - School for Oncology & Developmental Biology, Maastricht University Medical Centre, the Netherlands. [11]Department of Gynecology and Obstetrics, GROW - School for Oncology & Developmental Biology, Maastricht University Medical Centre, Maastricht, the Netherlands. [12]Laboratory for Pathology Oost Nederland, Hengelo, The Netherlands. [13]Department of Obstetrics and Gynecology, Gynecological Oncology Center, Charles University in Prague - 1st Faculty of Medicine and General University Hospital in Prague, Czech Republic. [14]Department of Pathology, Charles University in Prague - 1st Faculty of Medicine and General University Hospital in Prague, Prague, Czech Republic. [15]Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland. [16]Department of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland. [17]Institute of Pathology, Medical University of Graz, Graz, Austria. [18]Department of Pathology, Hospital of the Sisters of Charity, Linz, Austria. [19]Department of Gynecologic Oncology, Oncology Center, Provincial Hospitals Gdynia, Gdynia, Poland. [20]Institut de Pathologie et de Génétique, Brussels, Belgium. [21]Department of Gynecological Oncology, UMC Utrecht Cancer Center, Utrecht, the Netherlands. [22]Department of Obstetrics and Gynecology, Radboud University Medical Centre Nijmegen, Nijmegen, the Netherlands. [23]Department of Obstetrics and Gynecology, University of Bonn, Bonn, Germany. [24]Institute of Pathology, University Hospital Cologne, Cologne, Germany. [25]Department of Women's and Children's Health, Karolinska Institutet at Karolinska University Hospital Solna, Stockholm, Sweden. [26]Department of Development and Regeneration, KU Leuven (University of Leuven), Leuven, Belgium. [27]Department of Oncology, Trace Platform, KU Leuven (University of Leuven) - UZ Leuven, Leuven, Belgium. [28]Department of Pathology, UZ Leuven - KU Leuven (University of Leuven), Leuven, Belgium. [29]Centre for Gynecologic Oncology Amsterdam (CGOA), Antoni Van Leeuwenhoek - Netherlands Cancer Institute, Amsterdam, the Netherlands.

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Translational Relevance

Uterine sarcomas are rare aggressive tumors characterized by high mortality rates and limited treatment options. Using an IHC screening approach, we aimed to investigate the expression of potential therapeutic targets in a large cohort of different human uterine sarcomas, encompassing all the main subtypes. We observed that p-S6^{S240}, reflecting mTOR pathway activity, is mainly expressed in high-grade sarcomas and that its presence correlates with shorter progression-free survival in leiomyosarcomas, the largest subgroup. Compounds targeting mTOR have shown limited success in leiomyosarcoma patients in clinical studies so far, possibly due to feedback-loop signaling activation. Here, we tested the efficacy of dual PI3K/mTOR inhibition on five different patient-derived leiomyosarcoma xenograft models. Our results provide preclinical evidence for the efficacy of dual PI3K/mTOR inhibition in uterine leiomyosarcoma patients and suggest that p-S6^{S240} could be considered as a predictive marker for response, opening new perspectives in terms of patients' treatment and stratification strategies.

Introduction

Uterine sarcoma is the general term referring to a heterogeneous group of rare neoplasms with diverse histologic features, that together account for 3,4% of all uterine corpus malignancies (1). Although rare, they entail substantial morbidity and mortality, with frequent recurrences and distant metastases, even after hysterectomy (2). Leiomyosarcoma is the most frequently diagnosed and a very aggressive subtype, accounting for 60% of all uterine sarcomas (1). Low-grade endometrial stromal sarcomas (LGESS) account for 20% of uterine sarcomas, and they usually follow a less aggressive disease course compared with leiomyosarcoma, with a more indolent growth and delayed recurrences (2). The remaining 20% of uterine sarcomas comprise high-grade ESS (HGESS), undifferentiated uterine sarcoma (UUS), and adenosarcomas. Smooth muscle tumors of uncertain malignant potential (STUMP) also arise from the myometrium, and represent a very rare entity that cannot be diagnosed as benign or malignant (3). Uterine sarcoma subtypes with HG histology are generally the most aggressive and are associated with poor prognosis. Adjuvant treatment is decided on the basis of the histologic subtype, but in general is scarce and of limited benefit, underlining the urgent need for new treatment options (2, 4).

During the past decade, our knowledge on the molecular aspects of sarcomas has expanded thanks to the advent of next-generation sequencing methods, which allowed biomarker identification and categorization into molecular and prognostic subgroups (5, 6). However, although efforts have been taken to identify therapeutic targets in sarcomas of the uterus, little consensus has been attained so far on their expression prevalence, mainly because of the limited sized sample sets available, variations in detection protocols, and different cutoffs for positivity. In this study, we present the results of an immunohistochemical screening of relevant targets performed on one of the largest human uterine sarcoma sample sets published so far. Through collaboration within the European Network of Individualized Treatment in Endometrial Cancer (ENITEC), we collected more than 300 human uterine sarcoma samples and corresponding clinical data, being able to perform disease course analysis and investigate correlations between potential targets and clinical parameters. For targets' analysis, we selected phosphorylated S6 ribosomal protein (p-S6^{S240}), the tumor suppressor and PI3K pathway inhibitor PTEN, platelet-derived growth factor receptor-α (PDGFR-α), erb-b2 receptor tyrosine kinase 2 (ERBB2/HER-2), and EGFR. Phosphorylated S6 is an important downstream player in the mTOR pathway, and PTEN inhibits the PI3K pathway upstream. PI3K/mTOR signaling has been implicated in leiomyosarcoma, confirmed by *in vitro* and *in vivo* studies (7, 8). PDGFR, ERBB2, and EGFR all have proven to be valuable targets in other cancer types. PDGFR, for example, is blocked by imatinib in gastrointestinal stromal tumors and dermatofibrosarcoma protuberans (9), whereas ERBB2 overexpression is tackled by the anti-ERBB2 antibodies trastuzumab and pertuzumab in breast cancer (10). Finally, EGFR is targeted by antibodies such as panitumumab in head and neck and colon cancer, and by tyrosine kinase inhibitors gefitinib and erlotinib in non–small cell lung cancer (11). To validate the results of such screening, we preclinically tested the most promising target in an *in vivo* context, using uterine sarcoma patient-derived xenograft (PDX) models. Of note, being established by implanting freshly isolated tumor fragments into immunocompromised mice, PDXs have proven high histologic and molecular similarity to the original tumor (12), together with high predictive value in terms of response to therapy (13).

