厚生労働科学研究費補助金

創薬基盤推進研究事業

高速シークエンサーを用いたnon-coding RNAまで包括されたトランスクリプトーム解析による新規安全性バイオマーカーの同定

平成２２年度　総括研究報告書

研究代表者　土屋　創健

平成２３（２０１１）年４月

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厚生労働科学研究費補助金（創薬基盤推進研究事業）
総括研究報告書

高速シークエンサーを用いたnon-coding RNAまで包括された トランスクリプトーム解析による新規安全性バイオマーカーの同定

研究代表者　土屋　創健　京都大学大学院薬学研究科助教

研究要旨

近年、生理作用や病態発現に重要な役割を果たす機能性non-coding RNAの存在が明らかとなり、non-coding RNAに着目した新たな生命科学研究に注目が集まっているが、現在までにnon-coding RNAに着目したトキシコゲノミクスの研究報告は皆無である。そこで、ギガシークエンサーを用いて、non-coding RNAまで包括されたデジタルトランスクリプトーム解析を世界に先駆けてトキシコゲノミクスに適用し、薬物毒性予測におけるnon-coding RNAの有用性を評価するとともに、薬物肝毒性に関わる新規安全性バイオマーカー、しいては肝細胞の細胞障害・細胞死を抑制するための治療標的分子候補の同定を試みた。

薬物肝毒性のモデルとしてトログリタゾンによる*in vitro*細胞生存評価実験系を構築するとともに、non-coding RNAまで包括されたデジタルトランスクリプトームデータの取得法及び解析法を構築した。これらを適用して解析を行った結果、トログリタゾンの毒性容量において発現変動する転写物の半分以上はnon-coding RNAに由来することを見いだした。さらに、バイオインフォマティクス解析からとりわけ肝毒性への関与が強く示唆されたnon-coding RNAである RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*)のsiRNAを用いたノックダウン実験を行ったところ、トログリタゾンによる薬物肝毒性の結果と合致して、*RMRP* siRNAの導入により細胞増殖の有意な低下が確認されたことから、*RMRP*は薬物肝毒性に関わる新規安全性バイオマーカー候補であるとともに肝細胞の細胞障害・細胞死を抑制するための治療標的分子候補であることが明らかとなった。

A. 研究目的

近年、transfer RNA や ribosomal RNA 以外のタンパク質をコードしない RNA、non-coding RNA が messenger RNA (mRNA) と同等数の２万種類以上転写されていることが明らかとなり、とりわけ、短鎖の non-coding RNA、microRNA が生理作用や病態発現に重要な役割を果たすことが見いだされ、non-coding RNA に着目した新たな生命科学研究が注目・展開されている。しかしながら、現在までに non-coding RNA に着目したトキシコゲノミクスの研究報告は皆無である。そこで本課題では、次世代型高速シークエンサーであるギガシークエンサーを用いて non-coding RNA まで包括したデジタルトランスクリプトーム解析を世界に先駆けて行い、薬物毒性予測における non-coding RNA の有用性を評価するとともに、薬物肝毒性に関わる新規安全性バイオマーカー、しいては肝細胞の細胞障害・細胞死を抑制するための治療標的分子候補の同定することを目的とする。

B. 研究方法

細胞培養：非動化（５６℃、３０分）された 10% (v/v) fetal bovine serum 含有の Dulbecco's Modified Eagle Medium (Sigma) を用い、コラーゲンコートディッシュにて 37℃、5% CO_2 の条件で HepG2 細胞を培養した。

WST-1 細胞増殖測定：WST-1 を基質とした Nicotinamide adenine dinucleotide (NADH) 還元酵素の活性を指標に、細胞増殖を測定した。HepG2 細胞をトログリタゾン (vehicle (0.1% dimethylsulfoxide; DMSO), 1μM、3 μM、100 μM) で刺激し、各０，６，１２，２４時間後に回収した。$1x10^4$ cells の HepG2 細胞に飽和 WST-1 溶液 (Roche) を加え、37℃、5 % CO_2 の条件下で 1 時間反応させた後、還元型 WST-1 (Formazan) の 460nm とバックグランド補正用の 650nm の吸光度をマイクロプレートリーダーで測定した。

