MicroRNA-493 inhibits the proliferation and invasion of osteosarcoma cells through directly targeting specificity protein 1

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Abstract. Osteosarcoma (OS) is the most common type of primary bone tumor and accounts for ~60% of all malignant bone tumors in children and adolescents. A large number of studies have proposed that the dysregulated and dysfunctional microRNAs may serve important roles in the occurrence, progression and metastasis of various types of human cancer, including OS. MicroRNA-493 (miR-493) has been identified to act as a tumor suppressor in several types of human cancer. However, little is known regarding the expression pattern and clinical significance of miR-493 in OS. In the present study, reverse transcription-quantitative polymerase chain reaction analysis revealed that miR-493 was markedly downregulated in OS tissues and cell lines and a low miR-493 level were associated with distant metastasis and clinical stage. Furthermore, functional experiments demonstrated that enforced expression of miR-493 led to a significant decrease in OS cell proliferation and invasion in vitro. Furthermore, through bioinformatics analysis, specificity protein 1 (SP1) was identified as a direct target gene of miR‑493 in OS. Its expression was upregulated in OS tissues and was negatively associated with miR‑493 expression levels. Inhibition of SP1 expression also suppressed the proliferation and invasion of OS, exerting a similar effect to that induced by miR-493 overexpression. These results suggested that miR-493 inhibited OS cell proliferation and invasion through negative regulation of SP1. Therefore, miR-493/SP1 may represent a potential therapeutic target for the treatment of OS.

Introduction

Osteosarcoma (OS), the most common type of primary bone tumor, accounts for ~60% of all malignant bone tumors in children and adolescents, with an incidence of 4-5 cases per million people (1,2). It originates from primitive transformed cells that exhibit osteoblastic differentiation and produce malignant osteoid tissue (3). Despite the development and improvement of modern treatment modalities, OS remains a fatal disease with a poor early diagnosis and low long-term survival rates (4,5). The principal reasons for poor prognosis include the occurrence of metastasis, recurrence of disease and chemo-resistance (6). The 5-year survival rate of patients with OS without metastasis is 60-70%; however, it is <30% for those with metastasis (7). Although a previous study indicated that certain molecular targets contribute to OS tumorigenesis and development, the mechanisms of this have not been fully identified (8). Therefore, elucidation of the effective molecules or signaling pathways that contribute to the formation and progression of OS are essential in order to develop novel therapeutic strategies and to improve the prognosis of patients with this malignancy.

MicroRNAs (miRNAs) are a group of conserved, endogenous and short non-coding RNA molecules between 18 and 25 nucleotides in length (9). miRNAs have been reported to negatively modulate the expression of their target genes through directly binding to the ‘seed sequence’ within the 3'-untranslated regions (3'-UTRs) of the mRNAs of their target genes, thereby resulting in either translation suppression or mRNA degradation (10,11). A previous study suggested that miRNAs are involved in the regulation of a broad array of critical cellular processes, including the cell cycle, apoptosis, differentiation, invasion and metabolism (12). A number of studies have proposed that the dysregulated and dysfunctional miRNAs may serve important roles in the occurrence, progression and metastasis of various types of human cancer, in which miRNAs may act as oncogenes or tumor suppressors (13-15). In this regard, miRNA-based therapeutic methods have been proposed as novel and efficient modalities for antitumor treatments, including the possible approaches of blocking oncogenic miRNAs using anti-miRNA oligonucleotides or replacing tumor suppressor miRNAs using miRNA mimics (16). Sp1 is a member of Sp-family and contains a glutamine rich region that...
can act as strong activation domain, it has also been reported that Sp1 can bind to some microRNAs (17).

Previous studies have reported that miR-493 is involved in the tumor formation and progression of several types of human cancer (18-22). However, little is known regarding the expression and clinical significance of miR-493 in OS. In the present study, miR-493 expression in OS was measured in order to evaluate the association between miR-493 expression and clinicopathological factors. The roles of miR-493 in the regulation of biological behaviors of OS cells were also investigated, in addition to attempting to establish their underlying mechanisms.

Materials and methods

Ethics statement. The present study was approved by the Research Ethics Committee of Changzheng Hospital (Shanghai, China), and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Changzheng Hospital. Written informed consent was obtained from all patients for the use of their clinical tissues.