Materials and Methods

Patient samples

After obtaining approval from the Medical Ethics Committee UZ/KU Leuven and Ethics Boards in collaborating centers, 303 archived formalin-fixed, paraffin-embedded sarcoma samples (6 of which are recurrences of included primary tumors), 52 benign uterine tumors, and 41 normal tissues were collected from 19 European hospitals, 13 of which are associated to ENITEC. A total of 307 unique tumor samples (malignant and benign), along with clinical data, were collected through ENITEC, with the following collaborating centers: UZ Leuven, Belgium ($n = 100$), Vall d'Hebron University Hospital, Barcelona, Spain ($n = 37$), MUMC Maastricht, Maastricht, the Netherlands ($n = 35$), Charles University in Prague—1st Faculty of Medicine, Prague, Czech Republic ($n = 23$), Turku University Hospital, Turku, Finland ($n = 23$), University Hospital Graz, Graz, Austria ($n = 23$), Haukeland University Hospital, Bergen, Norway ($n = 22$), Provincial

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Current address for K. Van de Vijver: Department of Pathology, Netherlands Cancer Institute, Amsterdam, the Netherlands; and current address for E. Wardelmann: Gerhard-Domagk-Institute of Pathology, University Hospital of Muenster, Muenster, Germany.

Corresponding Author: Frédéric Amant, KU Leuven (University of Leuven), University Hospitals Leuven, Herestraat 49, Leuven 3000, Belgium. Phone: 321-634-4273; Fax: 321-634-4629; E-mail: frederic.amant@uzleuven.be

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Hospitals in Gdynia - Oncology Center, Gdynia, Poland ($n = 11$), Radboud UMC, Nijmegen, the Netherlands ($n = 7$), University Hospital Bonn, Bonn, Germany ($n = 7$), UMC Utrecht, Utrecht, the Netherlands ($n = 7$), University Hospitals Köln, Köln, Germany ($n = 6$), and Karolinska University Hospitals, Stockholm, Sweden ($n = 6$). Remaining tumor samples were contributed by MST Enschede (Enschede, the Netherlands), ZGT Almelo and Hengelo and SKB Winterswijk, the Netherlands ($n = 26$), St. Jean, Ste. Anna-St. Remi and St. Etienne, Brussels, Belgium ($n = 11$), Yperman, Ieper, Belgium ($n = 2$), AZ Turnhout, Belgium ($n = 1$), Mariaziekenhuis, Overpelt, Belgium ($n = 1$), and Imelda Hospital, Bonheiden, Belgium ($n = 1$). The sample set included 157 leiomyosarcomas (4 recurrent matching to primary leiomyosarcoma), 68 LGESSs, 26 UUSs, 15 HGESSs (2 recurrent matching to primary LGESS), 17 adenosarcomas, 15 STUMPs, 5 HG uterine sarcomas, not otherwise specified (HG uSAR NOS), 44 leiomyomas, 8 endometrial stromal nodules (ESN), 23 healthy myometrial specimens, and 18 healthy endometrial samples. Of all collected tissue blocks, 6.5% was obtained from surgeries before 2000, 62.5% was obtained between 2000 and 2010, and 31% dated from 2010 or later. Patient follow-up ranged from 1 month to 30 years. In addition to the external classification of tumors, carried out in the center of origin by the local pathologist, all cases were reviewed and reclassified in a blinded manner by the dedicated central pathologist P. Moerman, uterine tumors expert, according to the WHO 2014 classification (14). Cases with discordant diagnoses were excluded, and only cases where concordance was reached by the two independent pathologists were included for the screening. For clinical data collection, the International Federation of Gynecologic Oncology 2009 system was applied for staging of all samples (see Supplementary Table S1 for clinical data and treatment modalities). HG cases were the following: all leiomyosarcoma, HGESS and UUS, HG uSAR NOS, and adenosarcoma with sarcomatous overgrowth. LG cases were all LGESS and LG adenosarcoma.

Immunohistochemical stainings

Paraffin slides (4 μm) were heated for 3 to 4 hours at 55°C, deparaffinized in toluol, and rinsed in ethanol. Tissues were blocked for endogenous peroxidases by 30-minute incubation in 0.5% H_2O_2 (107209, Merck Millipore) in methanol. After washing in TBS, epitopes were retrieved as displayed in Supplementary Table S2, which summarizes details of the IHC methods. Tissues were cooled down slowly in TBS, except after enzymatic retrieval of EGFR, which was stopped by 5-minute incubation in cold (4°C) TBS. Upon extensive washing, tissues were blocked with 1% milk powder, 2% BSA (A4503, Sigma-Aldrich), and 0.1% Tween-80 (822187, Merck Millipore) in TBS before antibody incubation. Blocking solutions were removed and tissues were incubated with antibody solutions in TBS (Supplementary Table S2). The following primary antibodies were used: anti-phospho-S6^{S240} (M7300, Dako), anti-PTEN (clone 6H2.1, M3627, Dako), anti-PDGFR-α (C-20, sc-338, Santa Cruz Biotechnology), anti-ERBB2 (A0485, Dako), and anti-EGFR (clone 31G7, 280005, Zymed). Tissues were washed, blocked for 15 minutes (except for PTEN staining), and incubated with secondary antibodies or, for PTEN, with EnVision-HRP (K4001, Dako). After washing, slides for EGFR and phospho-S6^{S240} stainings were incubated with streptavidin–HRP (P0397, Dako) for 30 minutes and washed again. All antibodies were visualized by 10-minute incubation in 3,3′-diaminobenzidine (DAB, D5905, Sigma) + 0.015% H_2O_2 (107209, Merck Millipore) in the dark. Nuclei were stained with Mayer's hematoxylin, and tissues were dehydrated in propanol, dipped in xylene, and mounted. Positive controls consisted of PDGFR-expressing ovarian carcinoma, PTEN-expressing normal endometrium, ERBB2-expressing breast carcinoma, EGFR-expressing tumor (grown in nude mice) from human HEC cells and S6-expressing endometrial carcinoma with S6 phosphorylation, confirmed by Western blot analysis. To ensure no staining was caused by a specific binding of secondary/tertiary molecules, control slides without addition of primary antibody were used.

Evaluation and scoring of immunohistochemical stainings

All stainings were evaluated semiquantitatively, using a scoring system (Supplementary Table S3) that takes into account both the staining intensity (0 = absent, 1 = weak, 2 = moderate, and 3 = strong) and the percentage of stained cells (0 = absent, 1 = less than 1%, 2 = 1%–10%, 3 = 11%–33%, 4 = 34%–66%, and 5 = 67%–100%; ref. 15). Both scores were added to obtain a maximum score of 8. Stainings were evaluated only in the cellular component where expression was expected. Tissues were considered positive at a cut-off score of 6, corresponding to strong positivity in $\geq$11% of cells, moderate positivity in $\geq$34% of cells, or weak staining in $\geq$67% of cells. This cutoff was deemed clinically relevant for therapeutic applications, as a targeted therapy would most likely be effective when a sufficient number of cells express the target. For ERBB2, this coincides with the generally applied scoring system approved by the FDA (16). Tissues were evaluated by the observer (T. Cuppens) and in randomly selected cases (25%) additionally by a second observer (A. Coosemans). For these specific cases, a concordance of >90% was reached between scorings by the two independent researchers. Photographs of representative cases were taken using the Axioskop microscope (MRc5, Zeiss) and the ZEN 2.0 software.