ギガシークエンスデータの取得：各濃度のトログリタゾン (vehicle, 1, 100 μM) で刺激した HepG2 細胞をそれぞれ０，６，２４時間後に回収し、acid guanidiniumthiocyanate-phenol-chloroform 法を用いて、total RNA を抽出した。次に Ribominus (Invitrogen) を用いて ribosomal RNA の anti-sense プローブにより ribosomal RNA の除去を行い、アルコール沈殿による精製後、アルカリ加水分解法により RNA を断片化した。それらを Rneasy micro kit (Qiagen) を用いて精製し、RNA アダプターを 5' 側と 3' 側にそれぞれ特異的に連結させた。3' 側のアダプター内の配列に相補的なプライマーをアニールさせて逆転写反応により 1st

strand DNA を合成し、引き続いてアダプター部位の配列を標的とするプライマー配列を用いてPCR増幅を行って、ギガシークエンサーによる解析を行うために必要な平均鎖長約 200 bp の２本鎖 DNA を調製した。そこでギガシークエンサーを用いて、PCR 反応により in situ で個々の増幅産物に特異的なクラスターを形成させ、蛍光標識塩基を用いた１塩基伸長反応を用いることにより、76 塩基の配列を約 2,000 万種同時に解読した。その中から、transfer RNA や ribosomal RNA にアライメントされるものを除去し、さらにヒトゲノムにアライメントされたもののみを更なる解析に使用した。

網羅的な遺伝子発現解析：NCBI の UniGene や Ensembl、ncRNA.org にアライメントされたリードに関して、R を用いて階層的クラスタリング解析、相関係数の計算、principal component analysis を行った。

薬物肝毒性における RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*)のノックダウン解析：*RMRP* の siRNA (Ambion)を HepG2 細胞に導入し、トログリタゾンの薬物細胞毒性における *RMRP* の機能・役割を調べた。遺伝子導入から４８時間後に *RMRP* のノックダウンによる細胞増殖への影響を調べるため、WST-1 法を用いて解析を行った。

（倫理面への配慮）

本課題の包括的デジタルトランスクリプトーム解析は商業的に販売されているヒト培養細胞を対象としていることから、倫理面での問題は特にないものと判断される。また、本課題を遂行するにあたり、組換えＤＮＡ実験を行うが、文部科学省並びに京都大学大学院薬学研究科施設の取り扱い規定に準拠し、組換えＤＮＡ実験安全委員会の承認を得るとともに、研究機関等における組換えＤＮＡ実験等の実施に関する基本指針に従う。

C. 研究結果

本研究プロジェクトでは non-coding RNA をトキシコゲノミクス解析に適用し、その有用性を評価するとともに新たな肝薬物毒性の安全性バイオマーカーを同定し、さらには薬物毒性の分子メカニズムを明らかにすることを主眼とする。そこで、薬物毒性を評価する上で最も重要な組織の一つである肝臓における薬物毒性発現の分子メカニズムを明らかにするとともに、non-coding RNA を包括したデジタルトランスクリプトームデータ解析の意義・有用性を評価するため、これまでに肝毒性を有する薬物として劇症肝炎などの肝障害を引き起こしたため臨床での使用が中止さ

れたトログリタゾン（チアゾリジン骨格を有するインスリン抵抗性改善薬、peroxisome-proliferator activated receptor γ のリガンド）と *in vitro* での肝毒性評価系として広範囲の薬物において高頻度に使用されているヒト肝癌由来細胞株 HepG2 を用いた *in vitro* の薬物肝毒性評価系を構築した。また、従来までの既存のギガシークエンサーを用いた手法では non-coding RNA が解析できず、転写された方向がわからない（正確なマッピングができない）という問題があったが、種々の改良を行い、non-coding RNA は包括し、転写方向性情報を有することで読まれたリードが正確にゲノムにアライメント出来る手法を確立し、上述の薬物肝毒性モデルにおける経時的（0、6、24 h）かつ用量依存的（vehicle、1、100 μM）な non-coding RNA 包括トランスクリプトームデータを取得した。

mRNA と non-coding RNA を同時に評価するために、まず UniGene（123,977 データ）にアライメントして解析を行った結果、薬物肝毒性刺激によって発現変動した UniGene の半分以上が non-coding RNA に由来している可能性が示唆された。さらに階層的クラスタリング解析を行った結果、non-coding RNA 包括トランスクリプトームデータは毒性と非毒性のデータを明確に分類するとともにこれらの principal component analysis を行ったところ、第二軸において毒性のサンプル群と非毒性のサンプル群のベクトルが分離され、この第二軸コンポーネントとトログリタゾンによる薬物毒性との強い相関が示唆された。この第二軸コンポーネントに強く影響する UniGene として１２個の UniGene が選出され、そのうち６つが mRNA に附属し、残りは non-coding RNA であると考えられた。

