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学位論文の題目	Double-faced CX3CL1 enhances lymphangiogenesis-dependent metastasis in an aggressive subclone of oral squamous cell carcinoma (高悪性度口腔扁平上皮癌において、CX3CL1 はリンパ管新生を増強しリンパ節転移に寄与する)
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学位論文内容の要旨

Introduction:

Oral squamous cell carcinoma (OSCC) is characterized by intratumoral heterogeneity and plasticity. On the other hand, Chemokines are small chemotactic cytokines with various effects on tumor microenvironment (TME) inside the TME. Their expression can be observed not only cancer cells, but also endothelial cells and immune cells. Therefore, chemokines can have multifaceted roles of tumor-inhibiting and tumor-promoting.

CX3CL1 is a member of the C-X3-C motif chemokine with high affinity to the chemokine receptor, CX3CR1. Due to the expression of CX3CL1 and CX3CR1 on numerous structures inside the TME, CX3CL1-CX3CR1 axis can influence cancer progression. Previous reports showed the involvement of CX3CL1 in cancer progression, recruitment of immune cells, endothelial cell growth, and trans-endothelial migration of cells. However, the role of CX3CL1 in the OSCC is unclear.

Since CX3CL1 involve in cancer progression as well as immune cell recruitment and endothelial cell formation, our study utilized Mouse oral squamous cell carcinoma (MOC) 1 and MOC2, the syngeneic murine OSCC cell lines. These cell lines are injectable into wild-type mice with competent immune systems. These cell lines allowed us to observe the full capacity of chemokine CX3CL1 on cancer cells and the supporting structures of TME.

In this study, we sought to identify the role of CX3CL1 in OSCC and its TME.

Materials and Methods:

We established the CX3CL1 overexpression models of MOC1 and MOC2, termed MOC1^{CX3CL1} and MOC2^{CX3CL1}. For the in-vitro experiments, we performed the MTS assay and transwell migration assay to access the cell proliferation and migration abilities, respectively. For the in-vivo experiments, we created the tumor models of MOC and CX3CL1 overexpressed MOC cells. Using the tumor tissue, we performed HE, immunohistochemistry and double fluorescence staining and analyzed using image J software. After observing the effect of CX3CL1

overexpression in TME, we created the CX3CL1 domain cleaved models of MOC1 and MOC2 further to discover the role of the functional domain of CX3CL1. We repeated the same in-vitro and in-vivo experiments using the domain cleaved models.

From there, we used the human cell line, HSC-3 and HSC-3-M3 cell lines to confirm the expression of CX3CL1 using immunocytochemistry staining and PCR analysis. Then, we used human OSCC patients with lymphatic metastasis who had no prior treatments. We performed immunohistochemistry staining on human tumor tissues and analyzed.

Statistical analyses were performed using GraphPad Prism 9.1.1 and P values less than 0.05 were considered significant.

Results:

MTS assay and Transwell migration assay showed that CX3CL1 can inhibit cell proliferation and promote cell migration. Similarly, the in-vivo results showed smaller tumor sizes in both MOC1 and MOC2, which correlated with the in-vitro results. On the other hand, MOC2 tumors showed increased metastases in the cervical lymph nodes (LNs). However, there was no change in the metastasis abilities of MOC1 tumors. Therefore, we analyzed the histopathological changes of the $MOC1^{CX3CL1}$ and $MOC2^{CX3CL1}$ tumors. $MOC1^{CX3CL1}$ and $MOC2^{CX3CL1}$ tumors recruited the $CX3CR1^{+}$ cell population into the tumor microenvironment. $MOC1^{CX3CL1}$ recruited cytotoxic CD8 T cells while $MOC2^{CX3CL1}$ recruited regulatory T cells. $MOC1^{CX3CL1}$ tumors showed increased keratinization after CX3CL1 overexpression, indicating a higher OSCC differentiation and a better prognosis. On the other hand, $MOC2^{CX3Cl1}$ tumors had increased invasion of lymphatic vessel structures. On a detailed analysis of lymphatic structures, the lymphatic vessels change from tube-like structures to complex mesh-like networks with increased transendothelial migration of $CX3CR1^{+}$ cells into lymphatic structures.

In CX3CL1 domain cleaved models, the increased in cell migration was canceled. In addition, CX3CR1 recruitment and lymphatic vessels formation were also cancel inside the tumor tissue of domain cleaved models. As the results, cervical LN metastasis were also reduced.

The highly aggressive cancer, MOC2, exhibits higher CX3CL1 expression than the indolent MOC1 cancer. CX3CL1 expression in MOC2 tumors was higher in metastasis sites. Similar change was also observed in the HSC-3-M3 cells compared to HSC-3 cells. Further, 45 patients with LN metastasis show that patients with CX3CL1 enrichment have a higher rate of lymphatic vessel formation near tumor nests. Also, CX3CL1 enrichment group has lower 12-year overall survival rates than CX3CL1 stable group.

