INTRODUCTION:
Ossification of the posterior longitudinal ligament (OPLL) of the spine is characterized by ectopic bone formation in the spinal ligaments. OPLL causes compression of the spinal cord and leads to various degrees of myelopathy. Various degrees of dysfunction, such as precise action and gait disturbance, lead to the restriction of activities involved in daily living and the deterioration of quality of life.

The occurrence and development of OPLL involve many environmental, systemic, and local factors. Genetic susceptibilities to OPLL have been identified by several groups.

According to the report of the Investigation Committee on OPLL of the Japanese Ministry of Health, Labour, and Welfare, ossification types have been classified into four types by latent plain radiographs: (1) continuous type; a long lesion extending over several vertebral bodies, (2) segmental type; one or several separate lesions behind the vertebral bodies, (3) mixed type; a combination of the continuous and segmental types, and (4) circumscribed type; mainly located posterior to a disc space.

OPLL often progresses after surgery, which may cause late-onset neurological deterioration. It was reported that the character of the patients who had OPLL progression related to age or OPLL type. As above, patients with mixed or continuous types of OPLL had the greatest risk for progression of ossification area.

Based on these observations, we made a hypothesis that mixed or continuous types of OPLL had a genetic risk factor of progression of ossification area. Then, we investigated genetic differences in osteogenic differentiation potency based on the classification.

METHODS:
Clinical diagnosis
The diagnosis of OPLL or non-OPLL was confirmed using X-rays and computed tomography of the cervical spine, preoperatively, by spine surgeons. Cases with OPLL were classified into four types; continuous, segmental, mixed or circumscribed type. We categorized into three groups; non-OPLL group (n=6), OPLL segmental group (segmental or circumscribed type) (n=6) or OPLL continuous group (continuous or mixed type) (n=6).

Spinal ligament primary cell culture
Paraspinous ligaments were harvested aseptically from patients during surgery to decompress the spinal cord for myelopathy, and cultured by the explant method. The fibroblast-like cells that migrated from the explants were harvested and replated in culture dishes for passage. Fibroblast-like cells were used for experiments after 5 passages.

Osteogenic induction, mRNA preparation, and cDNA synthesis
Cells were cultured in osteogenic medium (OSM: DMEM + 1% FBS + 0.1 µM dexamethasone) to induce osteogenesis for 24h, 48h, 3days and 7days or DMEM + 1% FBS (control). Total RNA was extracted from cells. The purity and integrity of the total RNA was verified. First strand cDNA was synthesized from 1 µg of total RNA using standard random hexamer priming techniques.

Real-time PCR analysis
Real-time PCR was carried out with Power SYBR Green PCR Master Mix on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The mRNA expressions of bone-related markers; BMP-2, Osterix and ALP were quantified by real-time RT-PCR. All samples were analyzed for G3PDH expression in parallel in the same run. Real-time PCR data were reported with the threshold level. To compare the different RNA samples in an experiment, we used the comparative Ct method.

ALP activity staining
Cells (OSM 0h, 3D and 7D) were stained by ALP activity stain kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer’s protocol. Number of stained cells/total cells was counted, and stained area was measured by ImageJ (http://rsb.info.nih.gov/ij/).

RESULTS:
In OPLL continuous group, mRNA expressions of BMP-2, ALP and Osterix were increased respectively after exposing to OSM as compared to OPLL segmental group and non-OPLL group. In OPLL segmental group and non-OPLL group, mRNA expressions of bone-related markers were increased, but not significantly (Figure 1). In ALP activity staining, both OPLL continuous group and OPLL segmental group had larger stained area than non-OPLL group. OPLL continuous group showed high osteogenic potency in comparison to OPLL segmental group (Figure 2).

DISCUSSION:
In this study, we surveyed genes being activated by OSM between OPLL continuous group and OPLL segmental group. In OPLL continuous group, mRNA expressions of bone-related markers were increased compared to OPLL segmental group and non-OPLL group. In OPLL continuous group, ALP activity stained cells/total cells and stained area was larger than OPLL segmental group and non-OPLL group. Until now, four types of OPLL have been studied as one condition, but we propose to distinguish OPLL continuous group from OPLL segmental group.