Establishment and validation of PDX models

Animal experiments were approved by the Animal Ethics Committee of KU Leuven (Leuven, Belgium). Mouse xenograft models were established in collaboration with the Trace Platform (UZ/KU Leuven). Small fragments of tumor tissue ($3 \times 3 \times 3$ mm), obtained during necessary surgery or biopsy upon informed consent, were implanted interscapularly in female NMRI nude mice of minimum 6 weeks old (Taconic) and expanded in several generations.

Treatment of PDX models

Mice were randomized according to tumor volume (when tumor volumes reached 200–250 mm^3) and treated for 19 to 22 days (5–9 mice/group for BEZ235- and placebo-treated groups, 3–7 mice for trabectedin-treated groups). Some mice in the trabectedin groups were excluded due to signs of toxicity around the tail vein. BEZ235 (also known as dactolisib; Novartis, through Selleckchem, S1009) was prepared in 10% N-methyl-2-pyrrolidone (sc-237581, Santa Cruz Biotechnology)/90% polyethylene glycol (90878, Sigma) and administered orally, daily, in a dose of 40 mg/kg. Placebo-treated mice received the same volume of vehicle as the BEZ235-treated group. Trabectedin (Yondelis) was acquired from the UZ Leuven Hospital Pharmacy, aliquoted in DMSO (102952, Merck Millipore), and diluted in saline. It was administered intravenously (0.15 mg/kg; tail vein), once weekly. Tumor volumes were measured with a caliper twice weekly

(calculated using the following formula: length × width × depth × π/6), and mice body weights were monitored. Treatment was discontinued after 3 weeks or when the tumor reached a volume of 2,000 mm^3. After sacrifice, all tumors were stained and scored for p-S6^{S240} level as before. Significant weight loss was defined as a loss of 15% of the body weight recorded at the beginning of the treatment.

Statistical analyses

IBM SPSS Statistics 20 was used for all statistical analyses except for the *in vivo* treatment experiments. Age and tumor size were considered continuous variables, whereas all other variables were categorical. The χ^2 test was used to compare staining results (portion of positive samples) between histologic subgroups. To determine potential associations between stainings and clinical variables (e.g., stage, age, tumor size) for primary versus recurrent tumors and LG versus HG histologies, univariate analyses were first carried out using χ^2 tests for categorical variables. Next, logistic regression was performed including only one variable for continuous and categorical variables, to permit direct comparison with multivariate logistic regression analysis, including all variables that showed a significant correlation in univariate analysis. Univariate survival analyses were carried out using the Kaplan–Meier method/log-rank test. In the *in vivo* treatment experiments, tumor volumes of different treatment groups were compared over time using two-way repeated measures ANOVA in GraphPad.

Results

HG uterine sarcomas are characterized by aggressive clinical behavior and poor prognosis

We collected and analyzed the following patient samples: leiomyosarcoma ($n = 153$), LGESS ($n = 68$), UUS ($n = 26$), HGESS ($n = 13$), STUMP ($n = 15$), adenosarcoma ($n = 17$), and HG uSAR NOS ($n = 5$), which could not be categorized in any conventional tumor group. Leiomyosarcoma, HGESS, UUS, and HG uSAR NOS are HG tumors. Of 17 adenosarcoma patients, 4 were diagnosed with an HG variant (with sarcomatous overgrowth). The remaining adenosarcoma were considered LG, as well as the LGESS. No grade was assigned to STUMP cases. The most important clinical data summarized per histologic subtype are shown in Supplementary Table S1. Information on disease-specific survival (DSS) and progression-free survival (PFS) was available for 242 and 210 patients, respectively. First, we pooled all patients with HG and LG tumors and compared their survival, confirming that HG tumors are clinically more aggressive (Supplementary Fig. S1A and S1B, both $P < 0.001$). For LG patients, the 5-year DSS and PFS rates were 86% and 64%, respectively, whereas for HG patients, after 5 years only 33% were alive and 18% showed no progression. Next, we determined survival rates for all separate subtypes (Supplementary Fig. S1C and S1D). The 5-year DSS rate was 0% to 22% in UUS patients, around 30% in HGESS patients, and 35% in leiomyosarcoma patients, contrasting with 85% in LGESS patients. Concurrently, the 5-year PFS rate was 0% to 10% in UUS patients, 0% to 29% in HGESS patients, 18% in leiomyosarcoma patients, and 63% in LGESS patients. Of note, patients diagnosed with STUMP had a significantly better PFS compared with patients diagnosed with leiomyosarcoma (median PFS = 41 months in STUMP and 17 months in leiomyosarcoma, $P = 0.023$), although the difference in DSS was not significant (median DSS = 52 months in STUMP and 35 months in leiomyosarcoma, $P = 0.086$), probably due to the low number of STUMP patients. For adenosarcoma patients, the 5-year survival estimation was not feasible due to the smaller sample set, with fewer events. The 3 HG adenosarcoma patients with available follow-up data died of disease within 26 months (100%). Of the patients with LG adenosarcoma, only 1 of 11 (9%) patients died of disease (after 25 months), and the follow-up time of other patients was between 19 and 119 months.

Overall, patient subgroups with HG tumors are characterized by a substantially worse prognosis, and the largest subgroup of uterine sarcoma presented in our study, as in the general population, is leiomyosarcoma.

The PI3K/mTOR pathway and PDGFR-α are potential targets in different uterine sarcoma subtypes

On the basis of available literature data and therapeutic potential (i.e., for which therapeutic agents are available and active in other cancer types), we investigated in our cohort of human uterine sarcoma samples the expression of the following druggable molecular targets: phospho-S6^{S240}, PTEN, PDGFR-α, ERBB2, and EGFR. Their expression levels were determined in a total of 396 samples, including malignant tumors (leiomyosarcoma, LGESS, HGESS, UUS, adenosarcoma, and HG uSAR NOS), tumors of uncertain malignancy (STUMP), benign tumors (leiomyoma and ESN), and normal myometrium and endometrium. Expression data for the five selected proteins are summarized in Table 1, per histologic subgroup, and for pooled HG and LG samples. Representative images for the stainings and a detailed description of the adopted scoring system are shown in Supplementary Fig. S2 and Supplementary Table S3, respectively. Tissues were considered positive at a score of 6 or higher, corresponding to weak staining in ≥67% of cells, moderate staining in ≥34% of cells, or strong staining in ≥10% of cells. Considering all uterine sarcomas and STUMP cases together, p-S6^{S240} was scored positive in 26% of samples. Loss of PTEN expression was seen in 34% of cases, with up to 50% loss in UUS samples. The most frequently expressed protein was PDGFR-α (82%), while ERBB2 and EGFR were detected in 5% and 9% of cases, respectively. EGFR was almost exclusively detected in the stromal component of adenosarcoma: 31% of LG adenosarcoma and 75% of HG adenosarcoma expressed EGFR. Remarkably, ERBB2 was mainly expressed in the epithelial component of adenosarcoma: 58% of LG adenosarcoma and 100% of HG adenosarcoma showed ERBB2 expression. Although this component is considered benign, it showed more frequent ERBB2 expression compared with normal endometrial epithelial cells ($P = 0.001$ for LG and $P < 0.001$ for HG, as determined by χ^2 test).

