Molecular Biomarkers of Response to Eribulin in Patients with Leiomyosarcoma

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ABSTRACT

Purpose: A randomized phase III study evaluated the efficacy of eribulin versus dacarbazine in patients with advanced liposarcoma and leiomyosarcoma. Improved overall survival (OS) led to approval of eribulin for liposarcoma, but not for leiomyosarcoma.

Experimental Design: We explored the molecular profile of 77 archival leiomyosarcoma samples from this trial to identify potential predictive biomarkers, utilizing low-coverage whole-genome and whole-exome sequencing. Tumor molecular profiles were correlated with clinical data, and disease control was defined as complete/partial response or stable disease (RECIST v1.1).

Results: Overall, 111 focal copy-number alterations were observed in leiomyosarcoma. Gain of chromosome 17q12 was the most common event, present in 43 of 77 cases (56%). In the eribulin-treated group, gains of 4q26, 20p12.2, 13q13.3, 8q22.2, and 8q13.2 and loss of 1q44 had a negative impact on progression-free survival (PFS), while loss of 2p12 correlated with better prognosis. Gains of 4q22.1 and losses of 3q14.2, 2q14.1, and 11q25 had a negative impact on OS in patients with leiomyosarcoma receiving eribulin. The most commonly mutated genes were *TP53* (38%), *MUC16* (32%), and *ATRX* (17%). The presence of *ATRX* mutations had a negative impact on PFS in both treatment arms; however, the correlation with worse OS was observed only in the eribulin-treated patients. *TP53* mutations were associated with longer PFS on eribulin.

Conclusions: Leiomyosarcoma has a complex genetic background, with multiple copy-number alterations and mutations affecting genes implicated in tumorigenesis. We identified several molecular changes with potential impact on survival of patients with leiomyosarcoma when treated with eribulin.

Introduction

Soft-tissue sarcoma (STS) is a group of rare, malignant tumors of mesenchymal origin, with more than 70 histologic subtypes described previously (1). Leiomyosarcoma accounts for approximately 14% of all STS, most commonly originating from the uterus, retroperitoneum, or large blood vessels, demonstrating a smooth muscle phenotype by morphology and IHC. Leiomyosarcoma is a clinically aggressive neoplasm with metastatic rates of 40%–45%, and with median survival from diagnosis of metastasis of around 20 months (2). For the treatment of patients with metastatic leiomyosarcoma, clinicians primarily use systemic anthracycline-based chemotherapy, with a median progression-free survival (PFS) of 5.3 months (range, 0.7–17.2; ref. 2). Multiple other drugs are used in consecutive lines of treatment, but not all of them are approved by regulatory agencies for sarcoma or have been adequately tested for this indication in large randomized trials.

Eribulin mesilate (Eisai) is a synthetic analog of halichondrin B, a natural polyether macrolide from a marine sponge *Halichondria okadai* (3, 4). Eribulin interacts with microtubules, inducing irreversible mitotic arrest (5, 6) and a strong inhibition of the microtubule growth (7). In breast cancer, eribulin also affects the epithelial–mesenchymal transition (EMT) switching back from mesenchymal to epithelial states, thereby decreasing migration and invasiveness (8), and it has an effect on vascular remodeling (9, 10).

A clinical phase II trial (EORTC 62052, clinicaltrials.gov: NCT00413192) demonstrated the efficacy and safety of eribulin in patients with advanced and/or metastatic STS (11). The efficacy signal detected in this study served as the scientific rationale for a randomized phase III trial (Eisai-309, NCT01327885) comparing eribulin with dacarbazine in patients with advanced liposarcoma and leiomyosarcoma. Overall survival (OS), the primary endpoint in this trial, was significantly improved in total study population of patients receiving eribulin (median OS, 13.5 vs. 11.5 months in dacarbazine-treated group), as assessed by RECIST version 1.1 (v1.1). Eribulin received broad approval for all STS in Japan, but because the therapeutic effect was more pronounced in patients with liposarcoma, regulatory agencies in the United States and European Union registered it to treat patients with advanced liposarcoma only, but not leiomyosarcoma (12). Nevertheless, a number of patients with leiomyosarcoma achieved objective responses with eribulin treatment in Eisai-309 trial which prompted us to work on identifying potential biomarkers which could help selecting patients with leiomyosarcoma with tumors sensitive to eribulin.

There is only limited data on potential biomarkers which may be considered to predict the response to eribulin. *In vitro*, EMT pathway expression correlated with eribulin sensitivity in a panel of breast cancer and endometrial cancer cell lines, such as an upregulation of vimentin in eribulin-resistant cell lines (13). Furthermore, reduced expression of tubulins and positive expression of transducin-like

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Translational Relevance

A recent randomized phase III trial (Eisai-309, NCT01327885) compared eribulin with dacarbazine in patients with advanced liposarcoma and leiomyosarcoma. On the basis of these results in the United States and European Union, eribulin received approval to treat patients only with advanced liposarcoma, but not leiomyosarcoma. Nevertheless, a number of patients with leiomyosarcoma achieved objective responses with eribulin treatment in the Eisai-309 trial. Our study identified several molecular alterations that should be further validated as predictive biomarkers, which could help select patients with leiomyosarcoma with tumors sensitive to eribulin.

enhancer of split 3 (TLE3) significantly correlated with eribulin sensitivity (14). In patients with breast cancer treated with eribulin, high levels of tumor-infiltrating lymphocytes were observed in the triple-negative breast cancer (TNBC) subpopulation, which was correlated with longer PFS and OS (15). In sarcoma, our group showed that 26 miRNAs were differentially expressed between responders (patients without progression at 12 weeks as defined by RECIST v1.0) and nonresponders in the earlier phase II EORTC 62052 clinical trial (NCT00413192). The high expression of *hsa-mir-106a*, *hsa-miR-17*, and *hsa-miR-34a* in nonresponders may have increased survival signaling and proliferation rendering these STS more resistant to eribulin (16). Until now no specific copy-number alterations (CNA) or gene mutations were identified as predictive biomarkers for eribulin response.

