



■ ONCOLOGY

Anti-tumour effect of tocilizumab for osteosarcoma cell lines

**T. Hagi,
T. Nakamura,
K. Kita,
T. Iino,
K. Asanuma,
A. Sudo**

From Mie University
Graduate School of
Medicine, Tsu, Japan

Aims

Tocilizumab, an interleukin-6 (IL-6) receptor (IL-6R) targeting antibody, enhances the anti-tumour effect of conventional chemotherapy in preclinical models of cancer. We investigated the anti-tumour effect of tocilizumab in osteosarcoma (OS) cell lines.

Methods

We used the 143B, HOS, and Saos-2 human OS cell lines. We first analyzed the IL-6 gene expression and IL-6R α protein expression in OS cells using reverse transcription real time quantitative-polymerase chain reaction (RT-qPCR) analysis and western blotting, respectively. We also assessed the effect of tocilizumab on OS cells using proliferation and invasion assay.

Results

The OS cell lines 143B, HOS, and Saos-2 expressed IL-6R. Recombinant human IL-6 treatment increased proliferation of 143B and HOS cells. Tocilizumab treatment decreased proliferation and invasion of 143B, HOS, and Saos-2.

Conclusion

In conclusion, we confirmed the production of IL-6 and the expression of IL-6R in OS cells and demonstrated that tocilizumab inhibits proliferation and invasion in OS cells.

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Keywords: Osteosarcoma, Tocilizumab, Anti-tumour effect, Interleukin-6, Interleukin-6 receptor

Article focus

- To investigate interleukin-6 (IL-6) production and IL-6 receptor (IL-6R) expression in osteosarcoma (OS) cells.
- To assess the effect of IL-6R-targeting antibody on OS cells.

Key messages

- The OS cell lines 143B, HOS, and Saos-2 express IL-6R α .
- Recombinant human IL-6 treatment increases proliferation of 143B, HOS, and Saos-2 cells.
- Tocilizumab treatment decreases proliferation and invasion of 143B, HOS, and Saos-2 cells.

Strengths and limitations

- The study highlights the potential of using tocilizumab in combination with other therapeutic agents.
- Only in vitro analysis was performed; further in vivo research is warranted

to confirm the applicability in clinical settings.

Introduction

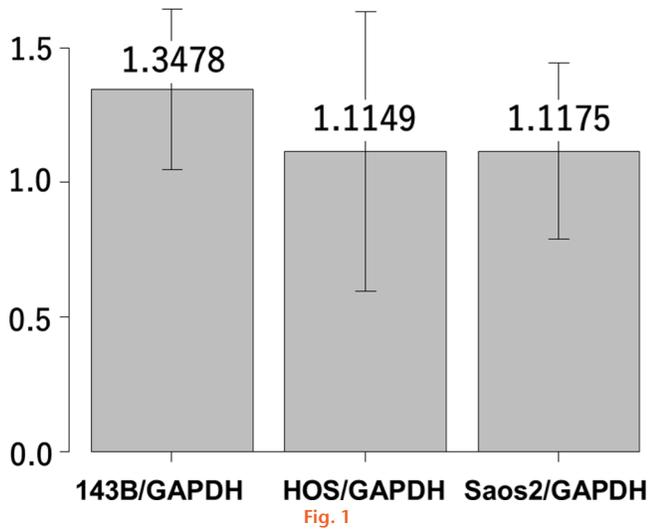
Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents.¹ The overall survival of non-metastatic OS has improved considerably since 1980, owing to the development of chemotherapy and surgical techniques;² however, the five-year survival rate remains at 60% to 80%.³ The treatment regimen of doxorubicin, cisplatin, methotrexate, and ifosfamide has not changed over the last two decades, thereby necessitating the development of new therapeutic strategies for improvement of survival in patients with OS.⁴

The cytokine interleukin-6 (IL-6) shows pleiotropic effects on various cell types in the tumour microenvironment and regulates the expression of signal transducer and activator of transcription 3 (STAT3), a pro-oncogenic transcription factor.⁵ IL-6 is produced in

