Prevalence of Alternative Lengthening of Telomeres (ALT) in Pediatric Sarcomas Determined by the Telomeric DNA C-circle Assay

C. Patrick Reynolds, Trevor A. Burrow, Balakrishna Koneru, Shawn J. Macha, Wenyue Sun, Frederic Barr, and Timothy Triche

1Texas Tech University Health Sciences Center School of Medicine
2National Cancer Institute Laboratory of Pathology
3Children’s Hospital Los Angeles Department of Pathology and Laboratory Medicine

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Abstract

**Background:** Alternative lengthening of telomeres (ALT) occurs in sarcomas and ALT cancers share common mechanisms of therapy resistance or sensitivity. Telomeric DNA C-circles are self-primed circular telomeric repeats detected with a PCR assay that provide a sensitive and specific biomarker exclusive to ALT cancers. We have previously shown that 23% of high-risk neuroblastomas are of the ALT phenotype. Here, we investigate the frequency of ALT in Ewing’s family sarcoma (EFS), rhabdomyosarcoma (RMS), and osteosarcoma (OS) by analyzing DNA from fresh frozen primary tumor samples utilizing the real-time PCR C-circle Assay (CCA).

**Methods:** We reviewed prior publications on ALT in pediatric sarcomas. DNA was extracted from fresh frozen primary tumors, fluorometrically quantified, C-circles were selectively enriched by isothermal rolling cycle amplification and detected by real-time PCR. **Results:** The sample cohort consisted of DNA from 95 EFS, 191 RMS, and 87 OS primary tumors. One EFS and 4 RMS samples were inevaluable. Using C-circle positive (CC+) cutoffs previously defined for high-risk neuroblastoma, we observed 0 of 94 EFS, 5 of 187 RMS, and 62 of 87 OS CC+ tumors. **Conclusions:** Utilizing the ALT-specific CCA we observed ALT in 0% of EFS, 2.7% of RMS, and 71% of OS. These data are comparable to prior studies in EFS and OS using less specific ALT markers. The CCA can provide a robust and sensitive means of identifying ALT in sarcomas and has potential as a companion diagnostic for ALT targeted therapeutics.

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1. Texas Tech University Health Sciences Center School of Medicine Cancer Center, Department of Pediatrics, Lubbock, TX, USA
2. Texas Tech University Health Sciences Center Graduate School of Biomedical Sciences, Department of Translational Neuroscience and Pharmacology, Lubbock, TX, USA
3. Texas Tech University Health Sciences Center Graduate School of Biomedical Sciences, Department of Cell Biology and Biochemistry, Lubbock, TX, USA
4. Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA
Abstract

ALT, alternative lengthening of telomeres; APB, ALT-associated PML bodies; ARMS, alveolar rhabdomyosarcoma; AU, arbitrary unit; CC+, C-circle positive; CCA, C-circle assay; DDR, DNA damage response; EFS, Ewing’s family sarcoma; ERMS, embryonal rhabdomyosarcoma; FN, fusion negative; FP, fusion positive; IF, immunofluorescence; IF-FISH, immunofluorescence-fluorescence in situ hybridization; LOF, loss-of-function; OS, osteosarcoma; PDCL, patient-derived cell line; PDX, patient-derived xenograft; RMS, rhabdomyosarcoma; TA, telomerase; TMM, telomere maintenance mechanism; TRAP, telomerase repeated amplification protocol; TRF, telomere restriction fragment; UTF, ultra-bright telomeric foci
Background: Alternative lengthening of telomeres (ALT) occurs in sarcomas and ALT cancers share common mechanisms of therapy resistance or sensitivity. Telomeric DNA C-circles are self-primed circular telomeric repeats detected with a PCR assay that provide a sensitive and specific biomarker exclusive to ALT cancers. We have previously shown that 23% of high-risk neuroblastomas are of the ALT phenotype. Here, we investigate the frequency of ALT in Ewing’s family sarcoma (EFS), rhabdomyosarcoma (RMS), and osteosarcoma (OS) by analyzing DNA from fresh frozen primary tumor samples utilizing the real-time PCR C-circle Assay (CCA).

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Conclusions: Utilizing the ALT-specific CCA we observed ALT in 0% of EFS, 2.7% of RMS, and 71% of OS. These data are comparable to prior studies in EFS and OS using less specific ALT markers. The CCA can provide a robust and sensitive means of identifying ALT in sarcomas and has potential as a companion diagnostic for ALT targeted therapeutics.