Leiomyosarcoma is known as a sarcoma with complex genomic alterations, characterized by multiple, nonspecific structural and numerical chromosomal aberrations. Cytogenetic studies have shown that losses of chromosomes 1p12, 2p, 13q, 10q, and 16q as well as gains of 17p, 8q, and 5p are the most frequent changes (17–19). *TP53, ATRX*, and *RB1* are frequently mutated in leiomyosarcoma, and *ATRX* mutations are correlated with poor differentiation of leiomyosarcoma and worse survival outcomes (18–20). Furthermore, the "BRCAness" phenotype, a molecular profile associated with downregulation of *BRCA1/2* expression causing defective DNA repair capacity, was recently postulated as a frequent and potentially actionable feature of leiomyosarcoma (19). "BRCAness" is known to be associated with resistance to other cytotoxic microtubule-stabilizing agents such as taxanes in TNBC (21, 22).

The aim of the current translational study was to explore CNA and somatic mutation profiles as potential predictive biomarkers of response to eribulin in patients with advanced leiomyosarcoma, based on archived tumor samples from patients with leiomyosarcoma collected in the framework of the phase III registration trial of eribulin (Eisai-309, NCT01327885).

Materials and Methods

Patient samples and clinical data

A total of 82 archival, primary, or metastatic leiomyosarcoma samples were available for the biomarker analysis from North American patients participating in Eisai-309 (NCT01327885). Samples were collected prior to study entry as part of the original clinical trial protocol. Patients gave written inform consent and samples were provided by Eisai for the current translational study, which was reviewed and approved by an Institutional Review Board for scientific, legal, and regulatory compliance. The project was conducted in accordance with recognized ethical guidelines. Altogether, material from 80 formalin-fixed, paraffin-embedded blocks contained tumor cells and was subject to DNA extraction. In specimen where the tumor content was <75%, manual macrodissection was performed on the basis of hematoxylin and eosin staining.

Low-coverage whole-genome sequencing

Random DNA libraries were prepared using KAPA library preparation kit (KAPA), which were then sequenced on Illumina HiSeq2500 at low coverage ($\pm$ 0.1×), generating 50 bp reads to evaluate CNA. Raw sequencing reads were mapped to the human reference genome (NCBI37/hg19) using Burrows-Wheeler Aligner (BWA v0.5.8a). CNA profiles with 50 kb bin size were preprocessed using qDNAseq and segmented by ASCAT (23, 24). To determine significant recurrent alterations (losses and gains) within the sample cohort as a whole and in treatment-specific subgroups, a Genomic Identification of Significant Targets in Cancer (GISTIC) was used. It assigned a G-score to each aberration, that considered the amplitude of the aberration as well as the frequency of its occurrence across samples. False discovery rate (FDR) q values were calculated for the aberrant regions, using the Benjamini and Hochberg method to account for multiple testing (25, 26). A region was considered deleted if the logR value was <-0.1 and amplified when the logR was >0.1. Regions with q values below a value of 0.25 was used to select significant CNA. Alterations spanning >70% of a chromosomal arm were defined as whole-arm alterations, while CNA spanning <70% of a chromosomal arm were considered focal CNA.

Whole-exome sequencing

Random whole-genome libraries were enriched for exomic sequences using the Nimblegen SeqCap EZ Human Exome Library v3.0 kit following the manufacturer's instructions. These libraries were then sequenced by a HiSeq2500, generating 2 × 150 bp paired end reads. Raw sequencing reads were then analyzed using in-house pipeline, based on BWA for mapping to the human reference genome, Picard for duplicates removal, Genome Analysis Tool Kit (GATK) for local realignment and recalibration. Substitutions were identified using GATK haplotype caller and small insertions/deletions (indels) were recognized using Dindel (a quality score > 50 and at least 10× coverage). Low-quality mutations were excluded on the basis of mapping quality (quality score >30) and coverage (10×) for calling substitutions. Variants occurring in two (for SNPs) or three (for indels) alternative reads and with allelic frequency of at least 10% were kept for further analysis. Because there were no germline samples available for the analyzed cohort, a stringent filtering strategy based on publicly available databases was applied to limit the common SNPs. Mutations occurring in large databases (ESP, 1 kg, ExAC) with an allelic frequency >0.1% were removed. Mutations occurring in smaller, appropriate databases (bitsTrio, inhouseDB, cg69, GoNL, Illumina) were removed if they occur in > 1 individual. Cancer Consensus Genes (CCG) set were then selected using Catalogue of Somatic Mutations in Cancer databases (COSMIC v89) and analyzed further (27).

DNA sequencing data [low-coverage whole-genome sequencing (lcWGS) and whole-genome sequencing (WES)] have been deposited in the European Genome-Phenome Archive (EGA: https://ega-archive.org/) under the dataset ID EGAS00001005081.

Statistical analysis and clinical correlations

The response to the eribulin or dacarbazine treatment (analytic subcohorts) was evaluated in the clinical trial using RECIST v1.1 as

reported previously (12). Disease control, defined as complete, partial response or stable disease was seen in 43 patients involved in the current exploratory analysis; 34 patients had disease progression as best response. PFS was calculated from randomization to the first documented disease progression or death, and OS was defined from randomization until death or last follow-up date. Fisher exact test or χ^2 test was used to examine whether response groups were significantly enriched for certain alterations. Survival analysis with Kaplan–Meier estimates and comparison with log-rank test were used to assess the correlation between molecular findings and survival, while the prognostic value of CNA regions was evaluated using Cox regression analysis. Statistical analysis was performed using GraphPad Prism v6 and v8 and P values of < 0.05 were considered significant.

Results

Samples from 78 trial participants with leiomyosarcoma were available for analysis, one sample was excluded from the analysis for reasons described below. The patient population in this group included 66 females and 11 males, with an age at study entry ranging from 30 to 76 years (median 56). A total of 39 of these patients were treated with eribulin (with a median PFS and OS being 1.94 and 13.24 months, respectively), and 19 of them obtained disease control while 20 progressed on the treatment. In the dacarbazine treatment arm ($n = 38$ in this exploratory cohort, with a median PFS of 2.63 and OS of 14.48 months), 24 patients achieved disease control and 14 had progressive disease as best response. Basic demographic and clinical data of the analytic cohort are presented in **Fig. 1**.

Copy-number profiling of leiomyosarcoma

Genomic profiles

In all 78 samples subjected to lcWGS, we were able to map at least 5 million reads mapped was obtained, with an average of 12.3 million reads per sample. CNA were identified in all specimens. Examples of leiomyosarcoma genomic profiles are presented in Supplementary Fig. S1. In one of the analyzed samples, a high-level amplification of region 12q13-q21 was detected, with several amplicons, including *MDM2, CDK4*, and *GLI1*, that are specific for liposarcoma (Supplementary Fig. S1C). After review of the tissue diagnosis, this case was reclassified *post hoc* as liposarcoma and had to be excluded from subsequent analysis.

