INTRODUCTION
Methylthioadenosine phosphorylase (MTAP) is an important enzyme in the salvage pathway of adenosine and methionine synthesis. The MTAP is ubiquitously present in all normal cells and tissues derived, but deficient in a variety of malignant tumors. The enzyme deficiency is found to be caused by MTAP gene deletion. The MTAP gene consists of 8 exons and resides on chromosome 9p21 close proximity to the p16INK4a and p14ARF. We wondered whether it is possible to treat with chemotherapy targeting MTAP, using osteosarcoma cell lines.

For MTAP expression, 6 osteosarcoma cell lines were analyzed by Western-blot. Then, for the relationship between MTAP expression and sensitivity to the inhibitors of de novo AMP synthesis, chemosensitivity of osteosarcoma cell lines were examined by MTT assay.

Expression of MTAP is negative in 4 of 6 (66.7 %) osteosarcoma cell lines. In vitro, MTAP-negative cell was more chemosensitive to the inhibitors of de novo AMP synthesis than MTAP-positive cell.

For these results, it can be exploited for selective chemotherapy to MTAP negative osteosarcoma patients with de novo purine synthesis inhibitors.

MATERIALS AND METHODS

Samples. Six human osteosarcoma cell lines (SaOS-2, U2OS, HOS, KHOS, G292 and MG63) and one osteoblast cell line(hFOB) were used. U2OS, HOS, KHOS, G292 and MG63 were obtained from the American Type Culture Collection (ATCC). SaOS-2 was obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. hFOB was osteoblast cell line that we used as control in Western blot. These cell lines were grown in Dulbecco’s modified Eagle’s medium (Gibco-BRL Laboratory, Grand Island, NY) containing 10% fetal bovine serum in a 37°C humidified, 5%CO2 and 95% air.

Transfection with MTAP expression vector
G292 osteosarcoma cells were transfected with LIPOFECTAMINE 2000 (Invitrogen, Carlsbad, CA,USA) in growth medium according to the manufacturer’s instructions. After transfection with MTAP expression vector, the cells were diluted at 1:10 and were selected in DMEM containing Zeocin at 400µg/ml. Following incubation for 2 weeks in the presence of Zeocin, selected cells were gathered and subjected to cloning by limiting dilution.

Western-blot assay. Cells were cultured to sub-confluence in 100 mm dishes containing growth medium. Cells were collected using a cell scraper and lysed in RIPA buffer. The protein concentration was resolved using the BCA Protein Assay Reagent Kit (Pierce) and the protein amount was balanced. The samples (25µg) were boiled in 6 X sample buffer and resolved by electrophoresis on 10% polyacrylamide gels using the described discontinuous buffering system. Gels were soaked for 30 min in transfer buffer (running buffer containing 15% methanol) and then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a Bio-Rad transfer apparatus. The membrane was washed in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated with the mouse monoclonal anti-MTAP antibody (1:250) overnight at 4°C. The membrane was washed three times with TBS-T, and MTAP was visualized using a goat anti-mouse antibody labeled with peroxidase (1:5000) and NBIC/NST substrate (Kirkegaard & Perry Laboratory, Gaithersburg, MD, USA).

The monoclonal anti-MTAP antibody. The monoclonal antibody was produced by the procedure described by Galfre and Milstein. Recombinant MTAP was injection into BALB/c mice. Hybridomas were screened the antibody production by ELISA with recombiant protein as antigen. Selected hybridomas were cloned at least by limiting dilution method. The monoclonal antibody screening clones were propagated as ascitic fluid by procedure of Harlow and Lane. The isotyping of monoclonal antibody was performed with a mouse monoclonal isotyping kit according to the manufacturer’s instructions. Antibody was concentrated by ammonium sulfate precipitation of ascitis, and immunoglobulin G (IgG) antibody was purified by using protein A column.

Cell proliferation assay. After cells had been counted with a hemocytometer, six osteosarcoma cell lines (SaOS-2, U2OS, HOS, KHOS, MG63 and G292) and transfecteda were incubated in 100 µl of medium containing 1X 10^5 cells for 24h, using 96-well cell culture cluster (Corning Incorporated Corning, NY, USA). After 24h, medium containing various concentrations of the test drugs (MTX and L-alanosine) were changed, following incubation for 72h. Then, 10µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) reagent (MTT, 5mg/ml) was added, followed by incubation for 4h at 37°C. Formazan that formed was extracted with 100µl of acid-isopropanol. After 24h, it was measured at a wavelength of 595 nm.

RESULTS
MTAP expression in osteosarcoma cell lines. The MTAP expression in six osteosarcoma cell lines was analyzed using a Western blot. The MTAP expression is detected in hFOB (osteoblast cell line), SaOS-2 and U2OS, and is not detected in HOS, MG63, KHOS, and G292 (Figure 1).

Chemosensitivity in vitro. Chemosensitivity of six osteosarcoma cell lines and transfecteda to inhibitors of AMP synthesis (MTX and L-alanosine) was estimated by MTT assay. The effects of inhibitors of AMP synthesis differed between MTAP positive (SaOS-2, U2OS and MTAP expression vector transfecteda) and MTAP negative (HOS, MG63, KHOS and G292) osteosarcoma cell lines. The MTAP negative cell lines were more chemosensitive than the MTAP positive cell lines at various concentrations of MTX and L-alanosine(Figure 2,3).

DISCUSSION
Generally, the chemotherapy for malignant tumor is toxic not only to malignant cells but also to normal cells, resulting in strict side effects on patients. MTAP is usually present in all normal cells but is deficient in many human cancer cell lines and primary tumors. MTAP deficient is a precise metabolic difference at the molecular level between normal and malignant cells. This difference can be exploited for a selective chemotherapy. In this study, we report that in osteosarcoma cell line, the selective chemotherapy targeting MTAP can be performed. These results suggest that the inhibitors of de novo AMP synthesis including MTX and L-alanosine may have a selective effect on MTAP negative osteosarcoma patients.