学位論文内容の要旨
（Summary of dissertation）

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学 位 論 文 題 名
Studies on the roles of EMT-associated microRNAs in cervical cancer and aggressive endometrial cancer
（子宮頸癌及び高悪性度子宮体癌の上皮間葉転換における microRNA の役割とその作用メカニズムの解明に関する研究）

[Background and Objectives]

Cervical cancer (CC) is a common gynecological malignancy. Poorly-differentiated endometrioid adenocarcinoma and serous adenocarcinoma represent an aggressive subtype of endometrial cancer (EC). Cancer metastasis begins with the process of epithelial-mesenchymal transition (EMT), which converts well-polarized epithelial cells to non-polarized mesenchymal cells that acquire motility and invasion properties and exhibit cancer stem cell-like properties. The 90-kDa heat shock protein (HSP90) promotes EMT and tumor progression by protecting and stabilizing its client proteins. In addition, programmed death-ligand-1 (PD-L1) is known to exhibit a tumor cell-intrinsic function in mediating immune-independent tumor progression. MicroRNAs (miRNAs) function as post-transcriptional regulators of mRNAs by inhibiting the translation of their respective RNA targets or degrading their targets. Long non-coding RNAs (lncRNAs) can act as molecular sponges of miRNAs, thereby affecting the expression of target genes of miRNAs. In CHAPTER 1, we aimed to explore whether miR-361 directly targets HSP90 to inhibit EMT features and whether NEAT1 suppresses miR-361 expression and induces EMT and sphere formation in CC cells. In CHAPTER 2, we aimed to reveal the functional role of tumor cell-intrinsic PD-L1 expression in aggressive EC cells and the miRNA-associated mechanisms that regulate PD-L1 expression.

[Materials and Methods]

The levels of miR-361 or PD-L1 in one immortalized but non-malignant human endometrial cell line (EM), CC cell lines, and aggressive EC cell lines were measured by real-time PCR analysis or western blotting analysis. Cell functional assays were used to explore the effects of miR-361 or PD-L1 overexpression/knockdown on the malignant properties of cancer cells. Luciferase reporter assays were performed to verify the interaction between miRNAs and their target genes.

[Results]

[1] In CHAPTER 1, we showed that the levels of miR-361 in CC cell lines were significantly lower than those in EM cells. Lower expression of miR-361 was significantly associated with a poorer prognosis in CC patients.

[2] Overexpression of miR-361 induced an epithelial phenotype and significantly decreased cell invasion and sphere formation. Upregulation of miR-361 increased the expression of E-cadherin and inhibited the expression of Vimentin in CC cells.

[3] HSP90 expression was significantly higher in CC tissues and CC cells. CC patients with higher HSP90 expression had worse overall survival than those with lower HSP90 expression. Ectopic expression of miR-361 decreased, while inhibition of miR-361 increased the expression of HSP90 in CC cells. The luciferase reporter assays demonstrated that miR-361 directly targets and reduces HSP90 expression in CC cells.

[4] Knockdown of HSP90 reduced the mesenchymal phenotype of CC cells and significantly inhibited CC cell invasion and cancer stem cell properties. Silencing of HSP90 enhanced the expression of E-cadherin and reduced the protein expression of Vimentin.

[5] Our meta-analysis revealed a significantly higher level of NEAT1 in CC samples compared with normal samples. The expression of NEAT1 was significantly upregulated in CC cells

compared with EM cells. The depletion of NEAT1 by siRNA significantly upregulated miR-361 levels in CC cells. The luciferase reporter assays verified the direct binding relationship between NEAT1 and miR-361. The silencing of NEAT1 significantly suppressed the expression of HSP90 in CC cells. Cell invasion and sphere formation was decreased after NEAT1 knockdown. Our results supported that NEAT1 promotes CC cell invasion and sphere formation through upregulating HSP90 expression by binding with miR-361, a tumor suppressor that directly suppresses HSP90 expression.

[6] In CHAPTER 2, we found that expression of *PD-L1* in primary EC tissues was lower than that in normal samples. Higher expression of PD-L1 was associated with increased overall survival in EC patients. Our western blotting analysis confirmed that PD-L1 protein was expressed at lower levels in all aggressive EC cell lines compared to EM cells.

[7] Overexpression of PD-L1 in multiple aggressive EC cells significantly attenuated cell proliferation, migration, and invasion, while inducing cell apoptosis. The silencing of MCL-1 expression with MCL-1-specific siRNA largely reversed PD-L1 knockdown-induced mesenchymal cellular morphology and significantly inhibited the migratory and invasive ability of EC cells that was enhanced by the knockdown of PD-L1.

[8] Using qRT-PCR assays, we validated that miR-216a was significantly upregulated in aggressive EC cells as compared to EM cells. The luciferase reporter and western blotting assays demonstrated that miR-216a directly targeted and inhibited the expression of PD-L1 in aggressive EC cells.

[9] Furthermore, we showed that overexpression of miR-216a significantly induced the migration and invasion of HEC-50 cells, and cell migration and invasion were significantly reduced in SPAC-1-L cells following knockdown of miR-216a.

[10] The lncRNA MEG3 exhibited significantly lower expression in EC tissues as compared to normal tissues. High expression of MEG3 was associated with a favorable prognosis in EC patients. Knocking down MEG3 expression promoted miR-216a expression and reduced the protein expression of PD-L1 in aggressive EC cells.

[Discussion]

In CHAPTER 1, our findings support the complexity of miR-361-regulated signaling pathways that determine the phenotypes of human tumor cells and demonstrate that loss of miR-361 expression elevates HSP90 levels, leading to the acquisition of EMT and cancer stem cell-like phenotypes of CC cells. In addition, HSP90 could be secreted by cancer cells, and extracellular HSP90 promotes EMT and cancer cell invasion and stimulates metastatic spread. Whether secreted HSP90 acts as a pivotal regulator of CC progression and metastasis requires further investigation. Moreover, the overexpression of NEAT1 exerts its oncogenic functions in the majority of human cancers by functioning as a molecular sponge for miRNAs. In this study, we demonstrated for the first time that, by competitively binding to miR-361 and suppressing its expression, NEAT1 upregulates the expression of HSP90 to promote EMT, invasion, and sphere formation of CC cells. Future investigation will be required to determine the mechanisms by which NEAT1 performs this function in CC.

In CHAPTER 2, in line with previous reports describing an inverse correlation between the levels of PD-L1 and the degree of tumor malignancy in human EC, we found that the protein expression of PD-L1 was frequently lost in EC tissues compared with normal endometrium samples. Our results showed that tumor cell-intrinsic PD-L1 has tumor-suppressive functions in aggressive EC cells, at least through its negative modulation of EMT. Thus, the silencing of PD-L1 may be a molecular mechanism for inducing and maintaining the mesenchymal state of aggressive EC cells. It would be interesting to further determine the downstream targets of miR-216a and MEG3 in aggressive EC.

[Conclusion]

MiR-361 directly targets HSP90 to inhibit invasion and EMT features, and NEAT1 functions as an oncogenic lncRNA to suppress miR-361 expression in CC cells. In parallel, PD-L1 has a tumor cell-intrinsic role in suppressing the EMT features of aggressive EC cells. Our study identified MEG3 and miR-216a as critical upstream regulators of PD-L1. Together, these findings provide new insights into the roles of miRNAs implicated in the EMT of CC and aggressive EC.