Correspondence should be sent to Tomoki Nakamura; email: tomoki66@clin.medic.mie-u.ac.jp

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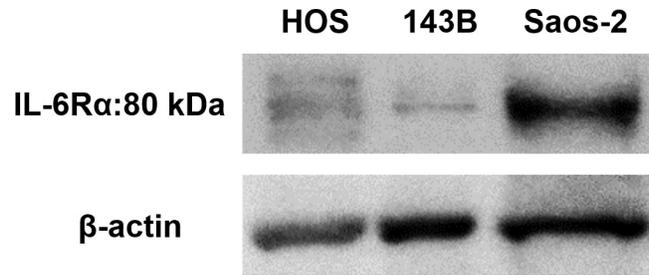
Reverse transcription real time quantitative-polymerase chain reaction (RT-qPCR) analysis showing interleukin-6 (IL-6) gene expression in 143B, HOS, and Saos-2 cells. Y-axis indicates the relative ratio of messenger RNA (mRNA). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

several cells, including fibroblasts, endothelial cells, macrophages, and cancer cells or tissues.^{6,7} Recently, several studies reported the association of high IL-6 levels with poor prognosis in patients with cancers such as in pancreatic cancer, breast cancer, colorectal cancer, small cell lung cancer, renal cell carcinoma, and soft tissue sarcoma.⁸⁻¹⁴ Moreover, serum IL-6 levels affected disease-free survival in patients with bone sarcoma.¹⁵ Tocilizumab (Chugai Pharmaceutical Co, Tokyo, Japan), an IL-6 receptor (IL-6R) targeting antibody, enhanced the anti-tumour effect of conventional chemotherapy in preclinical models of mucoepidermoid carcinoma.¹⁶ However, the effect of IL-6R-targeting antibody on OS has not yet been reported. Therefore, in this study, we investigated the anti-tumour effect of tocilizumab in OS cell lines. The study highlights the potential of using tocilizumab in combination with other therapeutic agents. Further in vivo research is warranted to confirm the applicability in clinical settings.

Methods

Osteosarcoma cell lines. We used the 143B, HOS, and Saos-2 human OS cell lines in this study; 143B and HOS were cultured in minimum essential medium (MEM; Gibco, Carlsbad, California, USA) containing 10% fetal bovine serum (FBS). Saos-2 was cultured in McCoy's 5A (modified) medium (Gibco) containing 15% FBS. Cells were maintained as attached monolayers and incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Reverse transcription real time quantitative-polymerase chain reaction analysis. Total RNA was isolated from the OS cell lines using the mirVANA Isolation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and reverse transcribed using random primers and the First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) for



Representative western blot images showing expression of interleukin-6 receptor-alpha (IL-6Rα) in 143B, HOS, and Saos-2 cells.

reverse transcription real time quantitative-polymerase chain reaction analysis (RT-qPCR). Real-time PCR for IL-6 expression was performed using the TaqMan Gene Expression Assays (Thermo Fisher Scientific) and ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Waltham, Massachusetts, USA). VIC-labelled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control.

Western blot analysis. The 143B, HOS, and Saos-2 cells were treated with 0 ng/ml, 10 ng/ml, or 100 ng/ml of recombinant human IL-6 (PeproTech, Rocky Hill, New Jersey, USA) for 24 hours, and lysed using radioimmunoprecipitation (RIPA) buffer (Millipore, Temecula, California, USA) supplemented with a protease inhibitor cocktail, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM Na₃VO₄. The 143B, HOS, and Saos-2 cells were treated with 100 ng/ml of recombinant human IL-6 for two hours, followed by 0 μg/ml, 10 μg/ml, or 100 μg/ml tocilizumab for 24 hours and lysed using RIPA buffer supplemented with a protease inhibitor cocktail, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM Na₃VO₄. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the samples were adjusted to the same protein concentration before loading. Proteins were transferred to a nitrocellulose membrane and blotted using the following antibodies (at the dilutions recommended by the manufacturer): IL-6Rα (1:200 dilution; Santa Cruz Biotechnology, Dallas, Texas, USA), STAT3 (1:2000; Cell Signalling Technology, Beverly, Massachusetts, USA), and phospho-STAT3 (1:2000; Cell Signalling Technology). β-actin was assayed as the loading control.