加えて、より統合・整理されたデータベースである Ensembl の転写データ（ヒトにおいて mRNA と non-coding RNA の両方の情報を含有、36,692 転写物分）を用いて同様の解析を試みた。principal component analysis を行ったところ、先ほどと同様に第二軸において毒性のサンプル群と非毒性のサンプル群のベクトルが分離され、この第二軸コンポーネントとトログリタゾンによる薬物毒性との強い相関が示唆された。Ensembl の場合においてはこの第二軸コンポーネントに強く影響する転写物は６個であり、そのうち４つが non-coding RNA であった。

これらの principal component analysis より薬物肝毒性への強い寄与が示唆された non-coding RNA の中から、リボソーム集合やテロメア機能、そしてアポトーシスや細胞周期

の制御に関与することが報告されている RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*)に着目して機能解析を行った。*RMRP* の siRNA を用いてノックダウンを行い、その際の細胞生存・増殖への影響を調べたところ、トログリタゾンによる薬物肝毒性の結果と合致して、*RMRP* のノックダウンにより細胞増殖・生存活性の有意な低下が確認された。

D. 考察

近年、数万種に及ぶ non-coding RNA の存在が見いだされたことから、これをトキシコゲノミクスに適用することでより高精度な毒性予測及び毒性発現分子メカニズムの解明が可能となることが予測されたが、現在までにトキシコゲノミクス分野における non-coding RNA の適用事例は皆無であった。そこで、本研究では、トログリタゾンで刺激した HepG2 細胞を肝毒性のモデルとして non-coding RNA 包括トランスクリプトーム解析を行った結果、mRNA と non-coding RNA の発現情報をデジタル情報として同時に取得できることにより、マイクロアレイを用いた解析では困難であった未同定の RNA の検出と各 RNA 内・間に渡る高精度なバイオインフォマティクス解析が可能となり、薬物毒性刺激により mRNA と同等数以上の non-coding RNA の発現量が変動していることを初めて明らかにした。さらに non-coding RNA 包括トランスクリプトームデータは UniGene と Ensembl のいずれにアライメントした際にも principal component analysis により第二軸コンポーネントにおいて薬物肝毒性と非毒性のサンプルを明確に分類し、合わせて１０種の non-coding RNA と８種の mRNA がトログリタゾンによる薬物毒性に強く関与している可能性と薬物毒性予測における non-coding RNA の有用性を示唆した。この選出されてきた mRNA には肝障害のマーカーとして報告されている遺伝子や、細胞外マトリックス成分で肝細胞のアポトーシスを抑制することが報告されている遺伝子、さらには脂質代謝に関わる遺伝子などが含まれていた。一方、同様の手法により選出された１０種の non-coding RNA のうち、*RMRP* のノックダウンによってトログリタゾンによる薬物肝毒性の結果が再現されたことから、non-coding RNA がトログリタゾンによる薬物毒性に実際に関与していることが明らかとなった。今後、*RMRP* は薬物肝毒性に関わる新規安全性バイオマーカーとして、そして肝細胞の細胞障害・細胞死を抑制するための治療標的分子として更に研究・開発が発展することが期待される。

non-coding RNA という新たな領域にトキシコゲノミクス解析を適用することによって得られた本研究成果は、まさに世界に先駆けるものであり、今後、他の肝毒性を有する薬物や、薬品の種類を問わず発現し、現れる臨床症状も多様である腎毒性、薬物の毒性評価を進める上で最も予測し難い要因であるアレルギー毒性などへの本解析手法の適用によってさらなる薬物毒性メカニズムの解明や高精度な毒性予測手法の確立が可能になるものと期待される。

