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Hypermethylation of potential tumor suppressor miR-34b/c is correlated with late clinical stage in patients with soft tissue sarcomas

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ABSTRACT

Soft tissue sarcomas (STSs) are comparatively rare malignant tumors with poor prognosis. STSs predominantly arise from mesenchymal differentiation. MicroRNA-34b/c, the transcriptional targets of tumor suppressor p53, possesses tumor suppressing property. Hypermethylation of miR-34b/c has been associated with tumorigenesis and the progression of various cancers. To determine whether aberrant miR-34b/c methylation occurs in STSs, we quantitatively evaluated the methylation level of miR-34b/c in 57 STS samples and 20 cases of peripheral blood from healthy volunteers serving as normal controls by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. We found that miRNA34b/c is more frequently methylated in STSs (0.157 ± 0.028) than in normal controls (0.098 ± 0.012, $p = 0.038$). Furthermore, the methylation levels of CpG_1.2.3, CpG_4.5.6.7, and CpG_11.12.13 of miR-34b/c were significantly higher in the STS group than in the normal control group ($p < 0.001$). No significant differences in the methylation levels within miR-34b/c were observed between specific reciprocal translocations in STSs and nonspecific reciprocal translocations in STSs (0.146 ± 0.039 vs. 0.168 ± 0.035, $p > 0.05$). The methylation levels of miR-34b/c in STSs were associated with clinical stage. The methylation levels of CpG_1.2.3, CpG_4.5.6.7, CpG_9.10, CpG_11.12.13, and CpG_14 in tumor-stage III/IV tissues were significantly higher than those in tumor-stage I/II tissues. Our findings indicated that DNA hypermethylation of the miR-34b/c is a relatively common event in STSs and is significantly correlated with late clinical stage in patients with STSs. Hypermethylation of the miR-34b/c may be pivotal in the oncogenesis and progression of STSs and may be a potential prognostic factor for STSs.

1. Introduction

Soft tissue sarcomas (STSs) are a group of mesenchymal origin malignant tumors characterized by highly biologic heterogeneity with respect to clinical behavior, morphological features, phenotype, and genetics. STSs encompass more than 50 distinct histological tumor subtypes. STSs occur relatively rarely and accounts for approximately 1% of malignant tumors (Ilaslan et al., 2010; Panotopoulos et al., 2014). Patients with STSs have poor prognosis because of rapid metastasis and poor response to treatment. Although therapy has improved over the years, mortality remains fairly high. Traditionally, STSs are mainly classified into two categories according to the characteristics of the tumor genetics. One type is specific reciprocal translocations in STSs (SRTSs) and is characterized by simple karyotypes, which are chromosomal specific translocations and fusion gene formation, such as synovial sarcoma (SS), Ewing's sarcoma/peripheral primitive neuroectodermal tumor (ES/pPNET), dermatofibrosarcoma protuberan (DFSP), alveolar soft part sarcoma (ASPS) and myxoid liposarcoma (ML). The other type is nonspecific reciprocal translocations in STSs (NRTSs) and is characterized by

Abbreviations: A, adenine; ASPS, alveolar soft part sarcoma; ASS1, argininosuccinate synthetase 1; BMP2, bone morphogenetic protein 2; C, cytosine; Da, Dalton; DFSP, dermatofibrosarcoma protuberan; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; ES/pPNET, Ewing's sarcoma/peripheral primitive neuroectodermal tumor; FFPE, formalin-fixed paraffin-embedded; FS, myxofibrosarcoma; G, guanine; HE, hematoxylin–eosin; LMS, leiomyosarcoma; LOH, loss of heterozygosity; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MDM2, mouse double minute 2 homolog; ML, myxoid liposarcoma; MiR-34b/c, micro-ribonucleic acid-34b/c; mRNA, messenger ribonucleic acid; MSP, methylation-specific polymerase chain reaction; NRTSs, nonspecific reciprocal translocations in soft tissue sarcomas; NSCLC, non-small-cell lung cancer; PAX3/7-FOXO1, paired box 3/7-forkhead box O1; PCR, polymerase chain reaction; PL, pleomorphic liposarcoma; PRMS, pleomorphic rhabdomyosarcoma; PTEN, phosphatase and tensin homolog deleted on chromosome ten; RNA, ribonucleic acid; SAP, shrimp alkaline phosphatase; SCLC, small-cell lung cancer; SRTSs, specific reciprocal translocations in soft tissue sarcomas; SS, synovial sarcoma; STSs, soft tissue sarcomas; T, thymine; U, uracil; UPS, undifferentiated pleomorphic sarcoma

