論文の内容の要旨

論文題目　Development of a novel miR-34-regulated oncolytic coxsackievirus B3 with minimal toxicity and strong anti-tumor activity.
（低臓器障害性で強力な抗腫瘍活性を有する miR-34 制御新規腫瘍溶解性コクサッキーウイルス B3 の開発）

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Lung cancer became the major cause of cancer deaths among Japanese, making up approximately 25% of all cancer deaths in men and 14% in women. Five-year survival of lung cancer patients is 39.1% for all stages, despite the use of intensive combined therapies and recent advances in molecular targeting therapies. To improve this poor prognosis, new therapeutic modalities are urgently required.

Oncolytic viruses (OVs) have emerged as new modalities for cancer treatment depending on tumor cell lysis by preferential replication of OVs in tumor cells followed by activation of host's anti-tumor immunity. Very recently, talimogene laherparepvec (T-Vec), a modified herpes simplex virus type 1 encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), is the first oncolytic virus approved by US Food and Drug Administration for the advanced melanoma. The success of this therapy encourages scientists to make further research in this new field. Till now, other types of OVs, such as adenoviruses, reoviruses, measles viruses, and Newcastle disease viruses, have been developed and pre-clinical and clinical studies for many cancers have been performed. However, tumor cells often become resistant to OVs due to downregulation of their receptors, resulting in insufficient proliferation and transmission of viruses. Therefore, novel OVs targeting different tumor-expressing receptors with strong proliferative ability would be useful in the clinical setting.

In order to obtain a novel OV, our lab previously screened 28 enterovirus strains and found that coxsackievirus B3 (CVB3), which uses coxsackievirus and adenovirus receptor (CAR) and decay-accelerating factor (DAF) as its receptors, was a novel OV with a strong ability to lyse human non–small cell lung cancer cells (NSCLC). CVB3 is a small, nonenveloped, single-stranded, positive-sense RNA virus and belongs to the *Enterovirus* genus within the *Picornaviridae* family. Its rapid replication and the broad expression of its receptors are considered to broaden the range of target cancers potentially susceptible to CVB3 therapy, resulting in a strong and sustaining anti-tumor effect. However, during subsequent characterization of CVB3 toward clinical translation of the virus, several non-negligible toxicities were observed in mouse tumor models due to its broad tropism, particularly in pancreas. Consequently,

elimination of these virulent features of CVB3 is essential for developing CVB3-based OVs in the clinical setting.

To inhibit viral replication-induced cytotoxicity in non-tumor cells, I adopted gene silencing mechanism of micro RNAs (miRNAs) by inserting target sequences of tumor suppressor miRNAs, which are abundantly expressed in non-tumor cells, in the genome of oncolytic CVB3. miRNAs mainly cause degradation or translational inhibition of mRNAs by guiding the RNA-induced silencing complex (RISC) to target sites. Among these miRNAs, I focused on miRNA-34 family, which consists of only three members of miR-34a, miR-34b and miR-34c and was reported to be broadly down-regulated in many tumor cells including NSCLC, to decrease off target replication of CVB3 in normal organ cells and keep vigorous virus replication in tumor cells.

Additionally, the insertable location of miRNA target sites (miRTs) is another important issue to suppress viral replication in normal organ cells, while maintaining oncolytic activity in cancer cells. Based on previous reports about insertion-tolerant sites of picornaviruses, I predicted that miRTs could be inserted in the 5' region immediately upstream of the start codon, as well as in the 3'region just downstream of the stop codon. Furthermore, insertion of four copies of miRT in viral vector genomes was reported to inhibit virus replication by complementary miRNAs. Therefore, I engineered miRNA-regulated CVB3s (miRT-CVBs) by inserting four tandem miR-34a target sites (miR-34aT) or miR-34c target sites (miR-34cT) into the 5'UTR (5-CVBs) or 3'UTR (3-CVBs) of the CVB3 genome.

First, I transfected synthetic miR-34a or miR-34c mimics to H1299 cells, which were expressing low levels of miR-34a/c compared to normal cells, and then inoculated the cells with miRT-CVBs or wild type (WT-) CVBs. After 72 h, the miR34a or miR-34c mimics-transfected cells exhibited much less cell lysis when infected with miRT-CVBs harboring complimentary miRTs, while in untransfected H1299 cells, all miRT-CVBs induced massive cell lysis as did in WT-CVB. These results indicate that insertion of miRTs made CVB3 less toxic only in cells expressing miR-34a/c.

The cytotoxic activity of miRT-CVBs was also evaluated using several human cancer cell lines and a human normal lung cell line, BEAS-2B, by MTS assay. The results showed that infection with miRT-CVBs in cancer cells retained its abundant viral replication in comparison with the parental WT-CVB, while its attenuated replication was observed in BEAS-2B expressing high level of miR-34a and miR-34c, depending on intracellular miR-34a or miR-34c levels.

Next, to investigate the anti-tumor activity of miRT-CVBs *in vivo*, I injected miRT-CVBs and WT-CVB into H1299 tumors in BALB/c nude mice, and monitored the tumor growth. In contrast to control mice showing continuous tumor growth, all WT- or miRT-CVB-treated groups exhibited complete tumor regression with no death during the observation period, indicating that miRT-CVBs preserved the anti-tumor activity of WT-CVB.