Taken together, our data show that PDGFR-α is a potential target in all uterine sarcoma subtypes, PI3K/mTOR targeting is an option in 26% of cases, mainly leiomyosarcoma, HGESS and UUS, and ERBB2/EGFR seem to be targetable in a minority of cases, mostly adenosarcoma. Recently, pazopanib, a multikinase inhibitor also targeting PDGFR, was approved for treatment of leiomyosarcoma after a successful randomized phase III trial (the PALETTE study; ref. 17), confirming the potential predictive value of such a histologic scoring system.

In addition, we assessed cyclin D1 expression and the presence of the t(10;17)(q22;p13) rearrangement, leading to the fusion gene YMHAE/NUTM2A/B, in HGESS and UUS cases because these alterations have been linked to HGESS and as the 14-3-3 oncoprotein, resulting from the translocation, has been suggested

Table 1. Expression of therapeutic targets in uterine sarcomas, benign tumors, and normal tissues

	p-S6^{S240}		PTEN		PDGFR-α		ERBB2		EGFR	
	Prim	**Rec**	**Prim**	**Rec**	**Prim**	**Rec**	**Prim**	**Rec**	**Prim**	**Rec**
All sarcomas + STUMP	60/261 (23%)	15/36 (42%)	160/249 (64%)	27/34 (79%)	219/261 (84%)	25/36 (69%)	9/264 (3%)	5/37 (14%)	22/261 (8%)	5/37 (14%)
Prim + Rec	77/299 (26%)		188/285 (66%)		245/299 (82%)		14/303 (5%)		27/300 (9%)	
Pooled HG	50/177 (28%)	14/26 (54%)	111/173 (64%)	20/24 (83%)	156/177 (88%)	19/25 (76%)	6/179 (3%)	2/26 (8%)	12/177 (7%)	2/26 (8%)
LMS	32/131 (24%)	11/22 (50%)	91/129 (71%)	17/21 (81%)	118/133 (89%)	18/21 (86%)	2/133 (2%)	2/22 (9%)	6/132 (5%)	1/22 (5%)
Prim meta	2/2 (100%)		1/2 (50%)		1/2 (50%)		0/2 (0%)		0/2 (0%)	
HGESS	3/13 (23%)	2/2 (100%)	7/13 (54%)	1/1 (100%)	12/13 (92%)	0/2 (0%)	3/13 (23%)	0/2 (0%)	1/13 (8%)	0/2 (0%)
UUS	14/25 (56%)	0/1 (0%)	11/23 (48%)	1/1 (100%)	19/23 (83%)	0/1 (0%)	0/25 (0%)	0/1 (0%)	1/24 (4%)	0/1 (0%)
HG AS stroma	0/4 (0%)		2/4 (50%)		3/4 (75%)		1/4 (25%)		3/4 (75%)	
HG AS epithelium	0/2 (0%)		1/1 (100%)		1/2 (50%)		2/2 (100%)		0/1 (0%)	
HG uSAR NOS	1/4 (25%)	1/1 (100%)	0/4 (0%)	1/1 (100%)	4/4 (100%)	1/1 (100%)	0/4 (0%)	0/1 (0%)	1/4 (25%)	1/1 (100%)
Pooled LG	6/69 (9%)	1/10 (10%)	37/61 (61%)	7/10 (70%)	54/69 (78%)	6/11 (55%)	3/70 (4%)	3/11 (27%)	9/69 (13%)	3/11 (27%)
LGESS	4/57 (7%)	0/9 (0%)	32/51 (63%)	6/9 (67%)	45/57 (79%)	5/10 (50%)	3/58 (5%)	2/10 (20%)	6/57 (11%)	2/10 (20%)
LG AS stroma	2/12 (17%)	1/1 (100%)	5/10 (50%)	1/1 (100%)	9/12 (75%)	1/1 (100%)	0/12 (0%)	1/1 (100%)	3/12 (25%)	1/1 (100%)
LG AS epithelium	1/11 (9%)		3/10 (30%)		10/12 (83%)		7/12 (58%)		1/12 (8%)	
STUMP	4/15 (27%)		12/15 (80%)		9/15 (60%)		0/15 (0%)		1/15 (7%)	
Benign tumors										
Leiomyoma	1/43 (2%)		18/26 (69%)		10/26 (43%)		0/42 (0%)		0/44 (0%)	
Endometrial stromal nodule	0/8 (0%)		2/6 (33%)		3/8 (38%)		0/8 (0%)		0/8 (0%)	
Normal tissues										
Myometrium	1/21 (5%)		9/16 (56%)		4/23 (17%)		0/23 (0%)		0/23 (0%)	
Endometrium stroma	1/17 (6%)		4/12 (33%)		11/16 (69%)		0/18 (0%)		2/18 (11%)	
Endometrium epithelium	4/17 (24%)		4/12 (33%)		11/16 (69%)		1/18 (6%)		0/17 (0%)	

NOTE: Displayed are numbers and proportions (%) of positive cases. The two primary metastatic leiomyosarcoma cases are excluded from the pooled analyses that are divided according to primary or recurrent tumors. Epithelial components of adenosarcoma cases are not considered as separate samples and are therefore not included in the pooled samples. STUMP cases do not have a grading system and are displayed as a separate category.

Abbreviations: AS, adenosarcoma; LMS, leiomyosarcoma; Prim, primary; Prim meta, primary metastasis; Rec, recurrent.

Table 2. Logistic regression: predictors of HG versus LG histology

Variable	N	Univariate OR (95% CI)	P	Multivariate OR (95% CI)	P
p-S6^{S240}					
Negative	120	1			
Positive	44	5.385 (1.803–16.082)	0.003	7.242 (2.294–22.866)	**0.001**
Tumor size	164	1.176 (1.076–1.286)	<0.001	1.158 (1.056–1.270)	**0.002**
Age	164	1.034 (1.008–1.061)	0.010	1.027 (0.998–1.057)	0.064

NOTE: Logistic regression with "LG histology" as a reference. OR > 1 and $P < 0.05$ indicate a statistically significant correlation of the variable with HG histology. *P*-values in bold indicate a significant effect in multivariate analysis.
Abbreviations: CI, confidence interval; OR, odds ratio.

as a therapeutic target (18–20). We confirmed that cyclin D1 was expressed more in HGESS (7/15; 47%) than in UUS (4/25; 16%), as shown by the χ^2 test ($P = 0.035$). Likewise, previous studies have reported 8 of 14 and 7 of 18 cyclin D1–positive HGESS cases (18, 21). Of 12 interpretable HGESS and 19 UUS cases, only 2 HGESS cases showed the t(10;17) translocation (one was confirmed by RT-PCR; the other case had no available RNA) and both had very strong (>90% positive nuclei) cyclin D1 staining, confirming the findings by Lee and colleagues (ref. 22; see Supplementary Methods and Supplementary Fig. S3). Our results are in line with previous studies that detected the translocation with FISH, where 4 of 14 and 4 of 16 cases were positive (18, 21). Although the portion of translocation-positive cases is higher in other studies (7/12 and 5/8), this may be explained by variability between methods, as exemplified by Micci and colleagues (20, 23). Cyclin D1 expression did not correlate with DSS, PFS, or any of the five investigated proteins.