Introduction

Telomeres

Telomeres are nucleoprotein structures at the ends of chromosomes that contain 5-10 kilobases of the canonical hexanucleotide (5'-TTAGGG-3') repeat sequence encased in shelterin proteins. This complex protects genomic DNA from replicative erosion, shields the ends of chromosomes from aberrant fusion, and prevents DNA damage response (DDR) elements from errantly recognizing genomic DNA. Approximately 85-90% of all cancers achieve replicative immortality by utilizing the telomere maintenance mechanism (TMM) telomerase (TA), a ribonucleotide reverse transcriptase. The remaining 10-15% of cancer cases (250,000 U.S. patients annually) use a non-telomerase TMM called alternative lengthening of telomeres (ALT).

Prevalence of ALT Incidence of ALT varies amongst various sarcomas (Table 1), with the majority of cases arising from tissues of mesenchymal or neuroepithelial origin. Cancers with an estimated ALT frequency >40% include osteosarcoma (OS), diffuse and anaplastic astrocytomas, undifferentiated pleomorphic sarcomas, and pediatric grade 4 glioblastoma multiforme. Previously reported patient sample screenings have demonstrated a broad range of ALT frequency amongst pediatric cancers, from 0% in Ewing’s Family Sarcoma (EFS), up to 85% in OS. Recently, there have been calls for assessing patient samples with currently available ALT biomarkers to confirm historically reported ALT frequencies, especially for OS.

1.3 ALT Characteristics

The hallmarks of the ALT phenotype include absence of TA activity (TERT mRNA expression provides a suitable surrogate for TA activity) with the presence of high telomere content and heterogenous telomere length, non-canonical telomere variant repeats, extra-chromosomal telomeric repeats, ALT-associated PML bodies (APBs), ultrabright telomere foci by FISH, and telomeric DNA C-circles. These characteristic markers have been used to screen tumor sample cohorts to determine the frequency of ALT among various tumor histologies.

1.4 Identification of ALT

Historically, ALT has been identified by the telomerase repeated amplification protocol (TRAP) assay to demonstrate low TA activity and/or low TERT mRNA expression, since TA is mutually exclusive to ALT. Telomere content and heterogeneity have been evaluated by telomere restriction fragment (TRF) analysis and telomere fluorescence in situ hybridization for ultra-bright telomeric foci (UTF). UTF was combined...
with immunofluorescence (IF) of the PML protein, which was discovered to co-localize with telomeres in ALT samples to detect APBs, yielding an additional ALT feature.\textsuperscript{32} Recently C-circles, circular self-primed telomeric DNA repeats, have been shown to be a sensitive and specific biomarker for ALT in tumors\textsuperscript{24} that also circulate in patient plasma, potentially increasing the clinical utility of C-circles as a biomarker.\textsuperscript{33,34}

The Real-Time C-circle Assay (CCA)

After genomic DNA is extracted (Fig. 1) from fresh frozen tumor, or plasma, C-circles can be enriched and subsequently detected by blot or real-time PCR.\textsuperscript{35,36} First, C-circles are selectively amplified by \( \varphi \)-29 DNA polymerase via isothermal rolling-circle amplification (Fig. 1),\textsuperscript{35,37,38} which enriches the partially double-stranded telomeric DNA, termed C-circles, when compared to a reaction without \( \varphi \)-29. Subsequently, the telomeric signals for the \( \varphi \)-29 and no \( \varphi \)-29 reactions can be compared by real-time PCR (Fig. 1), which is then normalized to a single copy gene (e.g. VAV2) for the same \( \varphi \)-29 and no \( \varphi \)-29 reactions.\textsuperscript{35,36} This unique molecular diagnostic assay allows for high-throughput screening of DNA from fresh frozen tumor and plasma samples with as little as 1 ng of template input.\textsuperscript{13,35} Herein, we sought to assess the frequency of ALT in pediatric extracranial solid tumors using the real-time PCR CCA on DNA samples extracted from fresh frozen tumor.