Cell proliferation assay. The 143B, HOS, and Saos-2 cells were seeded (1 × 10⁴ cells per well) in 96-well plates in 100 μl medium and treated with 10 ng/ml recombinant human IL-6 (using 10 μl recombinant human IL-6 solution) and 0, 10, 100, or 200 μg/ml tocilizumab for 24 hours. Cell viability was measured using the Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, Wisconsin, USA). The IL-6R-targeting antibody tocilizumab was kindly donated by Chugai Pharmaceutical Co. (Tokyo, Japan).

Cell invasion assay. Cell invasion assays were performed using the Corning BioCoat Matrigel Invasion Chambers

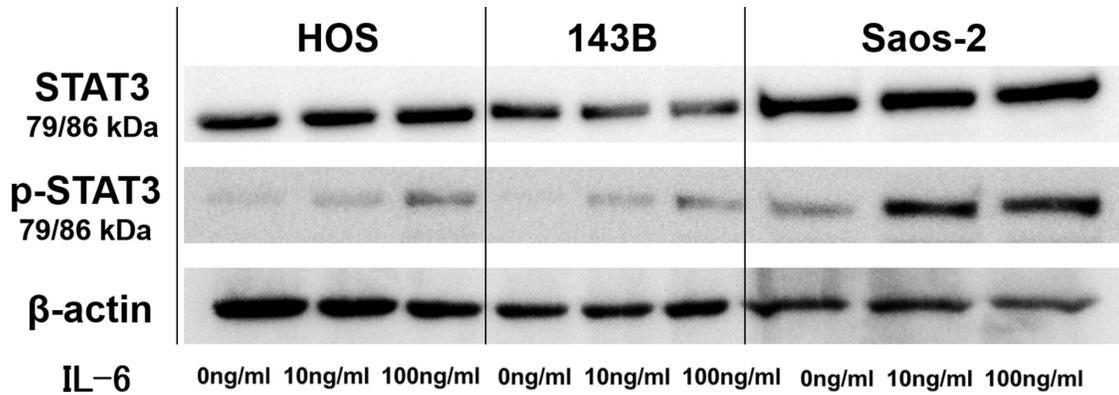


Fig. 3

Representative western blot images showing effect of interleukin-6 (IL-6) on phosphorylation of signal transducer and activator of transcription 3 (STAT3) in 143B, HOS, and Saos-2 cells. p-STAT3, phospho-STAT3.

(Corning, New York, USA). The 143B, HOS, and Saos-2 cells were seeded (3×10^4 cells/well) in the upper chamber, and serum-free medium containing IL-6 (10 ng/ml) with or without tocilizumab (100 μ g/ml) was added to the lower chamber. After 24 hours of incubation, non-migrating cells were removed from the upper chamber using a cotton swab, and the cells on the lower surface of the insert were stained with haematoxylin and eosin. Invading cells were recorded and counted using Olympus cellSens ver.1.18 software (Olympus, Tokyo, Japan) and a BX50 microscope (Olympus) equipped with the DP74 camera (Olympus).

Statistical analysis. All in vitro experiments were repeated at least three times to ensure reproducibility. Data are expressed as means \pm SD (Figures 1 and 2). Differences between two groups were compared using the Mann-Whitney non-parametric analysis of variance test. A p-value < 0.05 was considered to indicate statistical significance.

