Hypoxia-induced Transmembrane Carbonic Anhydrase 9 Enzyme Inhibitor Suppresses Cell Proliferation, Migration And Invasion Of Osteosarcoma.

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Introduction: During solid tumor growth, subpopulations of tumor cells are exposed to variable conditions depending on the local and temporary supply of oxygen. Thus, the microenvironment surrounding the tumor cells is sometimes hypoxic (1) (2), and such hypoxic environment influences tumor proliferation, migration, invasion, metabolism, and viability (3) (4). Hypoxia is also associated with malignant progression and poor outcome in several cancers (5) (6), because of the suppression of apoptosis, increasing metastatic potential, chemotherapy and irradiation therapy resistance. Carbonic anhydrase (CA) 9 is one of the transmembrane enzymes that are induced by hypoxia (7). According to the previous report, CA9 is overexpressed in a wide variety of solid tumors, but is expressed in a limited way in normal tissues (8). The primary enzymatic function of CA9 is to catalyze the reversible hydration of carbon dioxide to bicarbonate and protons, and CA9 acts as a catalytic converter for the excretion of acid from cells. In addition to this role, there is evidence that CA9 contributes to cell proliferation, adhesion and migration (3) (4), which are vital for metastatic progression for the several malignant tumors. However, CA9 presence and function is unclear for osteosarcoma cells. CA9 has several roles in the high metabolic tumor such as osteosarcoma, and then we focus on CA9. In this study we investigate the effect of extracellular hypoxic conditions for human osteosarcoma cells, and quantify the CA9 inhibitor which will suppress the cell proliferation and migration.

Methods: To analyze the effect of different microenvironmental oxygen conditions for the cell growth and CA9 expression in human osteosarcoma cells, 5 osteosarcoma cell lines (143B, SaOS2, MG63, HOS and HuO9) were cultured in various oxygen concentrations (hypoxia 1% O2, normoxia 20% O2). Growth rate and expression of the CA9 proteins level were analyzed in each cell culture condition by MTS assay and western blotting. Furthermore, to examine the effect of CA9inhibitor for cell growth and migration, we tested CA9 specific inhibitor (215900-5MGCN; purchased from Calbiochem®).

In the proliferation assay, 4 osteosarcoma cell lines (143B, HOS, SaOS2 and HuO9) were analyzed. MG63 was excluded because of the low growth in 1% hypoxic condition. Each cell line was cultured in 96 well plates for each oxygen concentrations. Various concentrations of CA9 inhibitors were added, and MTS cell proliferation assay was performed. To investigate the effect of CA9 inhibitor for cell migration, we performed two assays (scratch migration assay and transewell chamber migration assay). For the scratch migration assay, osteosarcoma cells were seeded and grow until 80% confluent. Using 200μl pipette tip, a straight scratch wound was created. After incubation for 24hours, scratch line was photographed in 4 random areas, and cell-to-cell distance from each side of the scratch was calculated. Various concentrations of CA9 inhibitors were added after scratch and analyzed the difference of the distance. Transwell chambers containing polycarbonate membrane (8μm pore size, cell culture insert,
purchased from FALCON®) were used for the second assay. Osteosarcoma cells were cultured in each oxygen concentrations for 7 days. The cells were then seeded into the upper chambers at the density of 25000 cells/well and incubated for 24 hours with or without 10µM CA9 inhibitor. The cells that migrated to the bottom of the plates through the membrane were fixed and stained with Diff-Quick solution, and enumerated using a light microscope. We calculated the number of migrated cells and compared the differences with or without inhibitors in each oxygen concentration.

Results: Cell growth assay: The growth rate of osteosarcoma cells under hypoxic and normoxic conditions is shown in Figure 1A. MG63 cells did not grow well under hypoxia, whereas the other cells grew as well under hypoxia as under normoxia. HuO9 and HOS cells grew more rapidly under hypoxia than under normoxia. Thus, although growth rates of the osteosarcoma cells under hypoxic conditions differed between the tumor cell lines, hypoxic conditions did not suppress tumor cell growth, except for that of the MG63 osteosarcoma cell line. CA9 expression assay: CA9 expression was higher under hypoxic conditions than under normoxic conditions in all four cell lines, as previously reported in several cancers. CA9 expression of MG63 cells were also highly expressed under hypoxic condition, while the cells didn’t grow well in hypoxia (Fig. 1B). In all 4 osteosarcoma cell lines, 100µM CA9 inhibitors in each oxygen concentration significantly reduced the tumor growth except HOS in hypoxia (Fig. 1C).

Cell migration assay: In the scratch migration assay, cell-to-cell distance under the CA9 inhibitor condition was significantly larger than no inhibitor condition in all cell lines (Fig.2: SaOS2 and 143B data showed). The effect of CA9 inhibitor for cell migration was appeared even at a concentration of 1µM of it.

The number of invaded cells through transwell chambers was higher in hypoxic condition than in normoxic condition. In addition, the number of invaded cells with 10µM inhibitor in hypoxic condition was lower than that without inhibitor (Fig.3: SaOS2 data showed). From the results of migration assay, CA9 inhibitor suppressed the cell migration under normoxic and hypoxic condition, and the invasiveness used transwell chamber was suppressed especially in hypoxic condition.

Discussion: From the results of this study, the expression level of CA9 in osteosarcoma cell lines was promoted by hypoxic conditions, which are generally caused by high metabolism of tumor cells in high grade solid tumors. Tumor cells with high metabolism have the potential to be invasiveness, and such tumor cells would show high expression of CA9. An increase in hypoxia will increase the tumor cell migration as the cell migration study’s results showed, and the CA9 inhibitor has potential to suppress the cell proliferation and migration as this study showed. In other words, the expression of CA9 might correlate with hypoxia, and this increase in CA9 under hypoxia will stimulate cell migration, invasion and proliferation. We already reported that CA9 can become a intrinsic marker of hypoxia, and that CA9 correlates with tumor acidity and hypoxia, and that CA9 expression levels correlate with poor prognosis in osteosarcoma patients (9) (10). Previous papers also showed that CA9 has not only an enzymatic activity to induce the acidification of extracellular pH, but also physical function to perturb tight intracellular contacts linked to the cytoskeleton through competition with E-cadherin in binding with beta-catenin (11). Consistent with these date, our migration study showed that CA9 inhibitors suppressed tumor invasiveness under hypoxic condition in osteosarcoma cells. From the results, CA9 is highly expressed and CA9 inhibitor has a potential to suppress the tumor invasiveness or metastasis which leads to poorer prognosis in osteosarcoma patients.
**Significance:** Our study suggests that hypoxia can increases the cell migration potential and CA IX inhibitor suppresses the cell proliferation and migration.

![Figure 1A](image1.png) The growth rate of 5 osteosarcoma cell lines in each oxygen tensions at the time of the cells were 99% confluent in normoxia. 1B: The result of western blotting for the expression of CA9 in each oxygen tensions. 1C: The proliferation rate of 4 osteosarcoma cell lines in each oxygen tensions with or without CA9 inhibitor.

![Figure 2](image2.png) The results of scratch migration assay; cell to cell distance and the effect of CA9 inhibitor in each oxygen tension.
Figure 3: The transwell chamber migration assay: SaOS2 cells stained by Diff-Quick solution (left figure), and the invaded cell numbers in each oxygen tension with or without 10μM inhibitor (right table).

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