P-S6^{S240} expression correlates with recurrent and HG tumors and with shorter PFS

To identify links between protein expression and tumor characteristics, we checked for correlations with tumor grade and primary versus recurrent tumors. Remarkably, p-S6^{S240} was observed more frequently in HG tumors (66/205; 32%) than in LG tumors (7/79; 9%; $P = 0.004$) and was also detected more frequently in recurrent tumors (15/36; 42%) than in primary tumors (60/261; 23%; $P = 0.016$), as calculated by χ^2 test. Also, ERBB2 was expressed more frequently in recurrent tumors (5/37; 14%) than in primary tumors (9/264; 3%; $P = 0.006$). Subsequently, logistic regression analyses (correcting for other factors correlated with grade and recurrence) showed that p-S6^{S240} was independently associated with both histologic aggressiveness ($P = 0.001$; Table 2) and recurrence ($P = 0.019$; Table 3), whereas ERBB2 was associated only with recurrence ($P = 0.011$). Together, these findings suggest that mTOR pathway activation may be associated with disease progression in uterine sarcomas. Because leiomyosarcomas represent the largest uterine sarcoma subgroup, and are generally HG, we further focused our analyses on this subgroup. In leiomyosarcoma, phosphorylation of S6^{S240} was detected in 29% of cases, significantly more frequently than in LM ($P < 0.001$; χ^2 test) and healthy myometrium ($P = 0.018$). P-S6^{S240} was the only variable that was more often detected in recurrent leiomyosarcoma (11/22; 50%) than in primary leiomyosarcoma (32/131; 24%; $P = 0.014$; χ^2 test); hence, multivariate analysis was irrelevant. Of note, two primary metastatic leiomyosarcomas were included in the analysis and both showed p-S6^{S240} positivity. To assess the potential prognostic value of the investigated proteins, we carried out survival analyses in uterine sarcoma subgroups. Interestingly, p-S6^{S240} positivity correlated with shorter PFS in leiomyosarcoma patients ($P = 0.034$) and showed a trend toward shorter DSS in univariate analysis (Fig. 1A and B). Loss of PTEN, which negatively regulates PI3K signaling, correlated with shorter DSS ($P = 0.039$) in leiomyosarcoma patients, but not with PFS (Fig. 1C and D). Multivariate analysis was not feasible due to the small sample size obtained after filtering out cases with missing data.

Taken together, our data suggest that p-S6^{S240} correlates with HG and recurrent uterine sarcomas, an observation that was also confirmed in leiomyosarcoma cases, the largest uterine sarcoma subgroup. In addition, p-S6^{S240} and PTEN may have prognostic value in leiomyosarcoma patients.

Dual inhibition of mTOR and PI3K reduces tumor growth in p-S6^{S240}–positive leiomyosarcoma PDX models

The finding that p-S6^{S240} positivity is correlated with HG and recurrent uterine sarcomas suggests that mTOR pathway activation may play a central role in uterine sarcoma progression. To validate this observation, we decided to test the efficacy of mTOR pathway inhibition in clinically relevant PDX models of uterine leiomyosarcoma. Despite previous clinical trials with mTOR-targeting agents for treatment of leiomyosarcoma patients, so far, none of the tested compounds (e.g., ridaforolimus, temsirolimus) have been approved for leiomyosarcoma by the FDA (7). It has been suggested that the lack of clinical effect could be due to the feedback activation of AKT as a consequence of mTOR complex 1 (mTORC1) inhibition, which can sustain tumor growth through mTOR complex 2 (mTORC2) signaling (24, 25). For this reason, we selected a dual PI3K/mTOR inhibitor, BEZ235, also able to block mTORC2. Five PDX models were derived from uterine leiomyosarcoma of different patients, from which the clinical

Table 3. Logistic regression: predictors of recurrent versus primary tumor samples

Variable	N	Univariate OR (95% CI)	P	Multivariate OR (95% CI)	P
p-S6^{S240}					
Negative	222	1			
Positive	75	2.393 (1.162–4.929)	0.018	2.408 (1.156–5.016)	**0.019**
ERBB2					
Negative	283	1			
Positive	14	4.516 (1.423–14.336)	0.011	4.567 (1.406–14.827)	**0.011**

NOTE: Logistic regression with "primary tumors" as a reference. OR > 1 and $P < 0.05$ indicate a statistically significant correlation of the variable with recurrent samples. *P*-values in bold indicate a significant effect in multivariate analysis.
Abbreviations: CI, confidence interval; OR, odds ratio.