Results

Effect of recombinant human IL-6 on OS cell lines. We first analyzed the IL-6 gene expression and IL-6R α protein expression in the HOS, 143B, and Saos-2 cells using RT qPCR analysis and western blotting, respectively. The three OS cell lines showed different levels of IL-6 messenger RNA (mRNA) (Figure 1) and IL-6R α (Figure 2). Next, to determine the involvement of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway in IL-6 production, we treated the cells with recombinant human IL-6. STAT3 is constitutively activated via tyrosine phosphorylation in many human cancers. Therefore, we assayed STAT3 and phospho-STAT3 levels in HOS, 143B, and Saos-2 cells treated with different concentrations of recombinant human IL-6 using western blotting. All three cell lines expressed high levels of STAT3 and showed a dose-dependent increase in phospho-STAT3 levels (Figure 3). We next assessed the effect of different concentrations of recombinant human IL-6 on cell viability. Treatment with 100 ng/ml and 10 ng/ml recombinant

human IL-6 significantly increased the proliferation rates of HOS and 143B cells, respectively ($p < 0.05$, Mann-Whitney U test; Figure 4).

Effect of IL-6R-targeting antibody on OS cell lines. We next assessed the effect of different concentrations of tocilizumab, the IL-6R-targeting antibody, on the proliferation and invasion of HOS, 143B, and Saos-2 cells. Tocilizumab decreased cell viability in all three cell lines (Figure 5). For both HOS and Saos-2 cells, 100 μ g/ml and 200 μ g/ml tocilizumab significantly inhibited cell proliferation. In 143B cells, tocilizumab significantly decreased cell viability in a dose-dependent manner (Figure 5). We assayed the effect of tocilizumab to the STAT3 and phospho-STAT3 levels in OS cell lines under treatment with recombinant human IL-6 using western blotting. HOS and 143B cells expressed dose-dependent decrease in phospho-STAT3 levels, although there was no relationship between the expression of phospho-STAT3 and administration of tocilizumab (Figure 6). Cell invasion assays showed that the number of cells invading through the membrane was significantly lower for tocilizumab-treated cells, compared to that in the respective controls for all three OS cell lines ($p < 0.001$, Mann-Whitney U test; Figures 7 and 8).

Discussion

In the present study, we confirmed the expression of IL-6R in three OS cell lines. We also demonstrated that IL-6 promoted proliferation whereas tocilizumab, the IL-6R-targeting antibody, suppressed proliferation in OS cells. IL-6 plays an important role in the neoplastic process by affecting tumour cell adhesion, motility, proliferation, tumour-specific antigen expression, and thrombopoiesis.¹⁷ It regulates the crosstalk between tumour cells, inflammatory cells, and vascular endothelial cells and enhances the survival and plasticity of tumour stem cells.^{18,19} These findings provided the rationale for clinical trials investigating the efficacy of targeted IL-6 inhibitors in different cancers; these trials showed promising results.^{20,21} High IL-6 production and IL-6-mediated autocrine proliferation were reported in renal