Methods

2.1 Tumor Samples

The Children’s Oncology Group Biopathology Center provided 82 rhabdomyosarcoma DNA samples from residual stored DNA. These RMS specimens were collected from patients enrolled on a variety of Intergroup Rhabdomyosarcoma Study Group or Children’s Oncology Group Soft Tissue Sarcoma studies and received as de-identified samples. Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen, 51104), in accordance with the manufacturer’s instructions, and stored at -20 °C. PAX3-FOXO1 or PAX7-FOXO1 fusion status was previously determined on all samples without unambiguous embryonal RMS histology. Fusion status was determined by reverse transcriptase-polymerase chain reaction assays\textsuperscript{39} of RNA isolated using RNA STAT-60 (Tel-Test, Friendswood, TX). EFS, OS, and additional RMS DNA was isolated from primary tumors obtained under informed consent by Children’s Oncology Group and processed by the pediatric division of Cooperative Human Tissue Network at Nationwide Children’s Hospital. These anonymized samples were originally used for genomic analyses in the NCI SPECS program, and in the case of OS, also the NCI TARGET program, and, in both cases, exempt from Human Subjects Research per IRB review.\textsuperscript{40-42} DNA was stored in liquid nitrogen vapor until aliquoted and sent to TTUHSC on dry ice for use in the C-circle assay.

2.2 DNA Quantification

Fluorometric quantification of DNA samples was carried out on a Qubit 2.0 system with the Qubit dsDNA Broad Range Assay Kit (Invitrogen Cat. No. Q32853).

2.3 The Real-time PCR C-circle Assay (CCA)

The isothermal rolling circle amplification reactions were performed on an Eppendorf Vapo.Protect thermocycler at 30 °C for 8 hrs, 65 °C for 20 min, and held at 4°C. Reactions were comprised of: 32ng of template DNA, 2 μL BSA (2 μg/μL), 2 μL of 1% Tween, 0.8 μL DTT (100 μM), 2 μL of 10 mM dNTPs (NEB, Ipswich, MA, N0447L), 2 μL of \( \varphi \)-29 Buffer, 0.8 μL of \( \varphi \)-29 DNA polymerase (NEB, Ipswich, MA, M0269L), and nuclease-free water up to 20 μL. No \( \varphi \)-29 control reactions consisted of the aforementioned reagents with nuclease-free water in place of \( \varphi \)-29 DNA polymerase. After isothermal rolling circle amplification, all reactions were diluted with 20 μL nuclease-free water to a final volume of 40 μL. Subsequent real-time PCR amplification of telomere DNA (Forward Primer: 5’ - CGGGTTTGGGTGTTGGGTGTTGGGGGTTGGTTGGGTGTTGGGTT - 3’, Reverse Primer: 5’ - GGCGGCGTCTACCTACTCTACCTACCTACCTACCTACCT - 3’) and VAV2 DNA (Forward Primer: 5’ - TGGGCCATGACTGAGATGAC - 3’, Reverse Primer: 5’ - ATCTGGCCCTCACCTTCCCAA - 3’) (IDT, Coralville, IA) was performed using a 96-well Thermo-Fisher Quantstudio 3 Real-Time PCR System with the following cycling conditions: Telomere reaction: 95 °C for 15 min, 33 cycles
of 95 °C for 15 sec and 56 °C for 2 min, and VAV2 Reaction: 95°C for 15 min, 40 cycles of 95 °C for 15 sec, 57 °C for 30 sec, and 72 °C for 1 min. Real-time PCR reactions consisted of: 5 μL of diluted isothermal reaction product, 12.5 μL QuantiTect SYBR Green PCR Master Mix (Qiagen, 204445), 1 μL DTT (100 μM), 0.5 μL DMSO, 1 μL nuclease-free water, and 2.5 μL of primers (5 μM Tel, or 2 μM VAV2). All real-time reactions (Telomere φ, Telomere No-φ, VAV2 φ, VAV2 No-φ) were carried out in triplicate and assessed via arbitrary unit (AU) calculations and cutoff values. DNA from CHLA-90 and CHLA-20 cell lines were used for positive and negative controls, respectively. Samples were considered CC+ if they had ≥ 5 AU, after normalization to CHLA-90, as previously described.43-45

2.4 Statistical Analysis

The relationship between clinical characteristics and C-circle status (Table 3) was evaluated by Chi-square, or Fisher’s exact test, when appropriate. The Wilcoxon Signed Rank Test was used to analyze the telomere content of CC+ and CC- OS samples. Two-tailed statistical tests with P values ≤ 0.05 were considered significant.

Results

CCA Results

Of the 373 DNA samples received (n = 95 EFS, n = 191 RMS, and n = 87 OS), five samples (1 EFS and 4 RMS) did not amplify due to poor DNA quality. We observed 0 of 94 EFS (0%), 5 of 187 RMS (2.7%), and 62 of 87 (71%) OS tumors to be CC+.

