学位論文の内容の要旨

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論　文　題　目	Exploration of Alternative Mechanism for MiRNA-596-mediated Down-regulation of LGALS3BP in Oral Squamous Cell Carcinoma

（論文内容の要旨）

Abstract

MicroRNAs (miRNAs) are endogenous small non-coding RNAs negatively regulate gene expression via binding to the 3´-untranslated region (UTR) of transcripts. However, recent evidence has suggested that miRNA can also bind to the open reading frame (ORF) region within transcripts for its down-regulation. On the other hand, previous report demonstrated that *miR-596* has a tumor suppression function by targeting *LGALS3BP* through directly binding to its 3' UTR in oral squamous cell carcinoma (OSCC) cells. In this study, while four putative binding sites of *miR-596* were included within the ORF region of *LGALS3BP*, it was found that *miR-596* could not bind to those sites. Alternatively, results showed that the expression of LGALS3BP might be in part negatively regulated by proteasome system at the protein level. Thus, these findings may help clarify the molecular mechanism of the tumor-suppressive effect through down-regulation of *LGALS3BP* by *miR-596* in OSCC cells.

Introduction

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that regulate gene expression by interfering with the translation or stability of target transcripts via binding to the 3´-untranslated region (UTR), and function as a "fine-tuner" of numerous biological processes. However, a number of studies have suggested that miRNA can also bind to the ORF region within transcripts to down-regulate the expression of target gene. While dysregulation of miRNA expression is associated with tumor progression, including cell proliferation, invasion/metastasis, and chemoresistance, novel tumor-suppressive miRNAs have been previously identified in endometrial cancer, oral squamous cell carcinoma (OSCC), and esophageal squamous cell carcinoma (ESCC).

LGALS3BP (Galectin-3-binding protein) is a secreted galectin-3 ligand. Knockdown of *LGALS3BP* expression was revealed to decrease cell growth and tumor cell migration and invasion of OSCC cells. Furthermore, Endo et al. have previously found that *miRNA-596*

(miR-596) can negatively regulate *LGALS3BP* expression by binding to its 3'UTR, resulting in the growth inhibition of OSCC cells.

In this study, it was examined whether *miR-596* can bind to the ORF region of LGALS3BP for its down-regulation. Unexpectedly, it was found that *miR-596* could not bind to any of the four putative binding sites within ORF of *LGALS3BP* in luciferase reporter assay. Alternatively, possibility was showed that the level of LGALS3BP protein might be negatively regulated by the proteasome system. These findings may help clarify the molecular mechanism of the tumor-suppressive effect through down-regulation of *LGALS3BP* by *miR-596* in OSCC cells.

Materials and Methods

Cell culture and treatment with reagents

NA cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and were maintained at 37˚C with 5% CO_2. MG132 (Sigma) were added to the medium at 100 nM and treated for 12 h before lysates were collected.

Transfection of microRNAs (miRNAs)

The dsRNA mimicking mature human *miR-596* and nonspecific control miRNA (negative control #1) were obtained from Life Technologies and was transfected individually into cells at 20 nM of concentrations using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Western blotting analysis

Whole cell lysates were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes (GE Healthcare). After blocking with TBS containing 0.05% Tween-20 and 5% non-fat dry milk for 1 hour, the membrane was reacted with an antibody overnight. The dilutions for primary antibodies were: 1/5,000 for Flag and ß-actin (both from Sigma). The membrane was washed and exposed to horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit IgG antibodies (both at 1/4,000) for 2 hours. The bound antibodies were visualized in LAS3000 (FUJIFILM) using a Pierce ECL Western detection kit according to the manufacturer's instructions (Thermo Scientific).

Luciferase assay

Luciferase constructs were made by ligating oligonucleotides containing the wild-type or mutated sequence of each seed sequence of miR-596 within ORF of LGALS3BP downstream of the luciferase gene into the pMIR-REPORT luciferase vector (Ambion). All site-specific mutations were generated using the KOD -Plus- mutagenesis kit (Toyobo). Luciferase reporter plasmids and a pGL plasmid as an internal control were co-transfected in NA cells using Lipofectamine 2000

(Invitrogen) according to the manufacturer's instructions, and after 5 hours, 20 nmol/L of miRNA (*miR-NC* or *miR-596*) was also transfected. At 36 hours after transfection of miRNAs, *Firefly* and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated by normalizing *Firefly* luciferase by its corresponding internal *Renilla* luciferase control.

Statistical analysis

Differences between subgroups were tested by Student's t-test. A P value of < 0.05 was considered statistically significant.