E. 結論

トログリタゾンを用いた肝毒性モデルにおいて、non-coding RNA が薬物肝毒性に直接的に関与し、薬物毒性予測において極めて有用であることが示唆された。とりわけ *RMRP* は薬物肝毒性に関わる新規安全性バイオマーカー及び肝細胞の細胞障害・細胞死を抑制するための治療標的分子の有力な候補であることが見いだされた。

F. 健康危険情報

特記事項無し

G. 研究発表

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日本薬学会第131年会（静岡）2011年3月

H. 知的財産権の出願・登録状況

1. 特許取得
無し
2. 実用新案登録
無し
3. その他
無し

研究成果の刊行に関する一覧表

雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuchiya S., Fujiwara T., Sato F., Shimada Y., Tanaka E., Sakai Y., Shimizu K. and Tsujimoto G.	MicroRNA-210 Regulates Cancer Cell Proliferation through Targeting Fibroblast Growth Factor Receptor-Like 1 (*FGFRL1*).	***J. Biol. Chem.***	286	420-428	2011
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Sato F., **Tsuchiya S.**, Meltzer S. and Shimizu K.	MicroRNAs and Epigenetics.	***FEBS J.***	278	1598-1609	2011

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MicroRNA-210 Regulates Cancer Cell Proliferation through Targeting Fibroblast Growth Factor Receptor-like 1 (*FGFRL1*)*[S]

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The importance of microRNAs (miRNAs) in human malignancies has been well recognized. Here, we report that the expression of microRNA-210 (miR-210) is down-regulated in human esophageal squamous cell carcinoma and derived cell lines. Marked decreases in the level of miR-210 were observed especially in poorly differentiated carcinomas. We found that miR-210 inhibits cancer cell survival and proliferation by inducing cell death and cell cycle arrest in G_1/G_0 and G_2/M. Finally, we identified fibroblast growth factor receptor-like 1 (*FGFRL1*) as a target of miR-210 in esophageal squamous cell carcinoma and demonstrated that *FGFRL1* accelerates cancer cell proliferation by preventing cell cycle arrest in G_1/G_0. Taken together, our findings show an important role for miR-210 as a tumor-suppressive microRNA with effects on cancer cell proliferation.

MicroRNAs (miRNAs)[2] are evolutionarily conserved small noncoding RNAs (20–23 nucleotides) that bind to complementary sequences in the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs) and regulate gene expression by the cleavage of target mRNAs and/or translational inhibition (1). Currently, >800 human miRNAs have been identified and registered in the miRNA database, miRBase (2). miRNAs play important roles in the differentiation of various cell types and in the initiation and progression of cancer, and it has been shown that the expression of some miRNAs is altered during cell differentiation and in malignancies (1, 3, 4).

In a recent study, we identified microRNA-210 (miR-210) as one of the miRNAs that is markedly differentially expressed during the process of epithelial differentiation (3). It has been reported that miR-210 expression is down-regulated during epithelial-mesenchymal transition, the aberrant activation of which triggers cancer pathology (5). Carcinomas are derived from epithelial cells, and poor prognosis in patients with carcinoma is associated with the disruption of characteristics of differentiated epithelial cells, such as cell junctions and polarity (6–8). Hence, given that the expression of miR-210 appears to be correlated well with epithelial differentiation, miR-210 might play a suppressive role in carcinomas. In support of this idea, allelic deletions at the miR-210 locus have been observed in 64% of cases of ovarian cancer (9), and ectopic expression of miR-210 represses tumor growth when human cancer cell lines are implanted into immunodeficient mice (10). However, the clinical roles of miR-210 in carcinomas and the mechanisms by which it represses tumor growth remain unknown.

In this study, we investigated the functional role of miR-210 in the growth of carcinomas and the mechanism by which it acts using clinical samples as well as cell lines of esophageal squamous cell carcinoma (ESCC). ESCC is a highly aggressive malignancy with a 5-year survival rate of 10% worldwide. It has been used as a model to study the mechanisms of dysregulated epithelial differentiation and epithelial-mesenchymal transition in carcinomas (11, 12).

EXPERIMENTAL PROCEDURES

Specimens—All tumor samples were confirmed as ESCC by the Clinicopathologic Department at Kyoto University Hospital. All cases were classified according to the sixth edition of the pathologic tumor-node-metastasis (TNM) classification (13). Written informed consent for the research use was obtained from each patient before surgery. The study was approved by the Kyoto University Institutional Review Board.