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complicated karyotypes, which include the nonspecific chromosomal rearrangements, loss of heterozygosity and copy number alteration of DNA, such as pleomorphic rhabdomyosarcoma (PRMS), leiomyosarcoma (LMS), pleomorphic liposarcoma (PL), undifferentiated pleomorphic sarcoma (UPS) and myxofibrosarcoma (FS) (Mertens et al., 2010).

Previous studies have identified that genomic alterations, including the amplification or point mutation of oncogenes and the methylation or deletion of tumor suppressor genes (e.g., MDM2 amplification, P53 mutation, and PTEN methylation), are involved in the carcinogenesis of subtype-specific STSs (Barretina et al., 2010; Hettmer et al., 2014; Ware et al., 2014; Yin et al., 2013). Moreover, p53 mutations were detected in 25.6% of STSs, the date has suggested that p53 mutations may be involved in the oncogenesis of STS (Yin et al., 2012). By contrast, epigenetic alterations such as DNA methylation is an alternative mechanism of gene inactivation that occurs in the process of tumor progression and thus changes gene expression level without altering the DNA sequence (Liu et al., 2013; Zhu et al., 2012). Similar to genetic mutations, transcriptional silencing owing to gene promoter CpG unit methylation is stably inherited by the next cell generation and may therefore enable the clonal expansion of a cell population with a selective advantage during tumor progression. Existing studies have shown that various tumor suppressor genes are frequently methylated in STSs and contribute to tumorigenesis. For example, epigenetic silencing of tumor suppressor gene BMP2 via CpG hypermethylation fosters tumor growth and progression in rhabdomyosarcoma, and the suppression of BMP2 by epigenetic silencing may be critical in the genesis of rhabdomyosarcoma (Wolf et al., 2014). The hypermethylation of a novel tumor suppressor gene ASS1 promoter was detected and was strongly associated with ASS1 protein deficiency within myxofibrosarcoma, which was linked to increased tumor stage and grade, and independently predicted worse survival (Huang et al., 2013). From what has been discussed above, identifying the molecular markers and pathogenesis of STSs, especially the detection of abnormal methylation, may be useful for the diagnosis and therapeutic regimens of STSs.

MicroRNAs (miRNAs) are a highly conserved class of small non-coding single-stranded RNAs that transcriptionally or post-transcriptionally regulate the level of gene expression through specific binding at regulatory regions in multicellular organisms. MiRNAs are involved in many basic biological and physiological regulatory processes, including cell differentiation, proliferation, apoptosis, stress response, and inflammation (Anwar and Lehmann, 2014; Kishore et al., 2014). Epigenetic inactivation is one of the factors that influenced the regulatory mechanism of microRNAs. The transcription of miRNA as a protein-encoding gene might be inactivated by promoter hypermethylation. Widespread miRNA is dysregulated in STSs by changes in epigenetic silencing or protein expression, thus resulting in cellular malignant transformation (Diao et al., 2014; Karnuth et al., 2014). Among the various miRNAs, the miR-34b/c gene, which localizes to chromosome 11q23.1 and belongs to the miR-34 family, shares a common primary transcript and acts as a potential tumor suppressor that is directly regulated by p53 (Corney et al., 2007; Wong et al., 2011; Zhang et al., 2014). MiR-34b/c is involved in mediated cellular responses, such as cell cycle arrest (Yan et al., 2014), apoptosis, and metabolic regulation (Zhang et al., 2014). The reduced or deleted expression of miR-34b/c might contribute to diverse human cancers because of the aberrant CpG island methylation of its promoter (Beresneva et al., 2013; Nadal et al., 2013; Ng et al., 2014; Wu et al., 2014; Xie et al., 2014). However, only one study has reported the methylation of miR-34b/c gene promoters in STS cell lines and tissue samples (Vogt et al., 2011), but the methylation level of the miR-34b/c individual CpG unit and the exact function of DNA hypermethylation of miR-34b/c in STSs remains unclear.