The most significant whole-arm losses affected 16q and 10q, while whole-arm gains were observed in chromosomes 15 and 14 (**Fig. 2A**). A total of 111 significant focal events were detected, of which 55 were gains and 56 were deletions (**Fig. 2B**), the most significant being gains of 17p12 and 17p.11.2 and losses of 13q14.2 and 10q23.1 (**Table 1**; Supplementary Table S1). Furthermore, multiple cancer driver mutations affecting CCG set were affected by CNA in at least 40% of specimens, including genes encoding tumor suppressor genes (*TP53, MAX, FAT1*), DNA repair proteins (*RAD51, BARD1, MSH6, MSH2*), chromatin modifiers (*BRD4*), kinase signaling pathways (*FGFR1, MAP2K4, KRAS*), transcriptional regulators (*FOXO1, CDX2, TFEB*), etc. (Supplementary Table S1).

Chromosome 17p12 gains were present in 43 of 77 samples (55.8%), and involved in a high-level amplification in 26 specimens (**Fig. 1**). This region contains the gene encoding myocardin (*MYOCD*) suggested as a potential driver gene of the 17p12 amplification in leiomyosarcoma (28). Myocardin is a transcriptional cofactor of serum response factor, regulating smooth muscle development and differentiation.

In the eribulin-treated group, the most significant CNA identified in patients with disease control as best response were gains of 17p12, 17p11.2 and losses of 1p36.32, 2p25.3, and 13q21.1, while in samples from patients progressing on treatment, we observed deletions of 13q14.2, 2q37.3, 11q25, and 13q21.2, and gains of 13q34. In the dacarbazine-treated subcohort, samples from patients who achieved disease control revealed gains of 17p11.2 and 17p12, and losses of 13q14.2, 10q23.1, and 8p23.3, while tumors collected from patients progressing on this alkylating agent had frequently gains of 17p12 and 14q24.1 and deletions of 13q14.2, 2q37, and 11q25 (**Fig. 2C–F**; Supplementary Table S2).

We did not observe any correlation between cooccurrence of specific CNA in any of the analyzed (sub)cohorts.

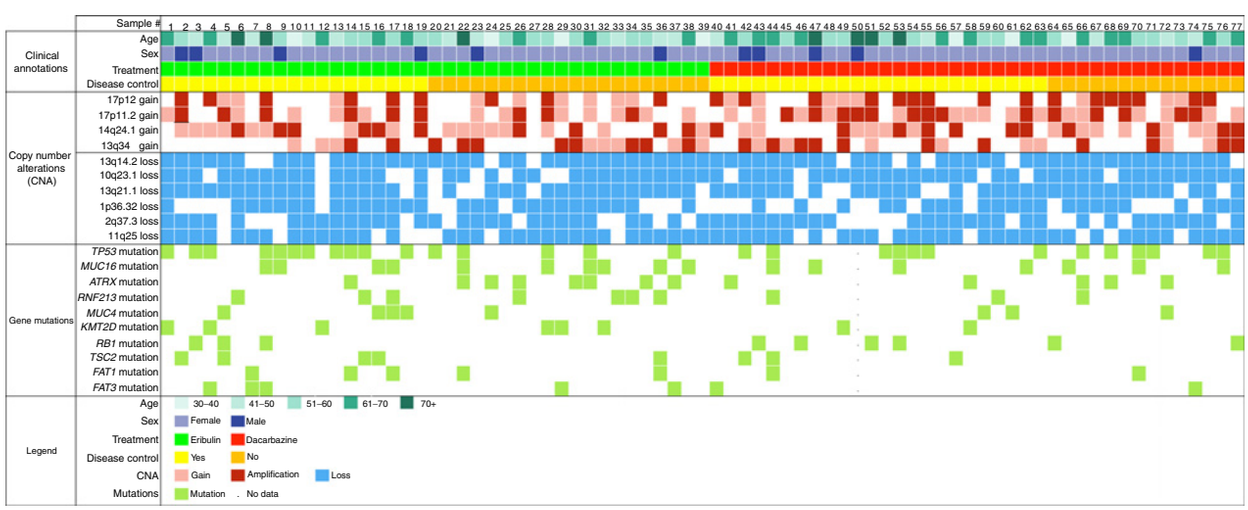

Figure 1.
Comprehensive profile of patient data, most significant CNAs, and most common gene mutations in 77 LMS. Both CNA and mutations are ordered on the basis of their significance, as analyzed with GISTIC.