Cell Culture and Transfection—HE3 cells, which are human primary normal esophageal epithelial cells, were established and cultured in keratinocyte SFM (Invitrogen) containing human recombinant epidermal growth factor (Invitrogen) and bovine pituitary extract (Invitrogen). HEEpiC cells were purchased from ScienCell Research Laboratories (Carlsbad,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3.
The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE20637).
[1] To whom correspondence should be addressed. Tel.: 81-75-753-4523; Fax: 81-75-753-4544; E-mail: gtsuji@pharm.kyoto-u.ac.jp.
[2] The abbreviations used are: miRNA, microRNA; ESCC, esophageal squamous cell carcinoma; FGFRL1, fibroblast growth factor receptor-like 1; miR-210, microRNA-210; ncRNA, negative control RNA; ncsiRNA, negative control small interfering RNA; NEST, normal esophageal squamous tissue; PI, propidium iodide; qRT-PCR, quantitative reverse transcriptase-PCR.

CA) and were cultured according to the manufacturer's instructions (14). The human ESCC cell lines KYSE-150, -170, -190, and -590 were maintained as described previously (14, 15). KYSE-170 cells were transfected with oligoribonucleotides for miR-210 or negative control RNA (ncRNA) (Ambion, Austin, TX) using HiPerFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol for overexpression. KYSE-170 cells were also transfected with *FGFRL1* (fibroblast growth factor receptor-like 1) small interfering RNAs (siRNAs) or negative control siRNA (ncsiRNA) (Invitrogen) for knockdown of *FGFRL1*. The coding region of *FGFRL1* was isolated from complementary DNA (cDNA) from KTSE-170 cells, which was reverse transcribed using an oligo(dT) primer (Invitrogen), and ligated into the pcDNA3 expression vector. This *FGFRL1* expression construct was transfected into KYSE-170 cells using TransIT-LT1 transfection reagent (Mirus, Madison, WI). For inhibition of miRNAs function, KYSE-590 cells were transfected with a specific microRNA inhibitor for miR-210 (anti-miR-210) or its negative control RNA (anti-ncRNA) (Ambion) using HiPerFect transfection reagent.

RNA Extraction and Quantitative Reverse Transcriptase-PCR—Total RNA was extracted from the normal esophageal squamous tissues (NESTs), ESCCs, and cell lines by the acid guanidinium thiocyanate-phenol-chloroform method and then used for quantitative reverse transcriptase-PCR (qRT-PCR). To quantify mRNA expression levels, total RNA was reverse transcribed to cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen), and quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was quantified using standard curves that were generated using serially diluted reference samples and normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specificity of the PCR products was confirmed by gel electrophoresis and a dissociation curve analysis. Primer sequences are shown in supplemental Table 1. To quantify the levels of miRNAs and RNA U6 small nuclear 2 (*RNU6-2*), we used TaqMan MicroRNA assays (Applied Biosystems), which detect mature miRNAs specifically, following the manufacturer's protocol. The miRNA expression level was normalized to the expression level of *RNU6-2*.

Assay of Cell Proliferation, Cell Cycle, and Cell Death—The WST-1 assay to measure cell proliferation and flow cytometric assays to analyze the cell cycle and cell death were performed with KYSE-170 cells at 48 h after transfection as described previously (15). Calculations for the analysis of the cell cycle were performed with ModFit software (BD Biosciences). The bromodeoxyuridine (BrdU) incorporation assay, which measures cell proliferation, was performed using the BrdU Cell Proliferation ELISA kit (Roche Applied Science) according to the manufacturer's instructions.

Microarray Analysis—Total RNA from KYSE-170 cells that had been transfected with either ncRNA or miR-210 was labeled and prepared for hybridization to a human Oligo chip 25k (Toray, Tokyo, Japan) using standard methods. The GEO database accession code of the microarray data obtained is GSE20637.

Immunoblot Analyses—Cells were homogenized and centrifuged. The resultant supernatant was subjected to SDS-PAGE, and the separated proteins were transferred electrophoretically onto a PVDF membrane. An anti-FGFRL1, NDUFA4, GPR177, or LRP5L antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100) was used as the primary antibody, respectively, and a horseradish peroxidase-conjugated IgG antibody (1:5,000) was used as the secondary antibody. The membranes were stained using an ECL kit according to the manufacturer's instructions. An anti-β-actin antibody (Sigma-Aldrich) (1:1,000) was used as a control.