Tissue collection. OS tissues and associated adjacent non-tumorous tissues were obtained from 49 patients (31 male, 18 female; age range, 8-59 years old; mean age, 21 years) who had undergone surgical resection at the Bone Tumor Center, Changzheng Hospital between May 2012 and November 2014. Patients with OS enrolled in the present study had not been treated with chemotherapy or radiotherapy prior to surgery. All patients had undergone surgical resection at the Bone Tumor Center, Changzheng Hospital, before surgery. All patients were maintained at 37˚C in a humidified atmosphere with 10% FBS and Geneticin (400 µg/ml). Total RNA was isolated from tissues and cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For the detection of miR-493, U6 was used as an internal control, and cDNA was synthesized using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), and qPCR was performed using the TaqMan MicroRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The temperature protocol for reverse transcription was as follows: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. The cycling conditions for qPCR were as follows: 50˚C for 2 min, 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec; and annealing/extension at 60˚C for 60 sec. For the analysis of SP1, GAPDH was used as an internal control, reverse transcription was conducted using the Moloney Murine Leukemia Virus Reverse Transcription system (Promega Corporation, Madison, WI, USA), followed by qPCR using the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd., Dalian, China). The temperature protocol for reverse transcription was as follows: 95˚C for 2 min; 20 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 2 min; and 72˚C for 5 min. The cycling conditions for qPCR were as follows: 5 min at 95˚C, followed by 40 cycles of 95˚C for 30 sec and 65˚C for 45 sec. The primers were designed as follows: miR-493 forward, 5'-TTGATACATGTTAGGCTTATTT-3' and reverse 5'-AAC CATTATATTCTCCCCAGCC-3'; U6 forward, 5'-GCTTGG GCACGACATATACATAAT-3'; aCGTTTTTCGAGTTGCA-3'; miR-493 forward, 5'-UGGATTTTGCACTTCGTTAT-3'; miR-493 forward, 5'-GGAGCTTTGAGGTATTTGTT-3' and reverse 5'-GCCTT TCAGAATTTCACG-3'; miR-493 forward, 5'-GGAGCTTTGAGGTATTTGTT-3' and reverse 5'-GGAGCTTTGAGGTATTTGTT-3'. The 2ΔΔCt method was utilized to calculate the expression level of miR-493 and SP1 mRNA (23).

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to analyze OS cell proliferation. Cells were seeded onto 96-well plates at a density of 3,000 cells/well. After 6 h of incubation, cells were transfected with miR-493 mimics, miR-NC, SP1 siRNA or NC siRNA, prior to being incubated at 37˚C in a humidified atmosphere with 5% CO2. At each time-point, 10 µl CCK-8 reagent was added into each well and the cells were incubated for an additional 4 h at 37˚C. The absorbance of each well was measured at a wavelength of 450 nm.

Transwell invasion assay. A Transwell invasion assay was performed in order to evaluate the invasion capacity of OS cells using Transwell chambers (8 µm; Costar; Corning...
Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). After 48 h of transfection, cells were harvested and resuspended in FBS-free culture medium at a concentration of 2.5×10^6 cells/ml. Subsequently, 200 µl cell suspension was seeded into the upper chambers, and the lower chambers were filled with 500 µl DMEM containing 20% FBS. After 48 h incubation at 37°C, the non-invasive cells were removed using cotton swabs, and the invasive cells were fixed with 100% methanol at room temperature for 15 min and stained with 0.5% crystal violet at room temperature for 15 min. Images of the invasive cells were captured and the number of cells was counted in five random fields under an inverted phase-contrast microscope (Olympus IX83; Olympus Corporation, Tokyo, Japan) at x200 magnification.