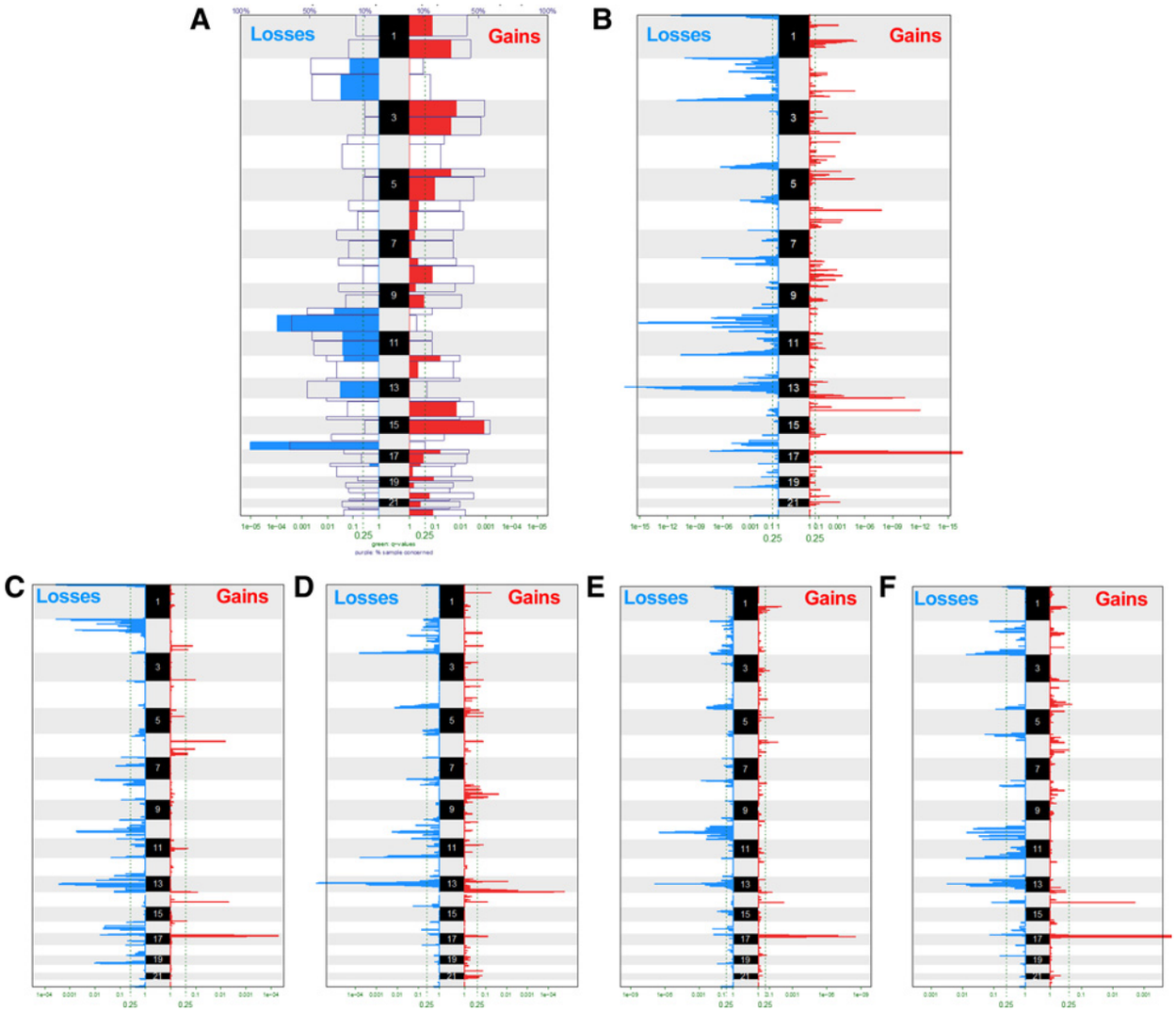

Figure 2.

Significant whole-arm level (**A**) and focal level (**B**) CNAs, detected in 77 leiomyosarcomas analyzed with GISTIC. Focal CNAs in eribulin-treated group, patients with disease control as best response (**C**) and with primary progression on treatment as best response (**D**), and CNA identified in the dacarbazine-treated group, in patients achieving disease control as best response (**E**) or progressing on treatment (**F**). *q* value for gains/losses by chromosome arm (short and long)—scale in green at the bottom of the plot. The threshold is set at *q* value: $-\log_{10}(q\text{-value}) = 0.25$ (i.e., <0.25 is significant)—dashed green line on the plot. Purple empty boxes: the percentage of samples affected by these gains/losses—scale in purple at the top of the plot.

Clinical significance of CNA

Amongst 111 significant focal CNA overrepresented in the 77 samples that were tested, seven regions correlated with PFS in the eribulin-treated subcohort. The most significant gain correlated with inferior PFS was found on chromosomal region 4q26 [$P = 0.009$; HR, 2.19, 97% confidence interval (CI), 1.36–5.24], while gain on 4q22.1 were most significantly associated with worse OS [$P = 0.011$; HR 2.64 (95% CI, 1.27–5.93)]. Among the significant CNA related to the eribulin therapy, 2p12 loss was mainly identified in patients with PFS longer than median (95% vs. 65%; $P = 0.044$), while 13q13.3 gain was observed in cases with shorter PFS (45% vs. 11%; $P = 0.031$). In the dacarbazine subcohort, loss of 19p13.3 correlated with worse PFS [$P = 0.018$; HR, 2.20 (95% CI, 1.24–6.00)], it was more frequently observed in patients with PFS shorter than median (58% vs. 21%; $P = 0.045$). On the other hand, loss of 20p13 was correlated with inferior OS [$P < 0.001$; HR, 2.99 (95% CI, 1.81–8.02)], and was recorded more often in cases with OS shorter than the median (68% vs. 26%; $P = 0.022$). All CNA that were significant for PFS and OS in patients treated with eribulin and dacarbazine are presented in **Table 2**.

WES to generate the mutation landscape of leiomyosarcoma

The average coverage after removing duplicates was 72.7× (range, 16–148). One sample with a coverage < 20× was removed from the analysis. Only 76 samples were considered for further evaluation. On average 93% (60%–97%) of the exome was covered ≥10×, with an average of 266.1 (143–626) nonsynonymous substitutions and 4.7 (0–16) indels per sample. A total of 912 variants affected 415 CCG, with an average of 11.5 (0–28) CCG variants per sample, and 206 mutated CCG were present in at least two samples.

Table 1. The most significant focal events identified in 77 leiomyosarcomas analyzed with GISTIC.

Chromosomal region, CNA	*q* value	Freq. (%)	Genes located in affected chromosomal region
17p12 gain	5.41E-24	55.8	*hsa-mir-744, DNAH9, MAP2K4, ZNF18, ARHGAP44, ELAC2,* ***MYOCD****, FLJ34690, MIR744,*
13q14.2 loss	7.09E-23	88.3	*RCBTB2, MLNR, LPAR6, FNDC3A, CYSLTR2, CDADC1*
17p11.2 gain	1.71E-16	67.5	*ALDH3A1, ALDH3A2, KCNJ12, MFAP4, MAPK7, MAP2K3, RNF112, TMEM11, ULK2, AKAP10, USP22, DHRS7B, B9D1, SLC47A1, SPECC1, SLC47A2, CDRT15L2, C17orf103, FAM27L, FLJ36000, LGALS9B, CCDC144NL, C17orf51, CCDC144C, KRT16P3, SNORA59B, SNORA59A, KCNJ18, MTRNR2L1*
10q23.1 loss	7.56E-16	83.1	*hsa-mir-346, ANXA11, GRID1, MAT1A, RGR, SFTPD, MBL1P, NRG3, LRIT1, GHITM, FAM190B, FAM213A, TSPAN14, C10orf58, DYDC2, CDHR1, DYDC1, EIF5AL1, LOC170425, LOC219347, PLAC9, ZCCHC24, LRIT2, SH2D4B, C10orf99, LOC439990, MIR346, LOC642361, LOC650623, SFTPA1,* ***SFTPA2****, LOC100288974, LOC100507470*
13q21.1 loss	6.01E-14	81.8	*hsa-mir-3169, hsa-mir-1297, hsa-mir-759, PCDH8, OLFM4, SUGT1, LECT1, CKAP2, PCDH17, PCDH20, TDRD3, DIAPH3, PRR20A, HNRNPA1L2, TPTE2P3, OR7E156P, PRR20B, PRR20C, PRR20D, PRR20E, MIR1297, MIR759, MIR3169*
14q24.1 gain	1.06E-12	66.2	***ACTN1****, ZFP36L1, RAD51B, DCAF5*
1p36.32 loss	2.36E-12	64.9	*116 genes, including* ***SKI****, TNFRSF14,* ***TP73***
2q37.3 loss	9.40E-12	71.4	*SEPT2*
11q25 loss	2.78E-11	81.8	*OPCML, IGSF9B, NCAPD3, ACAD8, B3GAT1, THYN1, NTM, JAM3, GLB1L2, VPS26B, GLB1L3, SPATA19, LOC283174, LOC283177, LOC100128239, MIR4697*
13q34 gain	5.11E-11	48.1	***COL4A1****,* ***COL4A2****, ING1, ANKRD10, RAB20, CARKD, CARS2, LINC00346*