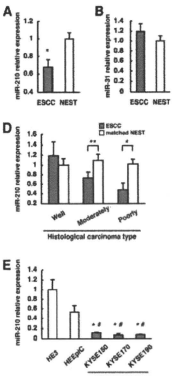
Immunostaining—Sections of ESCC and NEST were stained with an anti-FGFRL1 antibody (Santa Cruz Biotechnology) and ChemMate ENVISION kit (Dako, Glostrup, Denmark).

Luciferase Reporter Assay—The 3′-UTRs of *FGFRL1*, *WSB2*, and *GDI2* were isolated from cDNA from KTSE-170 cells. These 3′-UTRs and synthetic oligonucleotides of putative miR-210-target sites in the *FGFRL1* 3′-UTR ligated into the pGL3 basic luciferase expression (pGL3-Luc) vector (Promega, Madison, WI) at the 3′-end of the luciferase coding sequence (Luc), respectively. This pGL3-Luc vector containing 3′-UTR or miR-210 target sites and the pRL-TK internal control vector (Promega) were transfected into KYSE-170 cells using FuGENE HD transfection reagent (Roche Applied Science). Oligoribonucleotides for miR-210 or ncRNA were transfected 24 h after transfection of their vectors, and luciferase activity was measured 38 h after transfection of their vectors using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions.

Statistics—Results are expressed as the mean ± S.E. Student's *t* test was used to compare data between two groups. *p* values less than 0.05 were considered to be statistically significant. In Figs. 1*C* and 4*E*, each clinicopathologic parameter, with the exception of age, was evaluated using Pearson's χ^2 test.

RESULTS

MiR-210 Expression Is Significantly Down-regulated in ESCC, Especially in Poorly Differentiated Carcinomas—To investigate the role of miR-210 in human malignancies, we first examined the expression levels of miR-210 in clinical samples of matched NEST and ESCC by qRT-PCR (Fig. 1*A*). Compared with NEST, a significant down-regulation of miR-210 expression was noted in ESCC. On the other hand, the expression of miR-31, which has been reported to be not altered between ESCC and NEST (16, 17), showed no significant difference (Fig. 1*B*). For further analysis, 82 clinical samples of ESCC were divided into two groups (miR-210-high and miR-210-low) on the basis of their miR-210 expression levels, and the clinicopathologic characteristics of these two groups were assessed. A significant difference was observed between the miR-210-high group and the miR-210-low group with respect to histological type (*i.e.* well, moderately, and poorly differentiated), but there were no significant differences between the two groups with respect to gender, age, or



C

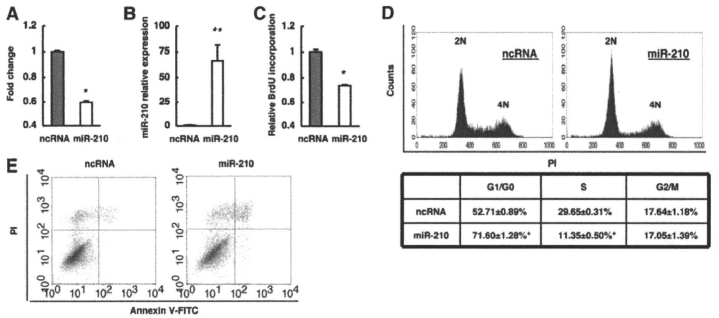
		miR-210-high	miR-210-low	*P*-value
Gender	Male	38	33	0.105
	Female	3	8	
Age	Mean	62.8	63.2	0.848
	Range	46 - 81	51 - 82	
TNM Stage	0	6	6	0.949
	I	5	4	
	IIa	8	6	
	IIb	15	15	
	III	2	4	
	IV	5	6	
Histological carcinoma type	Well differentiated	8	2	0.015
	Moderately differentiated	27	23	
	Poorly differentiated	6	16	