Discussion:

The role of CX3CL1 in MOC1, indolent cancer and MOC2, aggressive cancer was different because CX3CL1 influence on not only the cancer cell but also on the TME. Since MOC1 and MOC2 are the syngeneic mouse cancer cells, we can assess CX3CL1 effect in immunocompetent TME. The different type of cancer stroma changes and immune cell recruitment occurred in indolent cancer and aggressive cancer. This outcome may be due to MOC1 possess the immune-inflamed phenotype and MOC2 has the immune-excluded phenotype although further detailed

study is needed for this conclusion. In this study, we understood that the same chemokine effects changes drastically depending on cancer phenotype, which can help us better understand in studying chemokine in cancer.

Next, CX3CL1 promoted the lymphangiogenesis and changed shape of lymphatic structures inside TME. CX3CL1 increased transmigration of cancer cells, increasing lymphatic circulating cells in lymphatic vessels and promoting cervical LN metastases. The detailed mechanism in which CX3CL1 influences on the aggressive OSCC cancer was understood. We also confirmed the signal peptide and chemokine domains of CX3CL1 are essential in metastasis process of OSCC cancer. The increasing expression of CX3CL1, termed CX3CL1 enrichment in lymphatic metastasis was observed in the MOC2 tumor tissues, Human HSC-3 and HSC-3-M3 cell lines has prognosis values in predicting the lower overall survival rate, increasing distance metastasis rate and recurrence rate of OSCC patients.

Conclusion:

The multifaceted roles of CX3CL1 and its expression enrichment at the metastatic site can potentially be used as a prognostic predictor in OSCC with LN metastasis and can be used in long-term monitoring. Our research provides a better understanding of the dual roles of chemokines and their relationship with different cancer phenotypes, which can be used as the strategic approach to treating cancer.

論文審査結果の要旨

Introduction: Oral squamous cell carcinoma (OSCC) is characterized by high heterogeneity and plasticity. On the other hand, CX3C motif ligand 1 (CX3CL1) is a double-faced chemokine with anti- and pro-tumor functions. Due to the expression of CX3CL1 and CX3CR1 on various structures inside tumor microenvironment (TME), CX3CL1-CX3CR1 axis can influence cancer progression. This study sought to identify the role of CX3CL1 in OSCC and its TME.

Materials and Methods: The present study used mouse oral squamous cell carcinoma cell line: MOC1 and MOC2, human cell lines: HSC-3 and HSC-3-M3, to confirm the CX3CL1 role in cancer and tumor-supporting stroma. Regarding the research tools, microarray analysis, RT-PCR analysis, HE, IHC, double IHC staining are mainly used. Human patient samples were also used in this study.

Results: MTS assay and Transwell migration assay showed that CX3CL1 can inhibit cell proliferation and promote cell migration. In animal models, smaller tumor sizes were observed after CX3CL1 overexpression. However, $MOC1^{CX3CL1}$ tumors showed increased keratinization after CX3CL1 overexpression, indicating a higher OSCC differentiation and a better prognosis. On the other hand, $MOC2^{CX3CL1}$ tumors had increased invasion of lymphatic vessel structures. On a detailed analysis, the lymphatic vessels changed structurally with increased transendothelial migration of $CX3CR1^{+}$ cells in MOC2 tumors. In CX3CL1 domain-cleaved models, the lymphangiogenesis and metastasis were canceled, showing that CX3CL1 domains were necessary for metastasis. CX3CL1 expression change in metastasis cancer cells were observed in MOC2 tumors and HSC-3-M3 cells. Patients with LN metastasis showed that when CX3CL1 expression increased in metastasis site, the patients had lower 12-year overall survival rates and higher recurrence and distance metastasis rates.

Discussion: These results suggest that CX3CL1 effects on TME is dependent on the cancer phenotypes. Furthermore, the study established that CX3CL1 promoted cancer metastasis via lymphatic pathway by stimulating lymphangiogenesis. In addition, the present study demonstrates the prognostic value of CX3CL1 enrichment in long-term monitoring in OSCC.

This research provided insight into the cancer phenotypes' influences on CX3CL1 effects in TME, and CX3CL1 promotes cervical lymphatic metastasis via the lymphatic pathway in metastatic cancer. The results are satisfactory and potentially serve CX3CL1 as the prognostic predictor for future OSCC diagnosis and follow-ups. This paper has been accepted by JCI Insight and is internationally acclaimed. Therefore, the defense committee hereby agrees with this article as a doctoral dissertation in Dentistry.