Note: Relevant cancer consensus genes (CCG) identified in altered regions are underlined, genes implicated in leiomyosarcoma tumorigenesis and/or in (smooth) muscle development and differentiation are marked in bold.
Abbreviation: Freq., frequency, percentage of cases showing an alteration.

The most commonly mutated genes in the entire study cohort was the tumor suppressor gene *TP53* (in 29/76 samples; 38.2%). Furthermore, we observed that *MUC16*, which encodes the glycosylated protein mucin, was mutated in 24 of 76 specimens (31.6%). Of note, all *MUC16* variants were considered as benign based on cBioPortal (www.cbioportal.org). Other mutated genes encoded components of DNA damage/repair system *ATRX* (13/76 samples; 17.1%), chromatin remodeling/DNA methylation *KMT2D (*9/76; 11.8%), tumor suppressor genes *RB1 (*9/76; 11.8%) and *TSC2* (8/76; 10.5%), as well as *RNF213* (13/76; 17.1%); *MUC4* (12/76; 15.8%); *FAT1* (7/76; 9.2%); and *FAT3* (7/76; 9.2%; **Fig. 1**).

Clinical significance of mutations for treatment response

In the eribulin-treated patient subcohort, the most frequent mutations identified were *TP53* (16/39; 41%), *MUC16* (10/39; 25.6%) and *ATRX* (8/39; 20.5%). The presence of *TP53* mutations had a positive correlation with PFS [$P = 0.037$; HR 0.50 (95% CI, 0.25–0.93)] but no impact on OS (**Fig. 3A** and **B**). Interestingly, a *TP53* mutation was found more often in patients achieving disease control as compared with patients with primary progression, but this observation was not statistically significant, likely due to the small sample size (11/19 vs. 5/20). On the other hand, *ATRX* mutations showed a negative impact on both PFS [$P = 0.042$; HR, 2.23 (95% CI, 1.09–9.84)] and OS [$P < 0.001$; HR, 3.78 (95% CI, 2.79–34.11)] (**Fig. 3C** and **D**). They were also less common in patients obtaining disease control as best response (1/19 vs. 7/20; $P = 0.044$).

In dacarbazine-treated patients, mutations were most commonly found in *TP53* (13/38; 34.2%), *MUC16* (7/38; 18.4%), *RB1* (6/38; 15.8%), and *ATRX* (5/38; 13.2%). Mutations in *MUC16* [$P = 0.032$; HR, 2.44 (95% CI, 1.21–14.29)] and *ATRX* ($P = 0.03$; HR, 2.64 (95% CI, 1.25–20.54)] had a significant negative impact on PFS, but lost their significance on OS (**Fig. 3E** and **F**).

A "BRCAness" phenotype was present in the majority of leiomyosarcoma samples tested, with deleterious aberrations in chromosomal regions where homologous recombination repair (HRR) components are located, including *PTEN* (48%), *BRCA2* (43%), *FANCA* (39%), *ATM* (26%), *CHEK1* (34%), *XRCC1* (18%), and *XRCC3* (12%). Somatic mutations were identified in a limited number of leiomyosarcoma in *PTEN* (6%), *ATM* (6%), *ATR, BRCA1, FANCD2*, and *BAP1* (each 4%). Overall, 74 of 77 (96%) tumor samples presented genetic alterations in at least one HRR component and in half of them the molecular change was observed in more than five genes. The overview of the occurrence of mutations and CNA affecting genes, producing "BRCAness" phenotype in the analyzed cohort is presented in **Fig. 4**.

Discussion

Leiomyosarcoma is a malignant mesenchymal tumor with complex genetic abnormalities, in which surgical resection remains the only curative option in patients with operable disease and conventional chemotherapy and radiotherapy only have a limited therapeutic effect. The recent phase III trial Eisai-309 (NCT01327885) showed that eribulin prolongs OS as compared with the old cytotoxic agent dacarbazine in patients with advanced, metastatic leiomyosarcoma and liposarcoma. In this randomized trial, the treatment effect of eribulin was more pronounced in liposarcoma than in leiomyosarcoma, were the known activity of the comparator drug likely had an impact on the outcome of the survival analysis. Dacarbazine is an active agent in leiomyosarcoma and part of commonly used drug combinations in this sarcoma subtype (gemcitabine/dacarbazine; doxorubicin/ifosfamide/dacarbazine/mesna). A number of patients with leiomyosarcoma in the Eisai-309 trial achieved objective responses with eribulin, but the drug did not gain regulatory approval outside of Japan for patients with this common subtype of STS due to more convincing effects in liposarcoma as compared with dacarbazine.

We were interested to identify potential factors predictive for response to eribulin in leiomyosarcoma, making use of archival tissue samples collected as part of the phase III trial at study entry. Here we present genomic and mutational data to provide a better insight into molecular genetics of leiomyosarcoma and correlation with clinical

Table 2. Clinical significance of focal CNAs on PFS and OS in eribulin- and dacarbazine-treated patients with leiomyosarcoma.