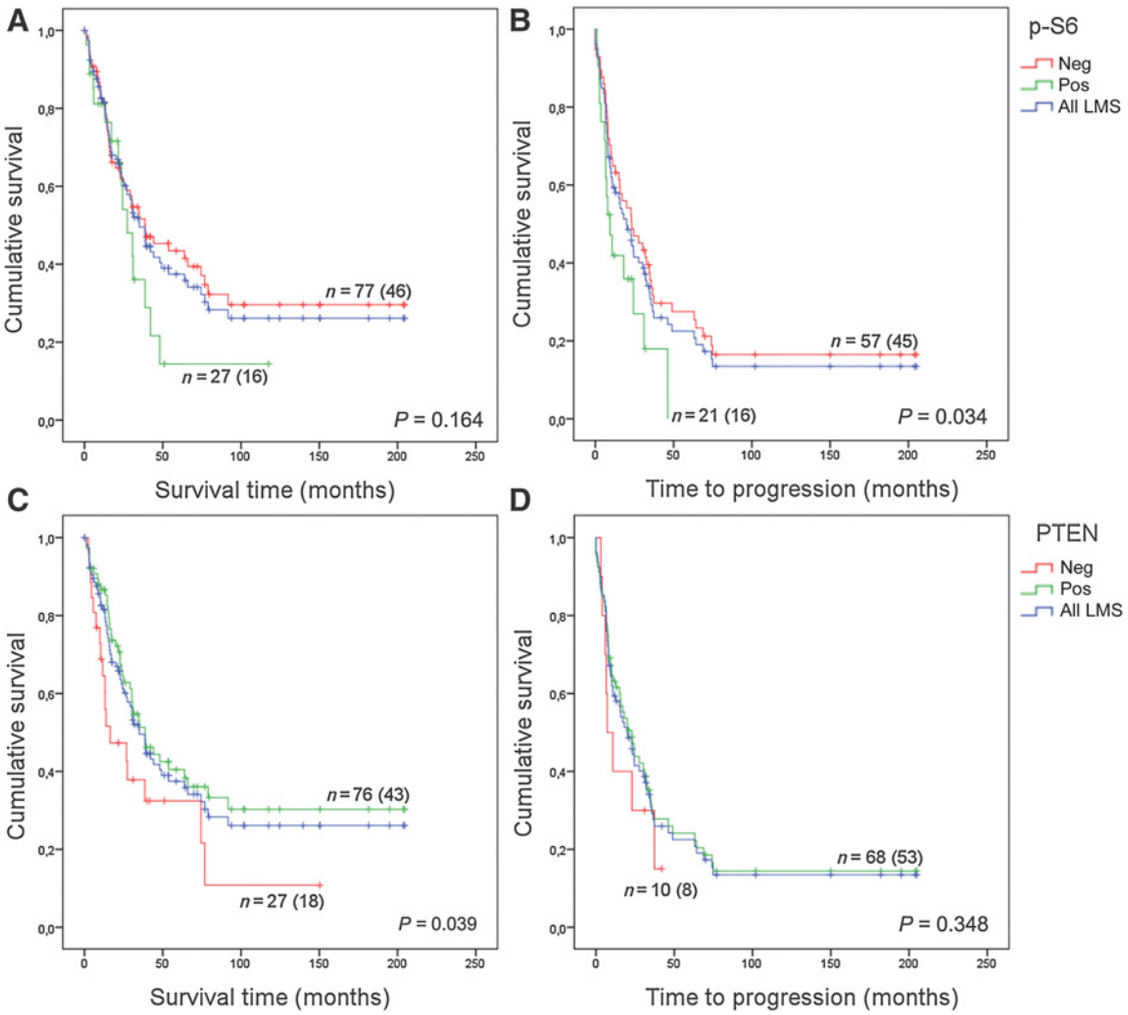

Figure 1.

Survival of leiomyosarcoma patients according to p-S6^{S240} and PTEN expression. **A–D,** Kaplan–Meier survival curves showing DSS (**A** and **C**) and PFS (**B** and **D**) of leiomyosarcoma patients. **A** and **B,** The log-rank test with corresponding *P* values applies to the p-S6^{S240}–negative (red) and p-S6^{S240}–positive (green) curves. Blue curves (all leiomyosarcomas) are depicted as comparison. The number of patients in the analyses is indicated next to the curve with number of events between brackets. **C** and **D,** The log-rank test applies to the PTEN-negative (red) and PTEN-positive (green) curves.

characteristics are shown in Supplementary Table S4. Each model was treated for 3 weeks with BEZ235, placebo, and trabectedin (Yondelis), an alkylating chemotherapeutic agent approved for leiomyosarcoma treatment after failure of anthracyclines. We chose trabectedin as a chemotherapy control as it is the youngest, most recently approved chemotherapy. Its antiproliferative properties rely on multiple mechanisms, including the inhibition of transactivated transcription and the interaction with DNA repair proteins (26). Of five treated leiomyosarcoma models, four showed response to dual PI3K/mTOR inhibition (Fig. 2). Whereas the tumor volume was stabilized in EMC029, tumor growth was slowed down in EMC050. Furthermore, tumor shrinkage was observed in EMC036 (21% reduction, compared with placebo) and EMC041 (35% reduction, compared with placebo). No response to BEZ235 was noted in EMC031, a recurrent, pretreated leiomyosarcoma. Response to trabectedin was noted in four models, while EMC029 showed a trend (nonsignificant) toward response after 8 days. No mice in any arms of the treatment experiments showed significant weight loss (data not shown).

Interestingly, the four responding models showed in their placebo-treated tumors expression of p-S6^{S240}, with mean scores between 6.3 and 7.8 (see Table 4 for mean scores; representative images are shown in Fig. 2), whereas all BEZ235-treated tumors were scored as negative. In the nonresponding model (EMC031), p-S6^{S240} staining in placebo-treated tumors was scored as negative, with a mean score of 5.1. These findings suggest that p-S6^{S240} expression can be used to predict response to PI3K/mTOR blockage in leiomyosarcoma.

To extend our testing of dual PI3K/mTOR inhibition beyond BEZ235, EMC041 was additionally treated with a combination of the mTORC1/2 inhibitor TAK-228, also known as sapanisertib, and the PI3Kα inhibitor alpelisib. The combination of TAK-228 and alpelisib was as effective as BEZ235 in inhibiting tumor growth (no significant difference between both treatment groups), supporting

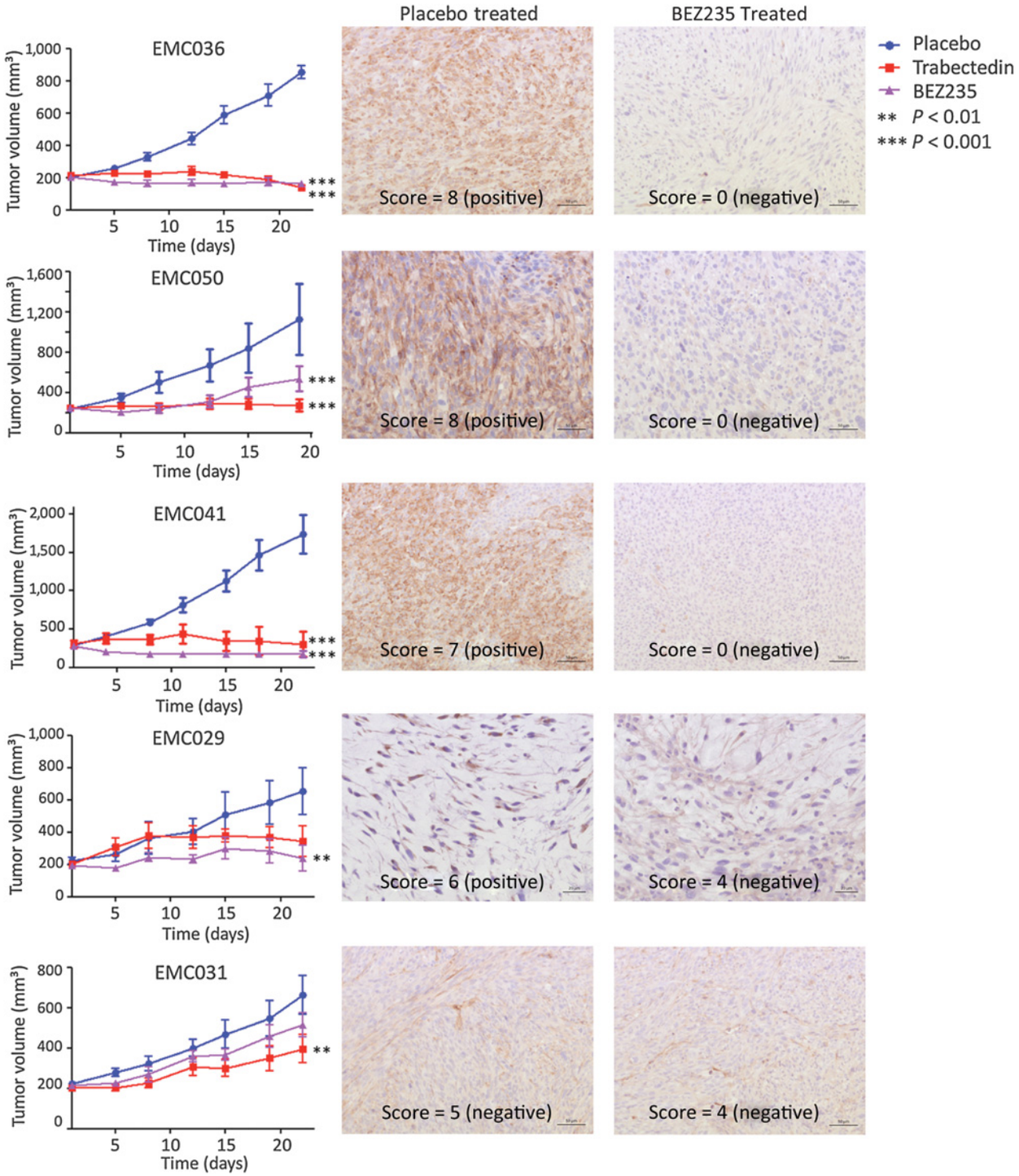

Figure 2.