Results

Down-regulation of exogenously expressed LGALS3BP protein by miR-596

A plasmid expression vector, Flag-tagged LGALS3BP (pCMV-3Tag3A- LGALS3BP), was constructed by inserting only the ORF region of LGALS3BP without 3'UTR into pCMV-3Tag3A vector. When Flag-tagged LGALS3BP and *miR-596* or miR-negative control (*miR-NC*) (both precursor miRNAs from Ambion) were sequentially transfected in NA cells, an OSCC cell line, the expression level of exogenously expressed Flag-tagged LGALS3BP was remarkably down-regulated by transfection of *miR-596*, not with *miR-NC* by Western blotting (P=9.76E-16). These findings suggest that *miR-596* may be able to bind to ORF region of *LGALS3BP* transcript for its down-regulation.

Examinations for binding to LGALS3BP ORF by *miR-596*

On NCBI database, it was found that 4 putative binding sites (R1, R2, R3 and R4) having a seed sequence of *miR-596* are included within the ORF region of *LGALS3BP*. Hence, to examine whether *miR-596* can directly bind to these sites, luciferase assays were performed using reporter plasmid vectors having wild type (WT) or mutant (Mut-R1.etc) seed sequences within the ORF in NA cells. The luciferase activity of the WT vectors was not reduced compared to the empty vector in *miR-596*-expressing cells, and was not shown any effect of those mutations. Furthermore, 4 plasmid expression vectors were constructed, having mutation within each seed sequence, Flag-tagged LGALS3BP Mutant-R1, -R2, -R3, or -R4. When these mutants and *miR-596* were sequentially expressed in NA cells, it was showed that the expression level of any mutants was remarkably down-regulated in *miR-596*-expressing cells compared with *miR-NC*-expressing cells, as well as that of Flag-tagged LGALS3BP WT. These results suggest that *miR-596* can remarkably induce down-regulation of LGALS3BP, however it was not due to direct binding through any four seed sequences within ORF of *LGALS3BP*.

Possibility of LGALS3BP down-regulation by *miR-596* at post-translational level

Next, it was considered that *miR-596* might induce down-regulation of LGALS3BP by

promoting protein degradation at post-translational level. Both proteasomal and lysosomal pathways are major systems for protein degradation. Therefore, the effect of two inhibitors, bafilomycin A1 (lysosomal inhibitor) and MG132 (proteasomal inhibitor) were examined on expression level of Flag-tagged LGALS3BP WT in NA cells. Although no effect of treatment with bafilomycin A1 was observed (data not shown), it was showed a partial restoration of *miR-596*-mediated down-regulation for Flag-tagged LGALS3BP WT in MG132-treated cells, compared with non-treatment. However, a significant difference was not shown. As expected, expression level of Flag-tagged LGALS3BP was reduced in *miR-596*-expressing cells under the non-treated condition (P=0.0006). These findings suggest that *miR-596* induces down-regulation of LGALS3BP by promoting several mechanisms including the proteasomal system for protein destabilization at the post-translational level.

Discussion

Previously, Endo et al. showed that *miR-596* was epigenetically silenced in OSCC cell lines and primary tumors, and its overexpression can down-regulate expression of LGALS3BP by directly binding to 3'UTR and lead to tumor growth inhibition in OSCC cells, strongly suggesting that *miR-596* is one of the tumor-suppressor miRNA. Furthermore, in the present study, it was firstly found that overexpression of *miR-596* could also induce down-regulation of Flag-tagged LGALS3BP expressed exogenously from a plasmid vector having only ORF region of LGALS3BP without 3'UTR. Hence, *miR-596* might be able to down-regulate LGALS3BP by directly binding to the ORF, not only to 3'UTR of LGALS3BP. Indeed, 4 putative binding sites including seed sequence of *miR-596* within the ORF of *LGALS3BP* were found. Unexpectedly, *miR-596* cannot directly bind to the ORF of LGALS3BP, but rather induce degradation of LGALS3BP protein by promoting several mechanisms including proteasomal systems for protein degradation. Additionally, there might be another possibility that unknown miRNA(s), which was indirectly up-regulated by *miR-596,* can down-regulate LGALS3BP by directly binding to the ORF of this gene. Thus, while the present findings provide the novel possibility for down-regulation of *LGALS3BP* by *miR-596,* these subjects require further study to clarify the tumor-suppressive mechanism of *miR-596* and the development of *miR-596*-mediated cancer therapy in OSCCs.