FIGURE 1. **Down-regulation of miR-210 expression in ESCC.** *A* and *B*, expression levels of miR-210 and miR-31 in NEST and ESCC assessed by qRT-PCR. The values are shown relative to the value obtained for NEST ($n = 82$; *, $p < 0.01$). *C*, clinicopathologic characteristics of 82 ESCCs divided into two groups ($n = 41$) on the basis of miR-210 expression levels. *D*, expression levels of miR-210 in the ESCCs and the corresponding NESTs in each histological type were compared by qRT-PCR. The values are shown relative to the value obtained for NEST in the well differentiated group (**, $p < 0.05$; *, $p < 0.01$). *E*, levels of miR-210 in HE3, HEEpiC, and KYSE cell lines analyzed by qRT-PCR. The values are shown relative to the value obtained for HE3 ($n = 3$; *, $p < 0.05$ *versus* HE3; #, $p < 0.05$ *versus* HEEpiC). *Error bars*, S.E.

pathologic TNM stage (Fig. 1*C*). Then, we compared the expression levels of miR-210 between ESCC and the matched NEST for each histological type. Significant differences were observed between moderately and poorly differentiated ESCC and the matched NEST (Fig. 1*D*). Moreover, as the degree of tumor differentiation decreased, the level of miR-210 showed a corresponding decrease in the ESCC ($p = 0.0875$, ANOVA) but not in the matched NEST ($p = 0.8728$, ANOVA), which indicated a strong correlation between the level of miR-210 and the degree of tumor differentiation in ESCC. This observation appears to agree well with the previous finding that miR-210 expression is up-regulated in parallel with epithelial cell differentiation (3, 18). Furthermore, when we compared the levels of miR-210 expression in normal human esophageal epithelial cells (HE3 and HEEpiC) with those in ESCC cell lines (KYSE-150, -170, and -190), we found that miR-210 expression was down-regulated significantly in the ESCC cell lines (Fig. 1*E*).

MiR-210 Inhibits Cancer Cell Proliferation by Inducing Cell Death and Cell Cycle Arrest in G_1/G_0 and G_2/M—Next, we examined the functional role of miR-210 in ESCC by adding synthetic miR-210 to the KYSE-170 cell line, in which miR-210 expression is low (Fig. 1*E*). Given that miR-210 expression was correlated with the level of differentiation in ESCC (Fig. 1, *C* and *D*), we examined the effect of miR-210 on the proliferation of ESCC cells. Transfection of miR-210 significantly decreased the proliferation of cancer cells, whereas a ncRNA had no inhibitory effect (Fig. 2*A*). Then, the introduced amount of miR-210 in cells transfected with miR-210 was a >60-fold increase compared with ones in cells transfected with ncRNA (Fig. 2*B*), which is within the comparable range for the differences between normal human esophageal epithelial cells and KYSE-170 cells (Fig. 1*E*). Moreover, we performed a BrdU incorporation assay and found that miR-210 significantly reduced the uptake of BrdU (Fig. 2*C*). These results suggested that miR-210 negatively regulates cancer cell proliferation. Next, we examined the effect of miR-210 on the cell cycle (Fig. 2*D*). Transfection of miR-210 resulted in a significant increase in the proportion of cells in G_1/G_0 phase as well as a significant decrease in the proportion of cells in S phase, whereas the proportion of cells in G_2/M phase was unaltered. These results indicate that miR-210 induces cell cycle arrest in both G_1/G_0 and G_2/M phases because cell cycle arrest only in G_1/G_0 phase decreases the proportion of cells in G_2/M phase. In addition, overexpression of miR-210 resulted in a significant increase in the proportion of FITC-annexin V-positive cells (Fig. 2*E*, *upper right* and *lower right*) and FITC-annexin V-negative/propidium iodide (PI)-positive cells (Fig. 2*E*, *upper left*), and a significant decrease in the proportion of FITC-annexin V-negative/PI-negative cells (Fig. 2*E*, *lower left*), which indicated that overexpression of miR-210 may lead to apoptosis and necrosis. Taken together, the results suggest that miR-210 may inhibit proliferation of ESCC cells mainly by inducing cell cycle arrest and apoptosis.