In vivo dual inhibition of mTOR and PI3K by BEZ235 in uterine leiomyosarcoma PDX models. Mice were treated with BEZ235, trabectedin (as a chemotherapy control), or placebo. Tumor volumes were measured twice weekly, and growth curves of treated mice were compared with placebo-treated mice using two-way repeated measures ANOVA. Data points and error bars represent mean values and SEM. Significant effects (compared with placebo) are indicated with ** and ***. Tumor growth curves are depicted with p-S6^{S240} stainings and scores of representative tumors of each model (left, placebo-treated tumor; right, BEZ235-treated tumor). Pictures were taken at ×20 magnification (scale bar, 50 μm) and at ×40 magnification for EMC029 (scale bar, 20 μm). A larger magnification was used for EMC029 to increase visibility as the cells show a small amount of cytoplasm. Numbers of mice for placebo, trabectedin, and BEZ235-treated groups are respectively: EMC036: $n = 6, 6, 5$; EMC050: $n = 6, 7, 6$; EMC041: $n = 6, 3, 6$; EMC029: $n = 5, 4, 5$; EMC031: $n = 7, 7, 9$.

Table 4. Response of PDX models to BEZ235 with p-S6^{S240} scores

Model	Response to BEZ235	p-S6^{S240} mean score placebo-treated tumors	p-S6^{S240} status placebo-treated tumors
EMC036	Decrease in tumor volume	7.8	Positive
EMC050	Decrease in tumor growth	7.7	Positive
EMC041	Decrease in tumor volume	7.0	Positive
EMC029	Stable tumor volume	6.3	Positive
EMC031	No response	5.1	Negative

NOTE: Five PDX models were treated with BEZ235, trabectedin, and placebo. Placebo-treated tumors were scored for p-S6^{S240} level. For each model, the mean scores of all placebo-treated tumors are depicted.

in general our approach of dual PI3K/mTOR inhibition in leiomyosarcoma (Supplementary Methods; Supplementary Fig. S4).

Thus, four of five uterine leiomyosarcoma models, which were p-S6^{S240} positive, responded to dual PI3K/mTOR inhibition, which can represent a new therapeutic option for leiomyosarcoma patients with p-S6^{S240}–positive tumors.

Discussion

We analyzed a large cohort of samples from uterine sarcoma patients for the expression of selected druggable therapeutic targets, to determine the subgroups for which specific targeted agents would be the most potentially effective.

Here, we show that PDGFR-α is expressed in the majority of samples, in all sarcoma subtypes. Importantly, after initiation of this study, pazopanib, targeting PDGFR, KIT, FGFR, and VEGFR, was approved for the treatment of leiomyosarcoma patients after a successful placebo-controlled phase III trial (17). Another recent phase II trial showed the addition of PDGFR-α inhibitor olaratumab to doxorubicin is beneficial in soft tissue sarcoma patients (including leiomyosarcoma; ref. 27). Our results confirm that PDGFR-α is frequently expressed in uterine leiomyosarcoma, but also other uterine sarcoma types show expression in at least 75% of cases, suggesting that pazopanib/olaratumab should also be tested in other uterine sarcoma subtypes. Of note, 2 LGESS patients have been reported to show response to imatinib in case reports, encouraging further studies (28, 29). Although one case expressed KIT (PDGFR status unknown), the other case showed no KIT expression or activating mutation, but was strongly positive for PDGFR, suggesting imatinib acted through PDGFR in the latter case. Indeed, because KIT is not mutated in uterine sarcomas (6), imatinib may exert its effect by PDGFR blocking in uterine sarcomas (9).

ERBB2 and EGFR, although being important targets in other cancer types, have not been studied frequently in uterine sarcomas (7). An exception is the study by Movva and colleagues (6), describing that ERBB2 is rarely overexpressed in leiomyosarcoma and ESS. In our sample set, ERBB2 and EGFR were rarely detected, except in adenosarcoma. ERBB2 was expressed in the epithelial component in 58% of LG adenosarcoma and in 100% of HG adenosarcoma cases. Contrarily, EGFR expression in adenosarcoma was seen in a minority of epithelial cells, whereas it was expressed in the stromal component in 31% of LG adenosarcoma and in 75% of HG adenosarcoma cases. This stromal–epithelial distribution of EGFR and ERBB2 in adenosarcoma is in line with their expression pattern in carcinosarcomas (30–32). Only two other studies reported on the expression of EGFR (2/6 positive cases) and ERBB2 (0/6 and 0/10 positive cases) in adenosarcoma, but without evaluating the epithelial component (30, 32). In addition, we show that in uterine sarcomas, ERBB2 is more frequently detected in recurrent samples than in primary tumors, suggesting that ERBB2 may play a role in sarcoma progression.

The PI3K/mTOR pathway has been implicated in the pathogenesis of leiomyosarcoma, and preclinical studies have shown effect of mTOR-targeting agents (7, 8). A negative regulator of PI3K/mTOR signaling, PTEN, is frequently deleted in leiomyosarcoma (6, 33). In our cohort, absence or low expression of PTEN was noted in 28% of leiomyosarcoma samples. This is concordant with earlier findings, showing decreased expression of PTEN in 20% to 38% of leiomyosarcoma cases (6, 34). Another study reported PTEN loss by IHC in only 7% of uterine leiomyosarcoma (35). This discrepancy is likely due to the use of different scoring systems. In leiomyosarcoma patients, we showed that PTEN loss correlates with shorter DSS. PTEN loss has been shown previously to have prognostic value in other gynecologic cancer types (36, 37). Next to its prognostic role, loss of PTEN may also guide therapy decisions. Indeed, PTEN-deficient tumors may be more sensitive to PARP inhibitors, due to PTEN's role in genomic integrity, with PTEN loss leading to defects in homologous recombination (38).