To address *in vivo* toxicity of those CVBs, blood biochemistry tests and pathological examination were performed for CVB-treated tumor-harboring mice. In WT-CVB treated mice, serum AST, ALT, LDH, and amylase levels were significantly increased, and histological signs of pancreatic injury were observed. While 5-CVBs failed to reduce the CVB3-associated pathogenicity, 3-CVB3 did not significantly increase the enzyme levels except ALT and induced less pancreatic injury. Because elevated LDH reflected the damage of many organs, the reduced LDH elevation in 3-CVBs mice were considered to be due to restricted virus replication in various organs. Between miR-34a and miR-34c target sites, miR-34a seemed to work better to reduce toxicities in mimics-transfected H1299 cells and in normal tissues, possibly because all organs including heart and pancreas, which are susceptible to CVB3 expressed miR-34a, while miR-34c expressed only in lung and brain. Another possibility is the difference in seed-matched sites of miRNA. Although miR-34a and miR-34c belong to the same family and share the same seed region, the type of miR-34aT, which contains an A at position 1 named 8mer-type site, is thought to induce miRNA inhibition much stronger than other types of sites. Although miR-34aT or miRT-34cT was successfully inserted in the 5'UTR and 3'UTR of CVB3 genome without losing anti-tumor effect of the original virus, 3-CVBs exhibited significantly lower pathogenicity than 5-CVBs. Stronger inhibition by inserting miRTs in 3'UTR was consistent with previous reports in which miRNA can inhibit a mRNA more efficiently when the target sequence is in the 3'UTR than in the 5'UTR. These results indicated that insertion of miRTs reduced WT-CVB-associated pathogenicity in normal cells without attenuating the anti-tumor activity, and the 3a-CVB (miR-34a inserted into 3'UTR of virus) was the best miRT-CVB in terms of anti-tumor effect and safety, while this virus still induced slight side effect, mild ALT elevation and less pancreas injury.

To further improve the safety of 3a-CVB, four tandem miR-34aT were inserted not only in 3' UTR but also in 5'UTR of CVB3 genome (53a-CVB) to increase miR-34a-sensitive sites. 53a-CVB showed the same level of cytotoxicity in H1299 cells as WT-CVB but significant lower toxicity in miR34a-transfected H1299 cells. As expected, the decrease of cytotoxicity was more significant than that of 5a-CVB (miR-34a inserted into 5'UTR of virus) or 3a-CVB. While 53a-CVB preserved the same level of anti-tumor effect in the xenograft mouse model, 53a-CVB did not cause significant elevation of all enzymes including ALT, which was elevated in 3a-CVB-treated mice. Histopathological examination also showed no findings of organ injuries by 53a-CVB treatment, suggesting nearly perfect suppressed CVB-induced organ toxicity by double insertion of miR34aT in both UTRs. To confirm the suppressed CVB replication in normal organs, copy numbers of CVB genomes in each mouse organ were determined by RT-qPCR. In mice treated with WT-CVB, pancreas was most heavily infected besides tumors, followed by spleen, heart, and lung. On the other hand, virus copies of all organs including pancreas were much less in 53a-CVB-treated mice than WT-CVB. Of note is that tumor of 53a-treated mice had similar virus load to that of WT-CVB-treated mice. These data were computable with the findings that 53a-CVB not only preserved anti-tumor effect but also obtained very low toxicity. To further evaluate the safety, I addressed effect of immunity on CVB3-relating toxicity by using immunocompetent C57BL/6J mouse model transplanted with syngeneic mouse lung

cancer TC-1 cells. There were no obvious signs of organ injuries by 53a-CVB treatment as observed in xenograft nude mice model suggesting that 53a-CVB would be a very efficient and safe and useful OV in the clinical setting.

To date, the target sequences of let-7 miRNA family members, which are another tumor suppressor miRNAs, have been used to reduce toxicity of OVs in normal tissues. Insertion of the let-7 targets in OVs, however, often decreases not only normal tissue toxicity but also anti-tumor activity due to the extensive overlap in target sequences among its various family members. Thus, I tried to find other miRNA-regulating systems with stronger on-target effects and milder off-target effects. In contrast to let-7, the miR-34 family consists of only three members, suggesting less interference among the family members. In this study, there was almost no reduction in the cytotoxicity when H1299 cells transfected with miR34a/c were infected with CVBs including different miR34a/cT, also suggesting excellence of miR34a/c-regulating system for controlling CVB replication.

Finally, I addressed why miR34aT-inserted CVB (miR34aT-CVB) could efficiently kill some tumor cells with high miR-34a. Although miR-34a was expressed more strongly in A549 cells than in normal lung cell line, 53a-CVB still exerted stronger cytotoxicity in these tumor cells with much lower titers than in non-tumor cells. To demonstrate mechanisms of this phenomenon, I focused on PI3K/Akt and MAP/ERK/MEK pathway because CVB3 replication depended on these pathways, which were often activated in cancer cells. In the presence of inhibitors for those pathways, cytotoxicity was significantly reduced by miR34aT-CVB in miR34a-high A549 cells but not in miR34a-low AsPC cells, suggesting that miR-34-induced inhibition of miR-34aT-CVB replication could be overridden by aberrantly activated PI3K/Akt and/or MAP/ERK/MEK pathways in tumor cells.

In conclusion, I successfully developed the first miR-34a/c–regulated CVB3 by inserting miR-34aT or miR-34cT in the 5’UTR or 3’UTR of the CVB3 genome, without attenuating its oncolytic activity. miRT-CVBs exerted significantly lower toxicity in normal tissues where miR-34a/c were highly expressed. CVB3 containing miR-34aT had less pathogenicity than CVB3 containing miR-34T, and insertion of the miR-34a/cT into the 3’UTR reduced pathogenicity to a greater extent than insertion into the 5’UTR. Finally, 53a-CVB, a double miR34aT-inserted CVB, was minimally toxic to normal tissues while maintaining full oncolytic activity in mouse xenograft model of lung cancer. This study successfully indicated that 53a-CVB is a promising OV that could be useful in the clinical setting due to its minimal toxicity with strong oncolytic effects.