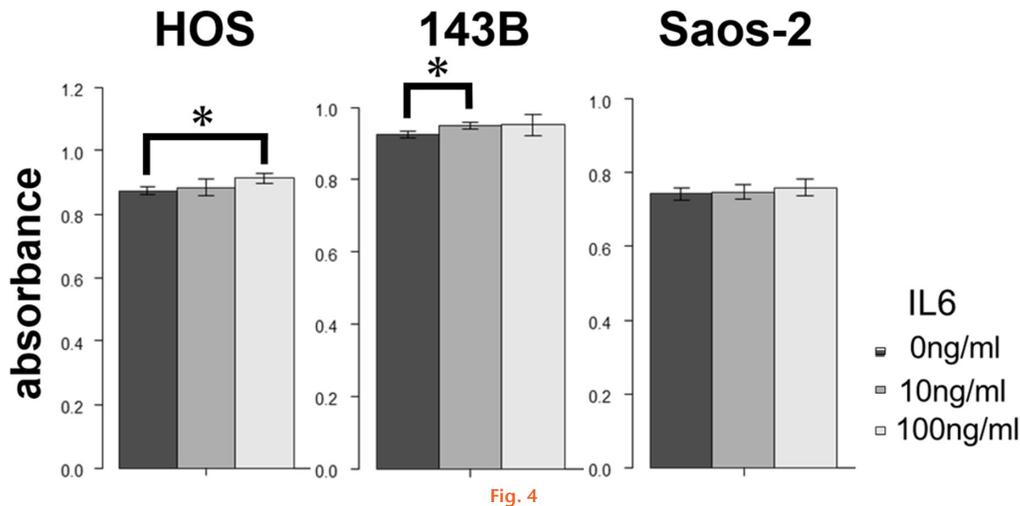


Fig. 4

Effect of treatment with recombinant human interleukin-6 (IL-6) on proliferation of 143B, HOS, and Saos-2 cells. * $p < 0.05$, Mann-Whitney U test.

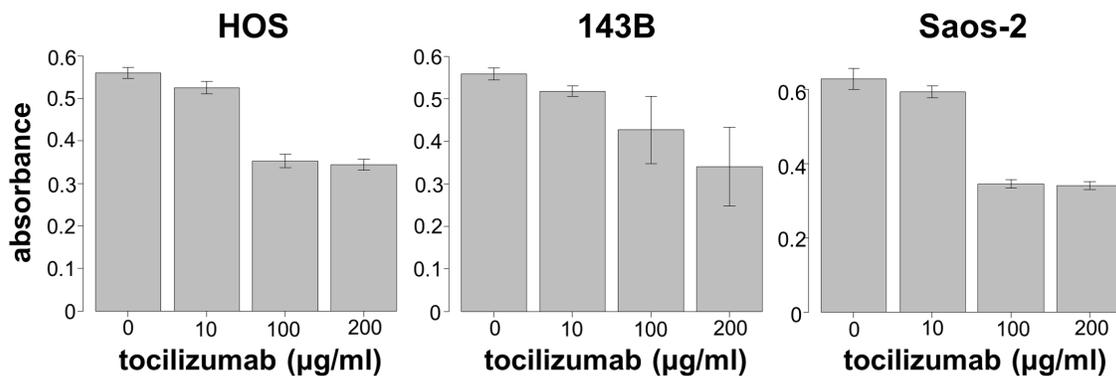


Fig. 5

Effect of tocilizumab treatment on proliferation of 143B, HOS, and Saos-2 cells.

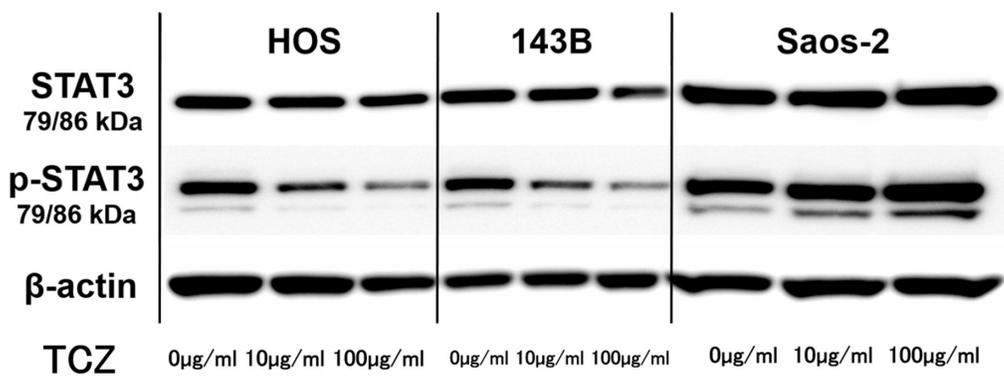


Fig. 6

Representative western blot images showing effect of tocilizumab (TCZ) treatment on phosphorylation of signal transducer and activator of transcription 3 (STAT3) in 143B, HOS, and Saos-2 cells with recombinant human interleukin-6 (IL-6). p-STAT3, phospho-STAT3.

cell carcinoma cell lines.²² Moreover, significant elevation of IL-6 expression in colorectal cancer tissues was associated with tumour invasion depth.²³ In the present study, we confirmed the production of IL-6 in 143B, HOS, and Saos-2 OS cell lines using RT-qPCR analysis.