Downstream to mTOR signaling, S6 kinases (S6K) are activated through phosphorylation. A well-known target of S6K is the S6 ribosomal protein, a component of the 40S ribosomal protein. Here, we used the phosphorylated form of S6 as a read-out for S6K activity, and thus mTOR pathway activation (39). The S6 protein can be phosphorylated at serines 235/236 and 240/244. Pende and colleagues (40) have described phosphorylation at S235/236 even when mTOR-activated kinases S6K1 and 2 are knocked out. In this situation, phosphorylation at S240/244 was obliterated, suggesting that mTOR-activated S6K1/2s are the only kinases responsible for phosphorylation at serines 240/244 in the S6 protein (40). Therefore, we chose to detect S6 phosphorylation at serine 240 using a phospho-site–specific antibody. In our dataset, 29% of uterine leiomyosarcoma samples showed p-S6^{S240} positivity, significantly more than in benign lesions and normal tissue. Similarly, Brewer Savannah and colleagues (35) reported 24% of uterine leiomyosarcoma to be strongly positive, and Hernando and colleagues (41) found 44% of soft tissue leiomyosarcoma samples to be p-S6^{S240} positive. Setsu and colleagues (34) found 74,5% of soft tissue leiomyosarcoma samples to be p-S6$^{S235/236}$ positive. However, the latter report did not include uterine lesions and used a lower cutoff for positivity.

In our study, p-S6^{S240} staining was observed more in HG and recurrent tumors, suggesting that S6 phosphorylation might be an event linked to disease progression. This finding is in line with the previous report of Brewer Savannah and colleagues (35), who observed higher levels of p-S6$^{S235/236}$ in recurrent and metastatic uterine leiomyosarcoma lesions. We are the first to report this finding in a large cohort of 153 uterine leiomyosarcoma patients. Furthermore, we show that p-S6^{S240} positivity correlates with

shorter PFS in leiomyosarcoma patients; hence, p-S6^{S240} could be a prognostic marker in leiomyosarcoma patients.

MTOR inhibition showed modest effectiveness in preclinical studies and in clinical trials on sarcomas, where leiomyosarcoma patients (origin not specified) showed minor response to ridaforolimus and temsirolimus (7, 42, 43). Taking into account their limited clinical effect, as well as the toxicities, the FDA has not approved mTOR inhibitors for leiomyosarcoma patients so far. This limited efficacy may be partly due to the absence of patient selection, as no predictive markers are currently available. In addition, these compounds only inhibit mTORC1, which may lead to feedback activation of AKT and sustained signaling through mTORC2 (25). New-generation inhibitors targeting also mTORC2, as well as PI3K, have not been tested in gynecologic sarcomas until very recently. SK-LMS-1, a vulvar leiomyosarcoma cell line, has proven to be sensitive to BEZ235, the same dual PI3K/mTOR inhibitor that we tested in our study (44). BEZ235 has also been shown to inhibit the proliferation of pazopanib-resistant retroperitoneal undifferentiated pleomorphic sarcoma (UPS) cells (45). However, in a genetically engineered mouse model of UPS, BEZ235 inhibited tumor growth in only 3 of 9 mice (46). BEZ235 inhibits various sarcoma cell lines, including rhabdomyosarcoma, Ewing sarcoma, osteosarcoma, and chondrosarcoma cells *in vitro*, although reported *in vivo* models show varying response (47, 48).

In contrast with the cell line–based *in vivo* models, which have been used in most studies on sarcomas, we have chosen to establish PDX models, which better represent the original tumor characteristics (13). Here, we show a strong response of uterine leiomyosarcoma PDX models to BEZ235. Unfortunately, after initiation of this study, BEZ235 development was discontinued by Novartis, mainly due to toxicity (49). BEZ235's clinical toxicity profile was unexpected because no such adverse effects were observed in our preclinical tests or in previous preclinical studies (47, 48). However, our results provide preclinical evidence for the efficacy of dual PI3K/mTOR inhibition in uterine leiomyosarcoma patients, supporting the use of other (less toxic) dual PI3K/mTOR inhibitors like gedatolisib (Pfizer), as well as combinations of PI3K inhibitors (e.g., alpelisib by Novartis) and mTOR inhibitors (e.g., TAK-228 by Takeda). Indeed, we here show that combined administration of mTORC1/2 inhibitor TAK-228 and PI3Kα inhibitor alpelisib results in an equal tumor inhibition as obtained by BEZ235, supporting our approach of dual PI3K/mTOR targeting in leiomyosarcoma. Intriguingly, models showing p-S6^{S240} expression responded better to PI3K/mTOR targeting, suggesting that p-S6^{S240} could be used as a predictive marker for response to PI3K/mTOR–directed agents. Iwenofu and colleagues (50) have previously suggested a role for p-S6$^{S235/236}$ in response prediction to ridaforolimus in sarcoma patients; however, no uterine sarcomas were included in their study (50). Taken together, our findings suggest that dual PI3K/mTOR targeting might be an effective strategy in uterine leiomyosarcoma.

In conclusion, the expression of five therapeutically relevant proteins was assessed in all uterine sarcoma subtypes, as well as in benign uterine tumors and normal tissues. In a set of 303 uterine sarcomas, we show that p-S6^{S240} expression identifies sarcomas with a poor prognosis and predicts response to dual PI3K/mTOR inhibition in PDX leiomyosarcoma models.

Disclosure of Potential Conflicts of Interest

Eva Wardelmann has received speakers bureau honoraria from Bayer, Menarini, Nanobiotis, and Novartis and is a consultant/advisory board member for MSD, New Oncology, and Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Cuppens, K. Van de Vijver, H.B. Salvesen, F. Amant
Development of methodology: T. Cuppens, N. ter Haar, G. Verbist, T. Bosse, F. Amant
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Cuppens, A. Coosemans, J. Trovik, N. ter Haar, E. Colas, A. Garcia-Jimenez, K. Van de Vijver, R.P.M. Kruitwagen, Mariël Brinkhuis, M. Zikan, P. Dundr, J. Huvila, O. Carpén, J. Haybaeck, F. Moinfar, H.B. Salvesen, M. Stukan, C. Mestdagh, R.P. Zweemer, L.F. Massuger, M.R. Mallmann, E. Wardelmann, M. Mints, G. Verbist, D. Thomas, E. Hermans, P. Moerman, T. Bosse, F. Amant
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Cuppens, J. Trovik, N. ter Haar, P. Dundr, O. Carpén, J. Haybaeck, F. Moinfar, F. Amant
Writing, review, and/or revision of the manuscript: T. Cuppens, D. Annibali, A. Coosemans, J. Trovik, N. ter Haar, E. Colas, K. Van de Vijver, R.P.M. Kruitwagen, Mariël Brinkhuis, M. Zikan, P. Dundr, J. Huvila, O. Carpén, J. Haybaeck, F. Moinfar, M. Stukan, R.P. Zweemer, L.F. Massuger, M.R. Mallmann, E. Wardelmann, M. Mints, D. Thomas, P. Moerman, T. Bosse, F. Amant
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Cuppens, M. Zikan, J. Haybaeck, M.R. Mallmann, E. Wardelmann, E. Gommé, E. Hermans, T. Bosse, F. Amant
Study supervision: T. Cuppens, K. Van de Vijver, T. Bosse, F. Amant
Other (minor revision): J. Huvila

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