Chromosomal region, CNA	Genes located in affected chromosomal region	*P*	HR (95% CI)
PFS in eribulin-treated subcohort			
4q26 gain	*FABP2, PDE5A, SEC24D, MYOZ2, USP53, METTL14, SYNPO2, FLJ14186, C4orf3, CEP170P1, LOC645513*	0.009	2.19 (1.36–5.24)
2p12 loss	33 genes[a]	0.015	0.42 (0.10–0.75)
20p12.2 gain	*JAG1, MKKS, C20orf94, LOC339593*	0.016	2.08 (1.24–4.65)
13q13.3 gain	75 genes[a], including *BRCA2*, *FOXO1*, *LHFP*, and *NBEA*	0.017	2.16 (1.25–6.24)
8q22.2 gain	45 genes[a], including *COX6C*, *PABPC1*, *UBR5*, and *NACAP1*	0.027	2.23 (1.15–4.34)
8q13.2 gain	*C8orf34, LOC100505718*	0.031	1.95 (1.15–4.16)
1q44 loss	69 genes[a]	0.039	2.00 (1.10–5.99)
OS in eribulin-treated subcohort			
4q22.1 gain	41 genes[a], including *NIN*	0.011	2.64 (1.27–5.93)
3q14.2 loss	*RCBTB2, MLNR, LPAR6, FNDC3A, CYSLTR2, CDADC1*	0.023	2.40 (1.21–8.91)
2q14.1 loss	*DDX18, ACTR3, DPP10, LOC389023, LOC440900, LOC100499194*	0.028	2.44 (1.11–5.00)
11q25 loss	*OPCML, IGSF9B, NCAPD3, ACAD8, B3GAT1, THYN1, NTM, JAM3, GLB1L2, VPS26B, GLB1L3, SPATA19, LOC283174, LOC283177, LOC100128239, MIR4697*	0.047	3.07 (1.04–5.60)
PFS in dacarbazine-treated subcohort			
19p13.3 loss	*hsa-mir-1302–11, HCN2, BSG, CDC34, GZMM, POLRMT, MADCAM1, PPAP2C, SHC2, FGF22, THEG, MIER2, RNF126, OR4F17, TPGS1, C2CD4C, ODF3L2, WASH5P, FLJ45445, FAM138F, FAM138A*	0.018	2.20 (1.24–6.00)
2q12.1 loss	*POU3F3, SLC9A2, IL18RAP, IL18R1, IL1RL1, TGFBRAP1, GPR45, MRPS9, C2orf49, MFSD9, TMEM182, LOC150568, LOC284998, SLC9A4, LOC100287010, LOC100506421, MIR4772*	0.026	0.48 (0.21–0.87)
19q13.43 loss	42 genes[a]	0.027	2.07 (1.16–5.07)
10p15.1 gain	*KLF6, AKR1E2, LOC338588, tAKR, LOC100216001*	0.032	0.489 (0.22–0.90)
18q11.2 gain	36 genes[a]	0.050	0.50 (0.18–0.97)
OS in dacarbazine-treated subcohort			
20p13 loss	*CSNK2A1, SOX12, TCF15, RBCK1, ANGPT4, TRIB3, NRSN2, DEFB126, FAM110A, ZCCHC3, SCRT2, C20orf54, TBC1D20, C20orf96, SRXN1, DEFB127, DEFB129, DEFB125, DEFB128, RSPO4, DEFB132*	<0.001	2.99 (1.81–8.02)
17p13.1 loss	81 genes[a], including *TP53*	0.014	0.43 (0.13–0.75)
3p26.3 loss	*CHL1, CNTN6*	0.033	2.03 (1.11–5.00)
22q13.33 loss	50 genes[a]	0.048	1.96 (1.03–4.20)

Note: Relevant cancer consensus genes (CCG) identified in altered regions are underlined.
[a]Full list of genes located in the described regions is presented in Supplementary Table S1.

data obtained in the prospective clinical study. Of note, we could only focus on material collected from North Americans, even though tumor specimens were collected virtually from all Eisai-309 trial participants.

The most significant CNA observed in leiomyosarcoma samples in the Eisai-309 trial were gains of region 17p12, found in 56% (43/76) of cases and in the majority (26/43) of cases high-level amplifications were found. The 17p12 region contains nine genes, including *MYOCD* and *MAP2K4*. *MYOCD* encodes myocardin, a serum response factor transcriptional cofactor necessary for cardiac and smooth muscle development with important functions in muscle cell differentiation and cell migration (29). *MYOCD* was previously suggested as a target of amplification in leiomyosarcoma, especially in cases with primary retroperitoneal location (28).

In addition, gains of 17p12 and 17p11.2 could lead to the disruption of *MAP2K4* (17p12), *MAPK7* and *MAP2K3* (both 17p11.2), which encode three members of the mitogen-activated protein (MAP) kinase family involved in directing cellular responses, regulating cell proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis, frequently disrupted in cancer (30, 31). A strong association between the expression levels of *MAPK7* and *MAP2K4* genes and clinical parameters of osteosarcoma was identified including a poor response to treatment. Furthermore, silenced *MAPK7* gene was effective at suppressing cell proliferation, inhibiting cell migration, and invasion (31).

On the other hand, recurrent loss of 1p36.32, detected in almost 65% of specimens analyzed, could affect the expression of tumor protein 73 (*TP73*) and *SKI* genes. Inactivation *TP73* tumor suppressor gene, encoding a functional and structural homolog of TP53, was previously observed in leiomyosarcoma (32), but its role in sarcomagenesis has not been elucidated. On the other hand, *SKI* encodes a protein which functions as a repressor of TGFβ signaling, and plays a role in, for example, skeletal muscle differentiation, but not in the determination of cells to the myogenic lineage. In transgenic mice, muscle-specific expression of the *Ski* induces hypertrophy exclusively in a subset of fast muscle fibers (33). Even though SKI is usually upregulated in cancer and its knockdown decreases cell proliferation and migration, for example, in osteosarcoma cells, there are studies showing that *SKI* can be also considered as a tumor suppressor gene (34). For instance, SKI expression had inhibitory effects on the growth, migration, and tumor formation of lung cancer cells *in vitro* and *in vivo*, it also had good effects on the overall survival of patients with breast cancer (35, 36). The potential role of *SKI* in leiomyosarcoma tumorigenesis is unknown and warrants further functional and clinical studies.