We showed that IL-6 promoted STAT3 phosphorylation in the 143B, HOS, and Saos-2 OS cell lines. Binding of IL-6 to IL-6R induces the homodimerization of the glycoprotein 130 (gp130), an IL-6 transducer, leading to phosphorylation of JAK1, which then induces the phosphorylation

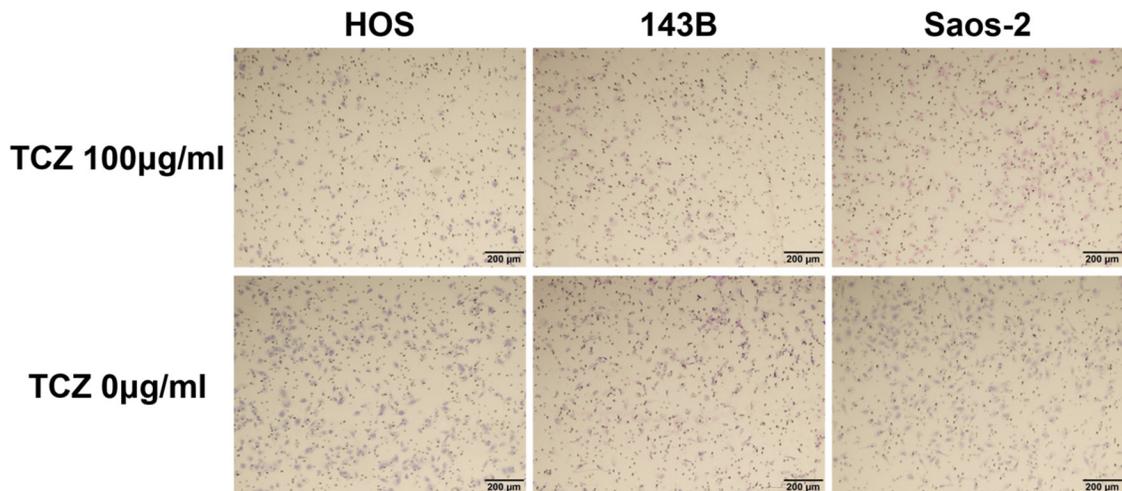


Fig. 7

Upper row shows the effect of tocilizumab (TCZ) treatment on invasion of 143B, HOS, and Saos-2 cells. Lower row shows the invasion of 143B, HOS, and Saos-2 cells without TCZ treatment.

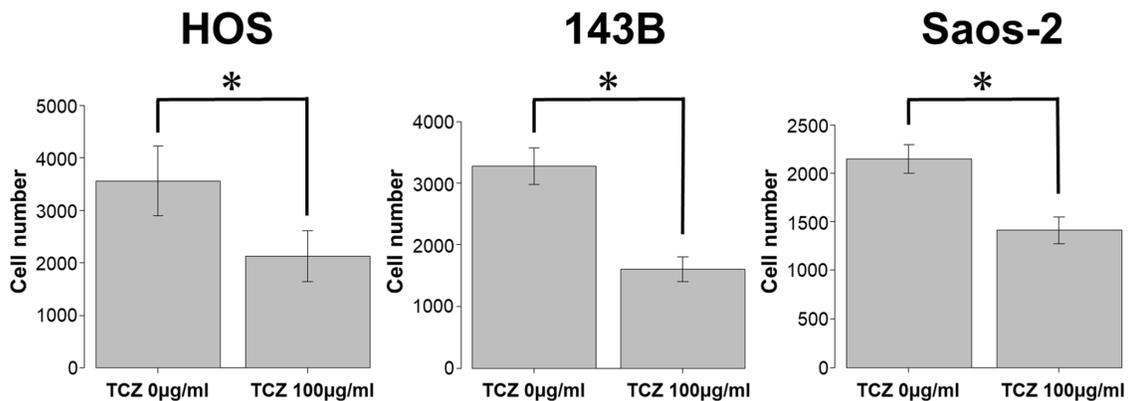


Fig. 8

Effect of tocilizumab (TCZ) treatment on invasion of 143B, HOS, and Saos-2 cells. * $p < 0.001$, Mann-Whitney U test.