In the present study, we accordingly therefore hypothesized whether miR-34 b/c CpG island aberrant methylation preferentially occurs in STSs and correlates with clinical parameters. To address these problems, we quantitatively evaluated the methylation level of the miR-34b/c individual CpG unit by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with 57 STS subjects, and then assessed the association between the methylation status of every CpG unit within the miR-34b/c and the clinicopathologic features of the patients with STSs. Results indicated that DNA hypermethylation of the miR-34b/c is a relatively common event in STSs and is significantly correlated with late clinical stage in patients with STSs. Hypermethylation of the miR-34b/c may be pivotal in the oncogenesis and progression of STSs and may be a potential prognostic factor for STSs.

2. Materials and methods

2.1. Ethics statement

Written informed consent was obtained from all participating patients in the study. This study was approved by the Research Ethics Committee at the First Affiliated Hospital of Shihezi University School of Medicine, PR China. The experiment was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. All specimens were handled and made anonymous according to the ethical and legal standards.

2.2. Sarcoma tissue samples

We analyzed a total of 57 eligible FFPE tissue samples from a collection of STS patients registered in the Department of Pathology, The First Affiliated Hospital of Shihezi University School of Medicine, China, during 1976 to 2010. No restrictions regarding sex, age and disease stage were set. We excluded such patients who had undergone surgery (except diagnostic biopsy), chemotherapy, radiotherapy or any blood transfusion in the preceding six months before recruitment. The diagnoses of all original slides, including hematoxylin–eosin (HE) and immunohistochemistry staining from each case, were confirmed by two experienced pathologists. Each paraffin block was reviewed to assure that at least 70% of the tumor cells were present before sectioning and DNA extraction. Patients with STS subtypes were composed of 28 cases of SRTSs (5 cases of SS, 11 cases of ES/pPNET, 8 cases of DFSP, 1 case of ASPS, and 3 cases of ML) and 29 cases of NRTSs (1 case of PRMS, 5 cases of LMS, 1 case of PL, 18 cases of UPS, and 4 cases of FS). A total of 20 cases of peripheral blood collected in ethylenediaminetetraacetic acid (EDTA) were obtained from healthy volunteers serving as normal controls. In this study, various clinic-pathological characteristics of patients with STSs were investigated as follows. There were 35 (64.1%) males and 22 (38.6%) females in the case group. The age was 45.74 ± 6.69 (mean ± SD) years for the STS samples. The samples included 14 (24.6%) well-differentiated patients (G1), 14 (24.6%) moderately differentiated patients (G2), and 29 (50.8%) poorly differentiated patients (G3). 30 (65.2%) were classified as stage I/II and 16 (34.8%) as stage III/IV.

2.3. Isolation of genomic DNA

Total genomic DNA was extracted from the 57 FFPE STS tissues by means of proteinase K lysis and subsequent phenol–chloroform extraction. Twenty sections (5 μm each) from each FFPE tissue specimen were shredded with 1.5 ml of xylene in a 2 ml centrifugation tube placed upside down. The sections were applied to mix up the samples for 5 min, followed by mixing at 10 min in a 55 °C water-bath. After centrifugation (5 min at 10,000 rpm), supernatants were removed. The cell pellets were washed with 1.5 ml of absolute ethanol and air-dried for evaporation of residual ethanol. The samples were lysed overnight at 60 °C with 250 μl of lysis buffer (50 mM Tris pH 8.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% [v/v] Tween 20) and 50 μl of proteinase K (20 mg/ml) in a thermomixer. Proteinase K was inactivated (5 min, 95 °C). High molecular weight DNA extraction was performed by