Role of HEY1 in osteosarcoma metastasis
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INTRODUCTION
The Notch pathway functions as an organizer in embryonic development. Notch proteins belong to a family of conserved transmembrane receptors that play a fundamental role in cell fate decisions including cell proliferation, differentiation, and apoptosis. Notch signaling is initiated by receptor–ligand interactions between neighboring cells resulting in the release of the intracellular domain (NIC), which translocates to the nucleus and binds RBP-Jc. RBP-Jc/NIC interactions result in the expression of various target genes including HES, HEY1, and HEY2. Recent studies have shown constitutive activation of the Notch pathway in various types of malignancies. However, it remains unclear the function of Notch pathway in human osteosarcoma. We previously reported that human osteosarcoma express elevated levels of Notch signal related genes. Inhibition of Notch pathway prevents osteosarcoma growth by cell cycle regulation in vitro and in vivo (Tanaka M.et al. British Journal of Cancer 2010). In this report, we examined the function of Notch target gene, HEY1. We found that HEY1 is up-regulated in human osteosarcoma. In addition, inhibition of HEY1 prevented osteosarcoma cell invasion and metastasis in vitro and in vivo.

METHODS:
Real-time PCR: To evaluate the expression of Notch pathway molecules, we performed real-time using human osteosarcoma cell lines.
Growth inhibition: We evaluate the effect of HEY1 and HEY2 knockdown by MTT assay.
Membrane assay: We evaluated osteosarcoma cell invasion by membrane assay following HEY1 and HEY2 knockdown.
RNA interference: HEY1 and HEY2 siRNA was purchased from Santa Cruz Biotechnology. An shRNA plasmid for human HEY1 was purchased from SABiosciences. Transfection of the plasmid was performed as supplier’s recommendations using FuGENE6.

Xenograft model of osteosarcoma lung metastasis:
143B cells were transfected with GFP lentiviral particles. Stably-GFP-expressing 143B cells (1 x 10^6) were mixed with a collagen gel in a 1:1 volume, and inoculated into the left knee joint of 6-weeks-old nude mice. Five weeks after inoculation, the mice were sacrificed. Metastatic nodules in the lungs were evaluated by direct microscopic visualization using an M165 FC microscope. Lung metastasis area was calculated by Lumina Vision.

RESULTS:
Over-expression of Notch pathway molecules in human osteosarcoma cell lines.
We have previously reported that Notch Pathway molecules are up-regulated in osteosarcoma biopsy specimens. We examined the expression of Notch pathway molecules in osteosarcoma cell lines. Real-time PCR revealed that osteosarcoma cell lines increased the expression of Notch2, Jagged1, Dll1, MAML1, HES1, HES5, HEY1, and HEY2.

Knockdown of HEY1 or HEY2 did not prevent osteosarcoma growth in vitro.
We examined the function of HEY1 and HEY2 because the expression of HEY1 and HEY2 were intensely up-regulated in the Notch target genes. To examine the function of HEY, we performed MTT assay following knockdown of HEY1 or HEY2. MTT assay revealed that knockdown of HEY1 or HEY2 did not prevent osteosarcoma growth.

Knockdown of HEY1 prevents osteosarcoma cell invasion in vitro.
To examine the function of HEY, we performed membrane assay following knockdown of HEY1 or HEY2. Membrane assay showed that knockdown of HEY1 prevents osteosarcoma invasion (Fig. 1). Knockdown of HEY2 did not prevent osteosarcoma cell invasion.

Knockdown of HEY1 prevents osteosarcoma metastasis in vivo.
Five weeks after inoculation, lung metastasis was evaluated. Seven of 7 control 143B cell inoculated mice showed lung metastasis. On the other hand, only 1 of 7 HEY1 shRNA transfected cell inoculated mice showed lung metastasis. Knockdown of HEY1 significantly decreased the lung metastasis in vivo (Fig. 2).

Knockdown of HEY1 decreased the expression of MMP9.
We examined the expression of epithelial-mesenchymal transition related genes following HEY1 knockdown. RT-PCR showed that knockdown of HEY1 decreased the expression of MMP9. ELISA assay showed that knockdown of HEY1 decreased the expression of MMP9 protein. These findings suggest that knockdown of HEY1 prevented osteosarcoma cell invasion via down-regulation of MMP9 expression.

DISCUSSION: We previously reported that inhibition of Notch pathway prevents osteosarcoma growth in vitro and in vivo. Our new findings suggest that Notch target gene promoted osteosarcoma cell metastasis via MMP9 expression.

SIGNIFICANCE: Our finding improves our understanding of osteosarcoma pathogenesis and suggests that inhibition of HEY1 may be regarded as an effective treatment for patients with osteosarcoma.