Bioinformatics predication and luciferase reporter assay. Bioinformatics analysis was performed in order to predicate the potential target genes of miR-493 using TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/). The 3'-UTR of the SP1-containing miR-493 binding sites, in addition to a mutant seed sequence in the SP1 3'-UTR, was produced by Shanghai GenePharma Co., Ltd. (Shanghai, China) and sub-cloned into the pMIR-REPORT vector (pMIR-SP1-3'-UTR Wt and pMIR-SP1-3'-UTR Mut; Shanghai GenePharma Co., Ltd., Shanghai, China). For the luciferase assay, 293T cells were seeded onto 24-well plates at 40-50% confluence. After 24 h, pMIR-SP1-3’-UTR Wt or pMIR-SP1-3’-UTR Mut, together with miR-493 mimics or miR-NC, were transfected into the 293T cells using Lipofectamine 2000, according to the manufacturer’s protocol. After 48 h incubation at 37°C, cells were harvested, and firefly and Renilla luciferase activities were determined using a dual-luciferase reporter assay system (E1910; Promega Corporation), according to the manufacturer’s protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis. Total protein was prepared using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and protein concentration was determined using a bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 µg) was electrophoresed on 10% SDS polyacrylamide gels, prior to being transferred to polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h, followed by incubation at 4°C overnight with a mouse anti-human monoclonal SP1 antibody (cat. no. sc-420; dilution 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or a mouse anti-human monoclonal GAPDH antibody (cat. no. sc-77242; dilution: 1:1,000; Santa Cruz Biotechnology, Inc.). Following washing in TBST three times, the membranes were incubated with a goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G secondary antibody (cat. no. sc-2005; dilution: 1:5,000; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Finally, the immunoreactive bands were visualized using an enhanced chemiluminescence western blotting kit (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as a loading control.

Statistical analysis. Data are expressed as the mean ± standard deviation, and were compared using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Differences were evaluated using Student’s t-test or one-way analysis of variance. Student-Newman-Keuls test was the post hoc test used following one-way analysis of variance. The chi-square test was used to assess the associations between miR-493 and clinicopathological factors of OS patients. Spearman’s correlation analysis was employed to determine the association between expression levels of miR-493 and SP1 mRNA in OS tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-493 in OS tissues and cell lines. In order to explore the biological roles of miR-493 in OS, the miR-493 expression level in OS tissues and adjacent non-cancerous tissues were measured. As demonstrated in Fig. 1A, miR-493 expression was low in OS tissues compared with that in adjacent non-cancerous tissues (P<0.05). Furthermore, miR-493 expression in four OS cell lines (MG63, HOS, SaOS-2 and U2OS) and the human normal osteoblastic hFOB1.19 cell line were determined using RT-qPCR. The results demonstrated that miR-493 was significantly downregulated in all examined OS cell lines when compared with hFOB1.19 (Fig. 1B; P<0.05).

Associations between miR-493 expression levels and clinicopathological factors in OS patients. In order to explore whether a low miR-493 expression level was associated with clinicopathological features in patients with OS, statistical
analysis was performed. The results demonstrated that the low expression level of miR-493 was associated with distant metastasis (P=0.030) and clinical stage (P=0.008) in patients with OS (Table I). However, there no significant association was observed between miR-493 expression and sex (P=0.961), age (P=0.336), anatomical location (P=0.856) or tumor size (P=0.308).

Upregulation of miR-493 inhibits the proliferation and invasion of OS cells. In order to further investigate the functions of miR-493 in OS, miR-493 mimics were transfected into HOS and U2OS cells, with miR-NC as the internal control group. The results from RT-qPCR analysis confirmed that miR-493 was markedly upregulated in HOS and U2OS cells following transfection with miR-493 mimics (Fig. 2A; P<0.05). A CCK-8 assay and Transwell invasion assay were performed in order to analyze the effects of miR-493 overexpression on OS cell proliferation and invasion. As demonstrated in Fig. 2B and C, HOS and U2OS cells transfected with miR-493 mimics had a significantly lower proliferation rate than cells transfected with miR-NC (P<0.05). Additionally, a Transwell invasion assay revealed that the invasive abilities of HOS and U2OS cells transfected with miR-493 mimics were significantly decreased compared with the miR-NC group (Fig. 2D; P<0.05). These results indicated that the upregulation of miR-493 inhibits OS cell proliferation and invasion in vitro.

SPI is a direct target of miR-493 in OS. The present study subsequently identified the molecular mechanisms of the tumor suppressive roles induced by miR-493 in OS by screening its target genes using bioinformatic analysis. Among these putative targets, SP1 became the principal focus (Fig. 3A), due to its associated with cancer cell proliferation, differentiation, migration, metastasis and invasion (4-6). In order to verify this prediction, a luciferase reporter assay was performed, which demonstrated that luciferase activities were markedly decreased in 293T cells following transfection with pMIR-SP1-3'UTR Wt and miR-493 mimics (Fig. 3B, P<0.05), while mutation of the binding sites abolished the inhibitory effect of miR-493. In order to confirm the regulatory effects of miR-493 on SP1 expression, SP1 mRNA and protein expression were examined following the transfection of miR-493 mimics or miR-NC into HOS and U2OS cells. As demonstrated

Table I. Correlation between microRNA-493 expression and clinicopathological factors of osteosarcoma.