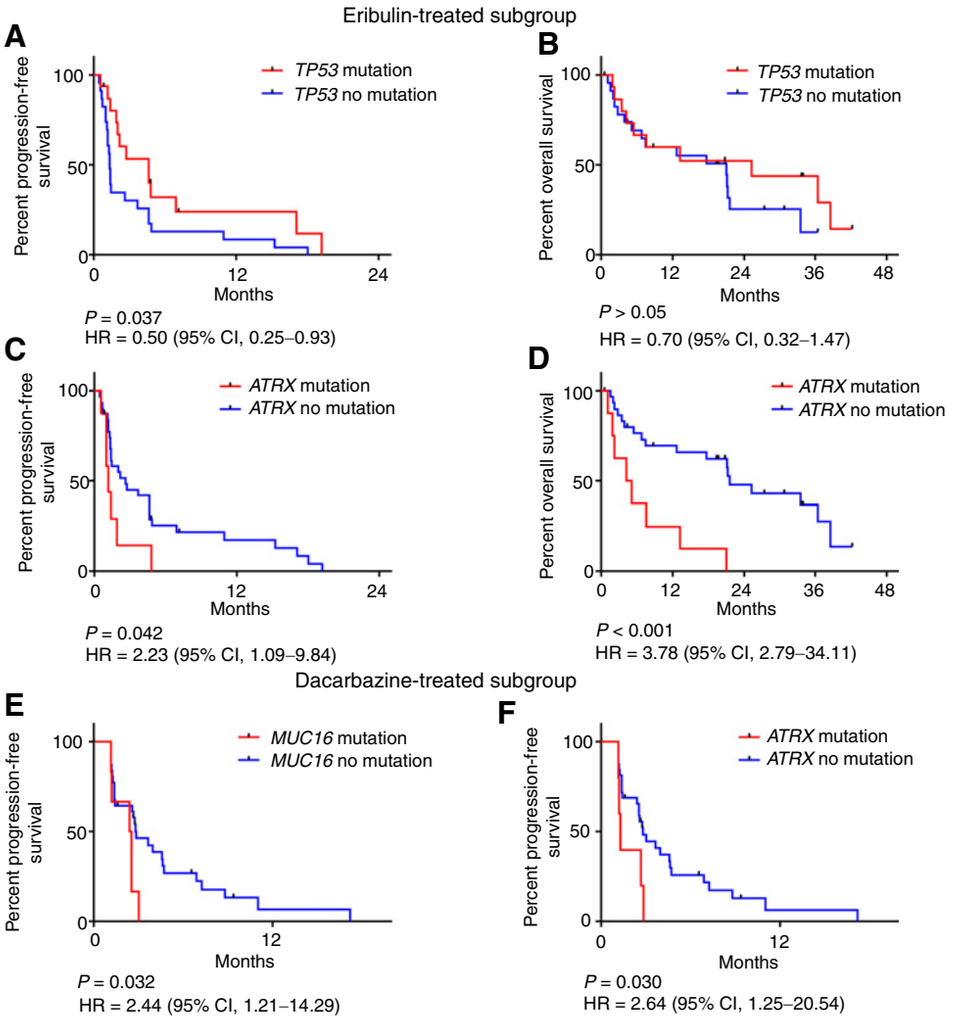

Figure 3.

Kaplan–Meier survival estimates in the eribulin-treated group correlation between the presence of TP53 mutation on PFS (**A**) and OS (**B**), and correlation between the occurrence of ATRX alteration on PFS and OS (**C** and **D**). In a dacarbazine-treated subcohort, the impact of the presence of MUC16 (**E**) and ATRX (**F**) mutations on PFS. HR, hazard ratio; 95% CI, 95% confidence interval.

In our cohort, the three most commonly mutated were *TP53* (38%), *MUC16* (32%), and *ATRX* (17%). These genes were also identified by others among top mutated genes in leiomyosarcoma, utilizing different technological platforms (19, 20, 37–40). Recently Chudasama and colleagues reported that even though the frequency of mutations in *TP53* and *RB1* was observed to be in the range of 50% or lower, inactivation of both tumor suppressors was occurring *via* diverse molecular mechanisms (CNA, indels, chromosomal rearrangements, and microalterations). In that study, biallelic inactivation was present in more than 90% of cases, making it a unifying feature of leiomyosarcoma development (19). We identified *TP53* biallelic inactivation in 23% cases, while an additional 55% of specimens showed either mutation or its *locus* loss *via* CNA. Similarly, *RB1* was inactivated by both mutation and genomic loss in 7% samples, and 43 of tumors revealed either mutation or 13q14 deletion. It is likely that other mechanisms of suppressor inactivation, not evaluated in this study (e.g., gene fusions or intrachromosomal rearrangements), may also be relevant in our cases.

In our leiomyosarcoma series, the presence of *TP53* mutations did not have an impact on patient survival. Interestingly though, in the eribulin-treated subcohort, *TP53* mutation was more frequently found in patients achieving disease control than progressing on therapy, though this was not a significant finding (11/19 vs. 5/20), likely due to a small sample size. Moreover, the presence of *TP53* mutation showed a positive correlation with PFS [$P = 0.036$; HR, 0.51 (95% CI, 0.26–0.93)] but had no impact on OS. It is widely accepted that the occurrence of *TP53* mutation is usually found in more advanced cancers and it is frequently correlated with poor outcome. However, because the wild-type TP53 leads to cell-cycle arrest in the presence of DNA damage, it is possible that mutated TP53 may sensitize tumor cells to DNA-damaging agents. Varna and colleagues showed that in TP53-mutated tumors, chemotherapy induced mitotic catastrophe and tumor death, leading to complete responses (41). Furthermore, an increased sensitivity to paclitaxel in *tp53* knockout mice and in a number of cell lines with altered *TP53* was observed (42, 43), which could explain a positive impact of *TP53* mutation in our study, as eribulin is primarily a tubulin inhibitor.

ATRX gene mutations were the third most common alteration detected in our study group, observed in 17% of tumors, which is comparable with what has been reported by others (19, 38–40). *ATRX* is located on chromosome Xp21.1 and encodes ATRX chromatin remodeling protein, which is responsible for proper chromosomal segregation in mitosis (44). *ATRX* mutations are present in developmental disorders as well as in a number of tumors, with the highest prevalence in lung and colon adenocarcinomas, and in brain tumors (45, 46). *ATRX* mutations are highly correlated with the

