Introduction: Osteosarcoma is a malignant tumor that most commonly affects adolescents and young adults. The development of effective chemotherapy and wide local excision has led to improve the prognosis of patients with osteosarcoma. But pulmonary metastases occur and are major reason for fatal outcomes. New strategy for the treatment is needed. We reported gene therapy of chondrosarcoma using retroviral vectors. Our model system using gene therapy demonstrated that the strong bystander tumoricidal effect was shown in vitro and tumor size was reduced markedly in vivo. In this study we examined gene therapy for pulmonary metastases in murine osteosarcoma in order to improve the prognosis of patients with osteosarcoma.

Materials and Methods:
Tumor cells and animals: A murine osteosarcoma cell line, LM8, which preferentially metastasizes to the lungs, was used for evaluation of the efficacy of gene therapy. Male C3H/He mice aged 6 weeks were used to determine in vivo experiments.

Production of recombinant retrovirus vectors and retrovirus Infection:
We produced recombinant retrovirus vectors (pLTRNL) bearing a herpes simplex virus thymidine kinase (HSV-tk) gene. The strategy is to infect osteosarcoma cells by virtue of HSV-tk gene expression, and to sensitize them to antitherpetic drug, ganciclovir (GCV). Ganciclovir is a guanosine analog, which is metabolized to a cytotoxic product by HSV-tk. In the other vector (LZRNL) the HSV-tk gene was replaced by Escherichia coli β-galactosidase gene (lacZ gene). To produce a transmissible virus, vector DNA was transfected with the calcium phosphate coprecipitation method into amphotropic producer cells (PA317). The murine osteosarcoma cells (LM8) were plated in 10 cm dishes and infected 24h later by exposure for 48h to virus from the PA317/LTRNL vector-producer line or the PA317/LZRNL in the presence of 8 μg/ml polybrene. These were cultured with previous medium containing the G418 (neomycin analogue). The G418-resistant clones of LM8/LTRNL (LM8-tk) and LM8/LZRNL (LM8-Z) were selected randomly from the surviving colonies and used in the following experiments. PA317 cells were plated into flasks (125cm2) and cultured at 32 °C, 5% CO2 for 72h. virus vectors were concentrated by centrifugation with 6000g, 4 °C, 16h twice. High titer retrovirus was purified and used in the following experiments. In vitro GCV sensitivity and bystander effect: The cytotoxicity of GCV was determined by using a tetrazolium-based colorimetric assay (MTT assay). LM8-tk and LM8-Z were cocultured in various ratios and in order to examine bystander tumoricidal effect in vitro.

In vivo bystander effect: LM8-tk and LM8 cells in various ratio in order to examine bystander tumoricidal effect were injected into C3H/He mice subcutaneously and GCV were administered, intraperitoneally. The animals were divided into four groups. Five mice were injected with 1×10⁷ LM8-tk cells subcutaneously (group A). Group B was treated with 1×10⁷ cells (LM8-tk: LM8=1:1). Group C was treated with 1×10⁷ cells (LM8-tk: LM8=1:2). Group D was treated with LM8 cells only. The tumor volume was examined on day 21. We checked the weight of mice, the wet weight of removed lungs and the metastatic nodules on their surfaces on day 21. Inhibition of tumor growth and pulmonary metastasis: LM8 cells in various ratios and in order to examine bystander tumoricidal effect were injected into C3H/He mice subcutaneously. The animals were divided into two groups. Eight mice were injected with 100 μl virus from the PA317/LTRNL vector-producer line into the lateral tail vein. An additional 8 tumors were injected in a similar manner with isotonic saline. On day 14 and 21, Eight mice were injected with 100 μl virus. One week after first injection, mice were injected twice daily intraperitoneally for 14 days with GCV at 150 mg/kg of body weight. Tumor size was measured with calipers twice a week from day 0 (first ganciclovir treatment) to day 28.

Results: The morphology of the murine osteosarcoma cell line was unchanged after retroviral vector-mediated transduction of lacZ or HSV-tk genes. In vitro GCV sensitivity: The cytotoxic activity of GCV was dose-dependent in the HSV-tk gene-transduced clones of the murine osteosarcoma cell line. However, no effect was show in cells without gene transduction. 47% of the gene transferred cells were killed at the concentration of 1 μM of GCV, and 86% of the cells at the concentration of 10 μM.

In vivo bystander tumoricidal effect: We observed the bystander effect at various cell ratios in cocultures of LM8-tk and LM8-Z. At a ratio of LM8-tk: LM8-Z cells of 1:0 to 1:2, 65% of cells were killed.

In vivo bystander effect: On day 28 the mean tumor size of group A was 175 mm³, group B was 985mm³, group C was 3883mm³, and group D was 4827 mm³. Group A and B were suppressed significantly (Fig 1).

Inhibition of tumor growth and pulmonary metastasis: The mean weight of mice after injected with virus vector was 22g and the weight of mice after injected with isotonic saline was 24g. The mean wet weight of lungs treated with virus was 165 mg and the mean wet weight of lungs treated with isotonic saline was 219 mg (Fig 2). The mean number of nodules on lung surface treated with retrovirus was null and treated with isotonic saline were 3.1.

Conclusion and discussion: Gene therapy has been applied to many incurable cancers. The transfer of HSV-tk gene seems to become an important tool for gene therapy for malignancy. When HSV-tk transduced cells was present, nontransduced tumor cells were also destroyed, showing bystander effect. The remarkable inhibition of tumor growth and pulmonary metastases were confirmed in vivo experiments.