<table>
<thead>
<tr>
<th>Clinicopathological factors</th>
<th>No. cases</th>
<th>miR-493 expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31</td>
<td>17</td>
<td>0.961</td>
</tr>
<tr>
<td>Female</td>
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<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
<td>0.336</td>
</tr>
<tr>
<td>&lt;20</td>
<td>23</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>≥20</td>
<td>26</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Anatomical location</td>
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</tr>
<tr>
<td>Tibia/femur</td>
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</tr>
<tr>
<td>Elsewhere</td>
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<td>8</td>
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</tr>
<tr>
<td>Tumor size (cm)</td>
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</tr>
<tr>
<td>&lt;8</td>
<td>24</td>
<td>15</td>
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</tr>
<tr>
<td>≥8</td>
<td>25</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
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</tr>
<tr>
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<td>25</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24</td>
<td>17</td>
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<tr>
<td>Clinical stage</td>
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</tr>
<tr>
<td>III-IV</td>
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*P<0.05. The χ² test was used to assess the associations between miR-493 and clinicopathological factors of OS patients.

Figure 2. miR-493 inhibited cell proliferation and invasion in HOS and U2OS cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-493 expression in HOS and U2OS cells transfected with miR-493 mimics or miR-NC. Cell proliferation was determined in miR-493 mimics or miR-NC-transfected (B) HOS and (C) U2OS cells using a Cell Counting Kit-8 assay. (D) The invasive ability of HOS and U2OS cells transfected with miR-493 mimics or miR-NC was analyzed using a Transwell invasion assay. P<0.05. miR, microRNA; NC, negative control.
in Fig. 3C and D, SP1 mRNA and protein expression were downregulated in HOS and U2OS cells following transfection with miR-493 mimics (both P<0.05). Taken together, these data demonstrated that miR-493 negatively regulates SP1 expression through binding directly to its 3′-UTR in OS.

**Upregulation of SP1 in OS tissues and its negative correlation with miR-493.** SP1 mRNA expression was subsequently measured in OS tissues in order to evaluate whether miR-493 was negatively correlated with SP1 expression. SP1 mRNA expression was significantly upregulated in OS tissues compared with associated adjacent non-cancerous tissues, as demonstrated in Fig. 4A (P<0.05). The association between miR-493 and SP1 mRNA was further illustrated using Spearman’s correlation analysis. The analysis indicated an inverse correlation between miR-493 and SP1 mRNA expression in OS tissues (Fig. 4B; r=-0.4713; P=0.0006).

**Knockdown of SP1 also inhibits the proliferation and invasion of OS cells.** As SP1 was identified as a direct target gene of miR-493, we hypothesized that SP1 may be involved in the inhibition of OS cell proliferation and invasion. In order to determine whether or not this was the case, SP1 siRNA was utilized to downregulate its expression in HOS and U2OS cells (Fig. 5A; P<0.05). Similar to the effects of miR-493 upregulation, the introduction of SP1 siRNA into HOS and U2OS cells resulted in decreased cell proliferation (Fig. 5B and C; P<0.05) and invasion (Fig. 5D; P<0.05), when compared with the NC siRNA group. These data further suggested that SP1 is a functional target of miR-493 in OS.
Discussion

An increasing number of studies have reported the important roles of miRNAs in tumorigenesis and tumor development (27-29). Furthermore, the ability to control cell growth, metastasis and survival may serve essential roles in preventing and treating various human malignancies, including OS (30).

The present study demonstrated that miR-493 expression was downregulated in OS tissues and cell lines. Low expression of miR-493 was associated with distant metastasis and advanced clinical stages in patients with OS. Functional analyses revealed that miR-493 inhibited cell proliferation and invasion in OS. Mechanistically, the data revealed that SP1 was the direct target of miR-493 in OS, in which SP1 was highly expressed in OS tissues and exhibited a negative correlation with miR-493. Additionally, SP1 downregulation was demonstrated to repress OS cell proliferation and invasion, in a similar way to the effects induced by miR-493 overexpression. These results suggested that miR-493 downregulation may serve crucial roles in OS formation and progression, and may be developed as a therapeutic target for the treatment of patients with this malignancy.