和文要旨

MicroRNA(miRNA)は、細胞内に存在する機能性ノンコーディング RNA の一つであり、一般的に、mRNA の 3'UTR に結合して、翻訳障害を起こすことにより、遺伝子の発現を負に制御する。しかしながら、最近、miRNA は、遺伝子の翻訳領域にも結合することで、標的遺伝子の発現を抑制することができることが明らかになった。一方、miRNA-596(miR-596)は、口腔癌で発現低下する癌抑制性の miRNA であり、標的として LGALS3BP(Galectin-3-binding protein)の発現を抑制する。本研究は、miR-596 が LGALS3BP のその他の発現抑制機構を探索することを目的として行った。まず、LGALS3BP の翻訳領域のみを含む発現プラスミドを作製し、細胞に発現させた後、miR-596 をトランスフェクションした。そ

の結果、LGALS3BP の発現が強力に抑制されることが分かった。このことは、miR-596 は、LGALS3BP の 3’UTR だけでなく、翻訳領域に作用することで発現を抑制することを示唆する。さらに、データベース検索により、LGALS3BP の翻訳領域には、４つの miR-596 結合予測配列が含まれることが分かった。

しかしながら、レポーターアッセイによる検討の結果、miR-596 による LGALS3BP 発現の抑制は、それらの４つの予測配列への結合に依存しないことが示唆された。他の可能性として、LGALS3BP のタンパク質分解・不安定性の検討を行ったところ、プロテアソーム分解の阻害剤での処理条件では、miR-596 による LGALS3BP の発現抑制がキャンセルされる傾向が認められ、miR-596 による LGALS3BP 発現の抑制はプロテアソームにおけるタンパク分解などの機構を介してなされる可能性が示唆された。これらの結果は、miR-596 の癌抑制機構を理解する上で重要な所見になると考えられた。

論文審査の要旨および担当者

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MicroRNA(miRNA)は、細胞内に存在する機能性ノンコーディング RNA の一つであり、一般的に、mRNA の 3'UTR に結合して、翻訳障害を起こすことにより、遺伝子の発現を負に制御する。しかしながら、最近、miRNA は、遺伝子の翻訳領域にも結合することで、標的遺伝子の発現を抑制することができることが明らかになった。

これまでの研究において、miR-596 は、口腔癌で発現低下する癌抑制性の miRNA であり、LGALS3BP の 3'非翻訳領域に結合することにより、LGALS3BP 発現を抑制することが明らかにされた。そこで李は、他の抑制メカニズムの可能性として、miR-596 の LGALS3BP に対する翻訳領域での発現抑制機構の探索を試みた。翻訳領域にも結合する可能性を求めたこの着眼点は､近年の研究の流れに沿った妥当なものであると評価された。まず、LGALS3BP の翻訳領域のみを含む発現プラスミドを作製し、細胞に発現させた後、miR-596 をトランスフェクションした。その結果、LGALS3BP の発現が強力に抑制されることが分かった。このことは、miR-596 は、LGALS3BP の 3'UTR だけでなく、翻訳領域にも作用することにより、その発現を抑制することができることを示唆していた。そこで次に李は、データベース検索を行って、LGALS3BP の翻訳領域には、４つの miR-596 結合予測配列が含まれることを確認し、結合部位を解析するために、ルシフェラーゼ遺伝子の 3'非翻訳領域にその結合予測配列や変異を挿入した配列を導入し、レポーターアッセイを用いて､その 4 つの配列への結合の可否を検討した。これらの研究手法は明快であり、周到な準備のもとに本研究がなされたものと評価された。

本研究によって得られた主な結果は以下の通りであった。

1)miR-596 は、LGALS3BP の翻訳領域のみを発現させるプラスミドの発現に対し、抑制的に作用していた。

2)データベース検索により、LGALS3BP の翻訳領域には、４つの miR-596 結合配列の存在が予想された。

3)レポーターアッセイによる検討の結果、miR-596 によってルシフェラーゼ活性は低下せず、従って、上記４つの予測配列には結合しないことがわかった。

4)ライソゾーム阻害剤であるバフィロマイシン A1 処理によって、miR-596 による LGALS3BP 発現抑制効果には、影響を与えなかった。

5)プロテアソーム阻害剤である MG-132 処理によって、miR-596 による LGALS3BP 発現

抑制効果は、一部阻害される傾向があったが、有意差は認められなかった。

以上の結果を基に、李は、miR-596 による LGALS3BP 発現抑制メカニズムとして、3'非翻訳領域への結合による抑制に加え、CCR4-NOT 複合体のような E3 リガーゼが誘導され、それによって LGALS3BP がユビキチン化されて分解される、あるいは、miR-596 によって LGALS3BP の ORF に直接結合できる何らかの miRNA が誘導される可能性を提唱した。最終的なメカニズムの同定には至らず、さらなる研究が必要ではあるものの、本研究から得られた推論として、妥当なものであると認められた。

以上の様に、本研究は、miR-596 の癌抑制機構を理解する上で、これまでのメカニズムでは説明のつかない新規な因子の存在の可能性を見いだしており、今後の歯科医学、癌治療生物学の発展に寄与するところが大きいと考えられる。よって、本論文は博士（歯学）の学位を請求するに十分値するものと認められた。