and subsequent nuclear translocation of STAT3. STAT3 is a critical mediator of oncogenic signalling and is activated in many human cancers.^{24,25} Furthermore, activation of STAT3 signalling induces the expression of certain anti-apoptotic genes and several genes involved in promoting cell proliferation and tumour growth.²⁶

Tocilizumab is a fully humanized monoclonal antibody against IL-6R and has been approved for treatment of patients with rheumatoid arthritis.²⁷ In Japan, tocilizumab has also been approved for the treatment of polyarticular-course juvenile idiopathic arthritis, systemic-onset juvenile idiopathic arthritis, and Castleman's disease.²⁸ Currently, its anti-inflammatory effect and potency as an anti-tumour agent are being widely investigated, as this drug was initially investigated in the field of oncology. Several studies have reported that IL-6 represents a potential therapeutic target for several tumours, including sarcomas. IL-6R-targeting antibody inhibited IL-6 signalling, suppressed tumour angiogenesis and growth, reduced spheroid formation, and inhibited

cell cycle progression in colon cancer cell lines.^{29,30} IL-6R-targeting antibody also decreased tumour progression in ovarian cancer cell lines.³¹ Consistent with these findings, the present study showed that tocilizumab treatment inhibited cell proliferation and invasion in OS cells. Our study also showed that tocilizumab decreased phospho-STAT3 levels in HOS and 143B cells. These findings indicate that tocilizumab may suppress tumour progression via the STAT3 pathway. However, phospho-STAT3 levels were not decreased by the administration of tocilizumab in Saos-2 cells. The present result may suggest that further study should be performed using HOS or 143B.

Recently, a phase I trial conducted in the Netherlands, involving carboplatin/doxorubicin treatment in combination with tocilizumab for patients with recurrent epithelial ovarian cancer, showed the possibility of providing a survival benefit.³² Similarly, a combination therapy of paclitaxel and tocilizumab was suggested to benefit patients with mucocpidermoid carcinoma.¹⁶ Thus, combining tocilizumab with chemotherapeutic

agents might enhance the anti-tumour effect in patients with OS. In conclusion, we confirmed the presence of JAK-STAT pathway-mediated production of IL-6 and the expression of IL-6R in OS cells, and demonstrated that tocilizumab inhibited proliferation and invasion in OS cells. Further in vivo research is warranted to confirm the applicability in clinical settings.

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Author information:

- T. Hagi, MD, PhD, Assistant Professor
- T. Nakamura, MD, PhD, Assistant Professor
- K. Kita, MD, Graduate Student
- T. Iino, MDT(Medical lab technologist), Research Assistant
- K. Asanuma, MD, PhD, Assistant Professor
- A. Sudo, MD, PhD, Professor
Department of Orthopaedic Surgery, Mie University Graduate School of Medicine, Tsu, Japan.

Author contributions:

- T. Hagi: Performed the experiment, validation, formal analysis, investigation, and visualization, Curated the data, Wrote the original draft of the manuscript.
- T. Nakamura: Conceptualized and supervised the study, Performed the methodology, validation, formal analysis, investigation, and visualization, Collated the resources, Curated the data, Wrote the original draft of the manuscript, Conducted the project administration, Acquired the funding.
- K. Kita: Performed the experiment and validation.
- T. Iino: Performed the experiment.
- K. Asanuma: Performed the validation, Curated the data.
- A. Sudo: Wrote, reviewed, and edited the manuscript.

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ICMJE COI statement

- There is no conflict of interest in this article.

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