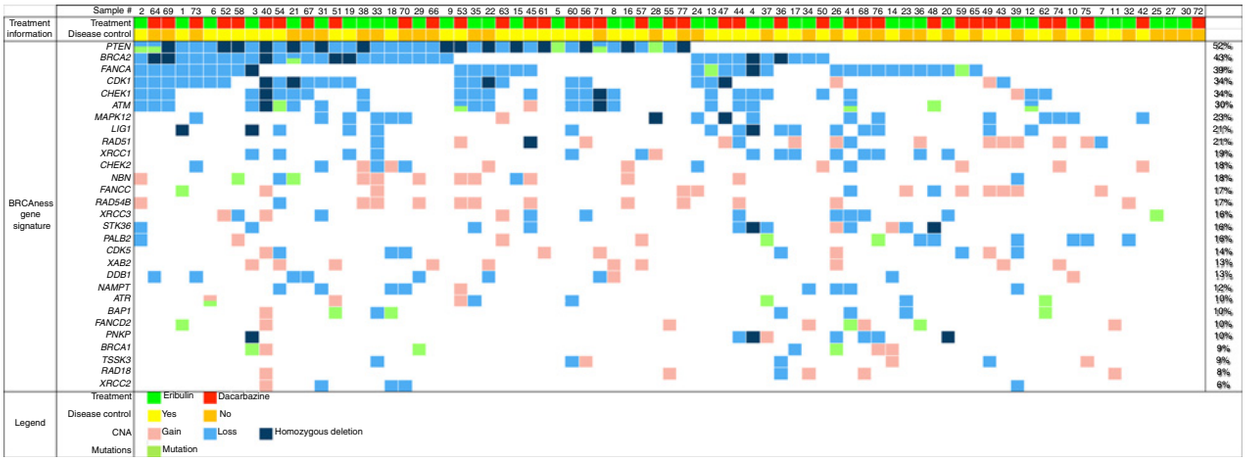

Figure 4.
Comprehensive "BRCAness" profile in 77 leiomyosarcoma cases.

alternative lengthening of telomeres (ALT) phenotype in leiomyosarcoma, a mechanism which is suggested as a major telomere-maintaining mechanism for sarcomas with complex genomic profile (18, 47). Yang and colleagues reported that patients with *ATRX*-mutated leiomyosarcoma had worse OS than those without *ATRX* alteration (38). In our study, these mutations had also a negative impact on both PFS and OS. Interestingly, *ATRX* mutations had a negative impact on PFS for both eribulin and dacarbazine when these groups were analyzed separately. However, a strong negative impact on OS was found only in the eribulin-treated group [$P < 0.001$; HR, 3.78 (95% CI, 2.79–34.11)]. Current theories suggest that mitotic checkpoint proteins are essential for proper cellular response to other tubulin inhibitors such as taxanes (48, 49). ATRX together with death domain-associated protein (DAXX) participates in chromatin remodeling and chromosomal segregation during mitosis (44, 45). Cell lines expressing increased levels of DAXX are sensitive to paclitaxel, while cells with low DAXX exhibit paclitaxel resistance in xenograft models (50). Although in our cohort, we detected a *DAXX* mutation in only one sample, we cannot exclude a potential interaction between mutated ATRX (and DAXX) in the resistance to eribulin, which warrants further studies.

"BRCAness" is a phenocopy of *BRCA1/2* mutation, causing defects in HRR, mainly through aberrations in individual genes that encode crucial components of this DNA reparation system (21). "BRCAness" is usually defined as an aggregation of a large number of molecular alterations in HRR-related proteins, that individually may occur in only a small percentage of cases in a given tumor type. In our study, we found that majority of leiomyosarcoma revealed a molecular profile suggesting an impaired HRR of double-strand breaks, similar to what was recently observed by others (19). Preclinical and clinical observations showed that low levels of *BRCA1* correlated with resistance to mitotic spindle poisons (e.g., taxanes) in TNBC (51–53). Conversely, tumors with impaired HRR of DNA double-strand breaks may be sensitive to PARP inhibitors (54). We believe that, even though none of the genes related to BRCAness phenotype correlated with eribulin activity, a more comprehensive mechanistic evaluation of the HRR pathway in leiomyosarcoma and genomic-guided clinical trials are warranted in this tumor type. A currently ongoing basket phase II trial is addressing this hypothesis, evaluating the combination of olaparib (PARP inhibitor) and temozolomide (MGMT inhibitor) in advanced, metastatic, or unresectable uterine leiomyosarcoma (NCT03880019). Furthermore, a basket phase II study is presently assessing durvalumab (inhibitor of PD-L1) with either olaparib or cediranib (inhibitor of VEGFR), relating the response to the changes in genomic and immune biomarkers (NCT03851614). Interestingly, HRR is a critical pathway for the repair of DNA damage caused by cisplatin as a DNA-binding agent, which was found to be inactive in uterine patients with leiomyosarcoma as a second line (55).

There are two major limitations in this study that could be addressed in future research. First, the study is based on a limited sample size, hence only a borderline significance for some biomarkers. Of note, we could base our analysis only on material collected from patients participating in North American study sites, even though tumor specimens were collected virtually from all Eisai-309 trial participants. The reason for this was the heterogeneity of the legal framework of clinical trials outside of the United States and the fact that health care legislation in Europe limits our ability to perform more comprehensive work with material collected from all trial participants. Second, no validation cohort was available to support our observations on correlations between molecular markers and response to the treatment. Furthermore, the access to a very limited biological material did not allow us to perform additional evaluations, for example, a confirmation of the most common mutations by Sanger sequencing or an assessment of the differential expression of proposed biomarkers on RNA/protein level. These limitations should be addressed in a new study, ideally analyzing samples collected before and after treatment with eribulin, supplemented by functional studies with appropriate preclinical models.

In conclusion, our work confirms the very complex genetic background of advanced leiomyosarcoma, with common presence of multiple CNAs and mutations affecting genes implicated in tumorigenesis. We identified several molecular changes with potential impact on disease control and survival of patients with leiomyosarcoma treated with eribulin. Further work will have to define whether these factors are predictive rather than prognostic, which will require further clinical validation.

Authors' Disclosures

A. Wozniak reports other from Eisai during the conduct of the study. B.A. Littlefield reports personal fees from Eisai Inc. outside the submitted work; in

addition, B.A. Littlefield has a patent for U.S. 6,214,865; "Macrocyclic analogs and methods of their use and preparation"; Littlefield BA, Palme MH, Seletsky BM, Towle MJ, Yu MJ, Zheng W. issued. P. Schöffski reports other from Eisai during the conduct of the study, as well as other from Eisai outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

A. Wozniak: Conceptualization, formal analysis, supervision, investigation, methodology, writing–original draft, project administration. **B. Boeckx:** Data curation, formal analysis, methodology. **E. Modave:** Data curation, formal analysis, methodology. **A. Weaver:** Resources. **D. Lambrechts:** Supervision, methodology. **B.A. Littlefield:** Conceptualization, resources, supervision, writing–original draft, writing–review and editing. **P. Schöffski:** Conceptualization, resources, supervision, writing–original draft, project administration.

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