Introduction: It is necessary for bone metastasis research to establish a human breast cancer cell line which metastasizes to bone. MDA-MB-231 is a human breast cancer cell line and likely to metastasize to bone, injected into arterial circulation of nude mice. So, we isolated the highly invasive cell line from this cell line using matrigel invasion chamber. The highly invasive cell line (MDA-231-S5) was analyzed compared to the parental cell line (MDA-231-P).

Material and Method: Isolation of highly invasive cell line: We used a Boyden chamber assay to isolate highly matrigel invasive phenotype from MDA-MB-231. Cells were cultured to subconfluence in Dulbecco modified Eagle’s medium (DMEM) supplemented 10% fetal bovine serum (FBS) at 37°C and 5% CO2/95% air atmosphere. Then they were suspended in serum free DMEM and seeded on filters of the culture insert at a density of 5 X 10^5/ chamber. The filters have 8 μm pores and were precoated with matrigel (11 μg/filter). The upper compartment of the chamber was filled with serum free medium and the lower compartment of the chamber was filled with DMEM containing 10% FBS. The surfaces of mediums in both chambers were adjusted to the same height. They were incubated at 37°C for 24 hours. Invaded cells which appeared on the lower surface of filter were collected with EDTA/Trypsin technique and cultured in the medium containing 10% FBS. When collected cells were grown to subconfluence, they were seeded on the filters precoated with matrigel again. This passage was repeated for five times. The cell line that invaded to matrigel for five times (MDA-231S5) was analyzed compared to parental cell line (MDA-231P).

Matrigel invasion assay: Matrigel invasion assay was carried out in Boyden chambers. Cells were suspended in serum free DMEM and seeded onto filters precoated with matrigel(11 μg/filter) at a density of 5 X 10^5/ chamber. The lower compartment of the chamber was filled with DMEM containing 10%FBS. The surfaces of mediums in both chambers were adjusted to the same height. Cells were allowed to migrate for 24 hours at 37°C in a humidified atmosphere containing 5% CO2. Cells on the upper side of the filters were removed mechanically. Those that had migrated to the lower surface of the filters were stained with Diff Quick and counted with a light scope. Each assay was carried in triplicate.

Phagokinetic track assay: Phagokinetic track assay was performed for quantitative analysis of cell motility. Coverslips precoated with BSA or matrigel were placed in dishes and hot gold particle suspension was layered on top. Then the gold particle-coated coverslips were washed several times in DMEM supplemented 10% FBS and placed in another dishes. 2000 cells suspended in the medium were plated in the dishes and incubated for 72 hours. The phagokinetic tracks were observed on a light microscope.

Zymographic analysis: Gelatinase activities of the conditioned medium were tested by zymography using SDS-PAGE containing 1mg gelatin/ml. Cells were cultured in a subconfluent manner, then cultured for 24 hours in serum free medium. After that, the supernatant of conditioned medium was collected and loaded onto 10% polyacrylamid gel. Electrophoresis was performed with the addition of gelatin (1mg/ml) in the running gel. After electrophoresis, the gel was rinsed and stained with Coomassie blue and unstained area corresponding to zones of digestion by gelatinase was visualized after destaining. RT-PCR analysis: RT-PCR analysis was performed for expression analysis of some matrix metalloproteinases (MMPs). MMP-1 (Collagenase-1), MMP-3 (Stromelysin-1), MMP-9 (Gelatinase B), and MMP-13 (Collagenase-3) were investigated. These MMPs were expressed by MDA-MB-231. Total RNA from subconfluent cells grown on plastic dishes was isolated and used for first strand cDNA synthesis, following the manufacture’s protocol. The reversetranscribed cDNA was used in the amplification reaction with Taq polymerase and appropriate primers for gene product. The amplification products were observed by ethidium bromide staining after 1.5% agarose gels electrophoresis and compared to control products.

Assessment of osteolytic bone metastases: 1 X 10^5 cells suspended in 0.1ml PBS were injected into the left cardiac ventricles of female 5-week-old nude mice that were deeply anesthetized with pentobarbital (0.05mg/g). Nude mice were getting weak. Radiographs of nude mice were taken when they died. Osteolytic bone metastases in lower extremities were assessed with radiographs. All procedures were performed according to protocols approved by the committee of animal research of Mie University.

Results: MDA-231-S5 was more invasive to matrigel. Cell motilities were not different on coverslips precoated with BSA, but on coverslips precoated matrigel MDA-231-S5 is significantly more motile than MDA-231-P. Zymographic analysis revealed that gelatinase activity was elevated in MDA-231-S5. RT-PCR analysis demonstrated that expression of MMPs was increased in MDA-231-S5 compared to MDA-231-P. Especially, the expression of MMP-9 was significantly increased. All mice died after from 24 to 35 days after inoculation. The average survival of mice injected with MDA-231-P and MDA-231-S5 were 31 days and 26 days, respectively (Table 1). Survival rate analysis based on the Kaplan-Meier method revealed survival rate of mice injected of MDA-231-S5 was significantly lower than that of mice injected of MDA-231-P. Average number of bone metastases in MDA-231-P-bearing nude mice and MDA-231-S5-bearing nude mice were 0.5 and 2.0, respectively (Table 1). Pathological fractures caused by bone metastases were observed only in MDA-231-S5-bearing nude mice (Fig 1).

Discussion: It is generally recognized that MMPs play an important role when malignant tumors metastasize to another site. Malignant tumors must cross multiple extra cellular matrix barriers as they traverse the epithelial basement membrane and interstitial stroma, enter blood vessels, and extravasate to form metastatic deposit at a distant site. Because of this requirement MMPs are thought to facilitate invasion and metastasis by degrading structural extra cellular matrix component. Virtually the relationship between MMPs and metastasis has been often reported, but bone metastasis has not yet been fully understood because of less number of bone metastasis models. We invented the new method and isolated highly invasive cell line in vitro. MDA-231-S5, which is highly invasive cell line, has high MMP activities and metastasizes to bone to a great extent to cause a pathological fracture. This present study suggested that high MMP activities promote bone metastases. This cell line will be a useful model by which we discuss the mechanism how MMPs play a role in bone metastases. Moreover this in vitro isolation method is so useful system to be applied to another cell lines.

49th Annual Meeting of the Orthopaedic Research Society
Poster #1002

Table 1. Average survival and Average number of bone metastasis of nude mice bearing MDA-231-S5 and MDA-231-P.

<table>
<thead>
<tr>
<th></th>
<th>Average Survival</th>
<th>Average Number of Bone Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-231-P</td>
<td>26days</td>
<td>2</td>
</tr>
<tr>
<td>MDA-231-S5</td>
<td>31days</td>
<td>0.5</td>
</tr>
</tbody>
</table>

FIG. 1. Radiographs of osteolytic bone metastases in hind limbs of nude mice. Arrows indicates pathological fractures.