Discussion

The prognostic value of ALT, and other TMM, is gaining traction,15,27,46 including in veterinary care.47 Studies have linked high telomerase expression with exceptionally aggressive tumors that can result in rapid progression and poor clinical outcomes.27,48 By contrast, ALT has been associated with indolent disease progression; yet, patients with various tumor types have been observed to have a worse overall survival.15,20,27,49 Recently, we observed that high amounts of ATM kinase activation (which promotes chemotherapy resistance) in patient-derived neuroblastoma cell lines (PDCLs) and patient-derived xenografts (PDXs),45 and also in PDCLs of other histologies (rhabdomyosarcoma, osteogenic sarcoma, triple negative breast cancer, and colorectal cancer) have the ALT phenotype.44 We have also observed that certain clinical stage drugs (an ATM kinase inhibitor45 and a p53 reactivator44) are active in reversing chemotherapy resistance in ALT PDCLs and PDXs. Thus, robust identification of ALT has the potential to be a prognostic biomarker and a companion diagnostic for ALT-targeted therapies. Generally, ALT is activated by loss-of-function (LOF) genetic alterations in the chromatin remodelers α thalassemia-mental retardation, X linked (ATRX)27 and death domain-associated protein 6 (DAXX).50 ATRX inactivating mutations are commonly observed among different tumor types, while DAXX mutations are primarily associated with pancreatic neuroendocrine tumors (PanNETs).50 ALT is less frequently associated with LOF alterations in H3F3A51,52 and SMARCA1 mutated tumors.53,54 Previous studies have used these genomic alterations as proxies to identify ALT, but depending on histology, as many as ½ ALT cancers can be wild-type for ATRX or DAXX.35,55 C-circles, TERT expression, high telomere content with heterogeneous telomere length, and APBs have been used to screen sample sets to establish ALT frequencies (Table 1) amongst sarcomas; however, each of these techniques have their own advantages and disadvantages (Table 2). Relatively fragile, C-circles can be degraded by excess freeze-thaw cycles, prolonged vortexing, and formalin-fixing; thus, proper sample handling and storage are required.56 Recently, ALT tumors have been shown protect C-circles from nuclease degradation in the blood by releasing C-circles within exosomes, which may provide a non-invasive blood-based biomarker for the detection and monitoring of ALT tumors in vivo.34 Although there is no standardized method for determining ALT status,50 C-circles are the only known molecule specific to ALT34 and the molecularly based real-time PCR C-circle assay can utilize DNA isolated for sequencing; thus, it is readily translatable to the clinical laboratory, and it’s for these reasons that we selected this approach.20,24,36,55 In concordance with previous reports (Table 1),10,14,15 there were no EFS CC+ cases identified, which is most likely due to the activation of TERT by EFS fusion proteins,33 and TERT activation is known to be essentially exclusive to
the ALT phenotype.\textsuperscript{24,33,34} The sharp contrast between ALT frequency in EFS and OS\textsuperscript{14,46} was observed in our cohorts, as OS had a CC+ frequency of 71%, which falls within the range of previous studies.\textsuperscript{15} ALT provides a potentially targetable mechanism present in the majority of OS patients, some of which have poor clinical outcomes.\textsuperscript{44,57,58} Expression of \textit{TERT} has been shown to portend an unfavorable clinical prognosis in OS patients;\textsuperscript{48} however, stage and clinical outcomes of ALT cases were shown to be equivalent to TA cases.\textsuperscript{59} Within the OS patient cohort, we did not observe statistically significant relationships between C-circle status and the clinicopathological data (Table 3), which aligns with previous studies that identified ALT through methods other than the CCA\textsuperscript{13,48,59} However, the observed incidence of CC+ OS cases (Fig. 2) further confirms OS as one of the histologies with the highest frequencies of ALT.\textsuperscript{15} Additionally, we observed CC+ OS to have a significantly higher \((p < 0.05)\) telomere content than CC- OS samples (Fig. 2B), as telomere content of ALT is generally higher than non-ALT samples across histologies.\textsuperscript{33} ALT is known to occur in RMS,\textsuperscript{13} the most common pediatric soft tissue sarcoma.\textsuperscript{60} Classically, pediatric RMS cases were generally categorized histologically as embryonal RMS (ERMS), which was linked with better prognoses, or alveolar RMS (ARMS), which was associated with poor clinical outcomes.\textsuperscript{61} Further, molecular identification of \textit{PAX3}, or \textit{PAX7}, fusions with forkhead box protein O1 (\textit{FOXO1}), is currently considered the preferred method of distinguishing the latter from the former.\textsuperscript{62} Instead of histologic criteria, which are inexact, fusion status of “fusion positive” (FP) identifies ARMS while “fusion negative” (FN) identifies ERMS.\textsuperscript{63} We observed a prevalence of 2.7% CC+ RMS in the sample cohort (Table 4), which is lower than the previously reported 6% that was determined by APB analysis.\textsuperscript{13} Of the 5 CC+ RMS samples identified, four were FN (ERMS) and one was FP (ARMS). The tested samples were from banked DNA extracted from fresh frozen tissue; thus, it is possible that the age of the samples, or excess freeze-thaw cycles, could have contributed to the lower ALT frequency due to the degradation of C-circles.\textsuperscript{35} APB analysis from a previous study\textsuperscript{13} has the advantage (Table 2) of using FFPE material, which enables distinguishing of tumor cells from stromal tissue; however, the APB assay is very labor intensive, not all ALT samples have APBs,\textsuperscript{27} and C-circles have been postulated to be more specific than other ALT markers.\textsuperscript{24} Ideally, future studies should evaluate the various methods for detecting ALT in the same histology within the same patient sample cohort, since each ALT marker is not necessarily present in every ALT sample or tumor model.\textsuperscript{13,27} However, our data confirm that RMS, and especially OS, have patient populations that may benefit from ALT targeted therapies.

Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

Acknowledgments

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References


7


**FIGURE 1**

**FIGURE 2**
assay

B. Telomere Content

C. OS Telomere Content

Tissue Histologies

C-circle Status
## TABLE 1

<table>
<thead>
<tr>
<th>Pediatric Sarcomas</th>
<th>Estimated Annual Cases</th>
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<th>Method</th>
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<td>6 6 0</td>
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<td>Non-Rhabdomyosarcoma STS</td>
<td>650</td>
<td>*See combined populations</td>
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## Adult Sarcomas

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<td>Myxofibrosarcoma</td>
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<td>76</td>
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### *Combined Populations*

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<th>Ref</th>
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<td>Malignant Peripheral Nerve Sheath Synovial</td>
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<td>Undifferentiated Pleomorphic/MFH</td>
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<td>77 65 63</td>
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<td>22 34 52</td>
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## TABLE 2
Advantages

- Heterogeneity in telomere length
- Direct measure of telomerase activity
- High-throughput, Quantitative

Disadvantages

- Demarcate stroma, High complexity, Large template input, Sensitivity/specificity, Some TA cells have long telomeres
- Demarcate stroma, False negative rate, Indirect ALT measure
- Demarcate stroma, Indirect ALT measure, Input/RNA
- High complexity, Low throughput
- High complexity, Low throughput Not all ALT have APBs

Real-time CCA

- Direct ALT measure, High-throughput, Plasma monitoring, Quantitative, Sensitivity and specificity
- Widely clinically translatable CC relatively fragile, Demarcate stroma, Not all ALT have CC

---

**TABLE 3**

<table>
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<th>Osteosarcoma</th>
<th>Osteosarcoma</th>
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<tr>
<td>Female</td>
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<td>NA</td>
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</tr>
<tr>
<td>&lt; 18 years</td>
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<td>&gt; 18 years</td>
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<td>Telangiectatic</td>
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<td>Osteoblastic &amp; Fibroblastic</td>
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### Table 1

<table>
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<td>Osteoblastic &amp; Sclerosing</td>
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<td>Osteoblastic &amp; Telangiectatic</td>
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<td>NA</td>
<td>44</td>
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**Response**

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<td>Responder</td>
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### Table 4

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<table>
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<th>PAX3/7-FOXO1 Fusion Status</th>
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**Figure and Table Legends**

**FIGURE 1.** The real-time PCR CCA. Self-primed telomeric C-circles are selectively amplified by φ-29 polymerase via rolling circle amplification. Subsequent real-time PCR detection of telomere content reveals an enriched telomeric signal, indicating the presence of C-circles.

**FIGURE 2.** Patient sample CC status and telomere content. (A) Normalized relative CC content was plotted by tumor histology. Samples above the previously established cutoff of 5 arbitrary units (AU) were considered CC+. (B) Telomere content, normalized to CHLA-90 at 5 AU, were plotted by histology. (C) Telomere content was plotted for CC+ and CC- OS samples.

**TABLE 1.** A review of published sarcoma and soft tissue sample screenings for ALT.

**TABLE 2.** Advantages and disadvantages of assays used to determine TMM.

**TABLE 3.** Clinicopathological data osteosarcoma sample cohort.

**TABLE 4.** PAX3/7-FOXO1 fusion status rhabdomyosarcoma sample cohort.