The aberrant expression of miR-493 has been reported to contribute to the initiation and progression of different types of cancer. For example, a study undertaken by Ueno et al (18) revealed that miR-493 expression was low in bladder cancer tissues and cell lines and that upregulation of miR-493 suppressed bladder cancer cell migration and motility. Furthermore, a study undertaken by Gu et al (19) reported that miR-493 was downregulated in non-small cell lung cancer. Additionally, restoration of miR-493 expression attenuated cell growth and invasion in vitro and in vivo (19). A study undertaken by Zhou et al (20) revealed that the expression level of miR-493 was decreased in gastric cancer and that low miR-493 expression was significantly associated with advanced clinical stages and lymph node metastases in patients with gastric cancer. Furthermore, the ectopic expression of miR-493 reduced gastric cancer cell growth and metastasis in vitro and in vivo (20). A study undertaken by Okamoto et al (21) indicated that the overexpression of miR-493 inhibited colon cancer cell liver metastasis and induced cell death in liver metastases. These observations suggested that miR-493 may have promising therapeutic values in treating these types of human cancer.

Previous studies have identified several direct targets of miR-493, including Ras homolog family member C (18), frizzled class receptor 4 (18), E2F transcription factor 1 (19), insulin-like growth factor 1 receptor (21) and mitotic arrest deficient 1 (22). It is typical for a miRNA to have numerous
target genes (11). In the present study, putative target genes were predicated using bioinformatics analysis. Among these potential target genes of miR-493, SP1 became the primary focus of the present study, due to its role as a regulator in cell proliferation, differentiation and metastasis (24-26).

To confirm this, a luciferase reporter assay was performed, which demonstrated that miR-493 directly targeted the 3’-UTR of SP1. RT-qPCR and western blot analysis indicated that miR-493 negatively regulated SP1 mRNA and protein expression in OS cells. SP1 expression was demonstrated to be increased in OS tissues and was inversely correlated with miR-493 expression. The effects of SP1 knockdown on OS cells were similar to those induced by miR-493 overexpression. Identification of cancer-specific miR-493 and its target genes is critical in order to fully comprehend their biological roles in the occurrence and development of OS (31,32).

SP1, located at 12q13.1, was the first transcription factor to be cloned from mammalian cells in 1983 (33,34). SP1 binds to GC/GT-rich promoter elements through its C(2)H(2)-type zinc fingers at C-terminal domains, thereby stimulating or inhibiting the activity of gene promoters (35). Previous studies reported that SP1 was increased in multiple types of human cancer, including colon (36), prostate (37,38), pancreatic (24), lung (39) and gastric cancer (40). Furthermore, studies have supported the biological roles of SP1 in regulating cell proliferation, differentiation, migration, metastasis and invasion (24-26). In the present study, miR-493 was revealed to target SP1 in order to inhibit the proliferation and invasion of cells in OS. Additionally, SP1 expression was upregulated in OS tissues. Downregulation of SP1 suppressed OS cell proliferation and invasion. Taken together, these results suggested that the miR-493/SP1 axis may be a novel and efficient therapeutic target for the treatment of this malignancy.

In conclusion, the results of the present study revealed that miR-493 was lowly expressed in OS, and was significantly correlated with distant metastasis and clinical stage. Upregulation of miR-493 decreased cell proliferation and invasion of OS through directly targeting SP1. This miR-493/SP1 axis may provide a novel molecular mechanism that underlies the carcinogenesis and progression of OS. However, the association between miR-493 and the prognosis of OS patients was not analyzed. In addition, the effect of miR-493 on OS cell growth in vivo was explored. In the future, these two limitations will be focused on.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JX, MQ and HG designed the work that led to the submission. XY and WY performed RT-qPCR analysis to detect miR-493 and SP1 mRNA expression. MQ, JZ and YL mainly carry out functional experiments to investigate the roles of miR-493 in OS. DP and ZL conducted western blot analysis. MQ and HG drafted the manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Changzheng Hospital (Shanghai, China), and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Changzheng Hospital. Written informed consent was obtained from all patients for the use of their clinical tissues.

Consent for publication

Written informed consent was obtained from all patients for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

References