Identification of Candidate Target Genes Degraded by MiR-210—To investigate the molecular mechanism by which miR-210 inhibits ESCC cell proliferation, we analyzed the target genes of miR-210 in ESCC. We performed comprehen-



	G1/G0	S	G2/M
ncRNA	52.71±0.89%	29.65±0.31%	17.64±1.18%
miR-210	71.60±1.28%*	11.35±0.50%*	17.05±1.39%

	Upper Left	*Lower Left*	*Upper Right*	*Lower Right*
ncRNA	10.76±1.84%	83.47±1.68%	4.00±0.22%	1.77±0.15%
miR-210	15.28±1.58%**	74.45±2.00%*	7.03±0.78%*	3.25±0.15%*

FIGURE 2. **Functions of miR-210 in KYSE-170 cells.** *A*, effect of miR-210 on cell proliferation was investigated by the WST-1 assay. Cells were incubated for 48 h after transfection with either ncRNA or miR-210, and viability was evaluated. The values are shown relative to the value obtained with ncRNA. *B*, expression levels of miR-210 in cells at 48 h after transfection with either ncRNA or miR-210 were investigated by qRT-PCR. The values are shown relative to the value obtained with ncRNA. *C*, 48 h after transfection, cell proliferation was evaluated by BrdU incorporation. The values are shown relative to the value obtained with ncRNA. *D*, effects of miR-210 on the cell cycle were investigated. The PI-stained DNA content of the cells was evaluated using a FACScan flow cytometer at 48 h after transfection. *E*, at 48 h after transfection, cells were stained with FITC-conjugated annexin V, and PI and cell death was evaluated using a FACScan flow cytometer. $n = 3$; **, $p < 0.05$; *, $p < 0.01$ *versus* ncRNA.

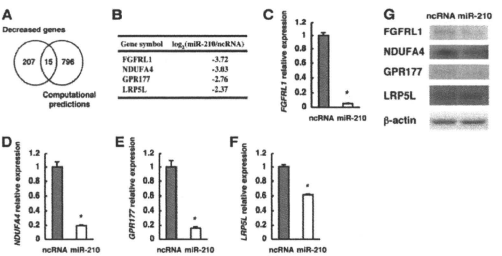
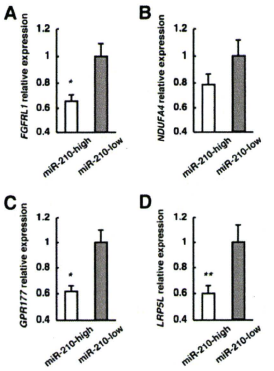


FIGURE 3. **Identification of candidate target genes degraded by miR-210 in KYSE-170 cells.** *A*, Venn diagram showing overlapping sets of genes whose expression was decreased >5-fold by transfection of miR-210 and computationally predicted target genes of miR-210. *B*, list of four potential miR-210 target genes with a signal value of more than 50 in ncRNA-transfected cells upon microarray analysis. *C–F*, expression levels of the four potential miR-210 target miRNAs assessed by qRT-PCR. The values are shown relative to the value obtained with ncRNA ($n = 3$; *, $p < 0.01$). *G*, Western blot analyses of FGFRL1, NDUFA4, GPR177, LRP5L, and β-actin proteins.

sive transcriptome analysis using RNA from ESCC cells transfected with either ncRNA or miR-210. Assuming that the expression of target genes of miR-210 might be down-regulated in cells transfected with miR-210, we selected 222 genes whose expression was decreased by more than 5-fold in miR-210-transfected cells compared with ncRNA-transfected cells (supplemental Table 2). Fifteen of the 222 genes were predicted to be miR-210 target genes by the target prediction programs microcosm (2), TargetScan (19), and PicTar (20) (Fig. 3*A* and supplemental Tables 2 and 3). Four (*FGFRL1*, *NDUFA4*, *GPR177*, and *LRP5L*) of these 15 genes had a signal value of more than 50 in ncRNA-transfected cells (Fig. 3*B* and supplemental Table 2). Furthermore, their expression was confirmed by qRT-PCR and Western blotting; the mRNA expression of all four genes was significantly down-regulated by transfection of miR-210, although the degree of decrease of



E

		FGFRL1-high	FGFRL1-low	P-value
Gender	Male	35	36	0.746
	Female	6	5	
Age	Mean	63.6	62.4	0.547
	Range	51 - 81	46 - 82	
TNM Stage	0	8	4	0.687
	I	4	5	
	IIa	6	8	
	IIb	13	17	
	III	3	3	
	IV	7	4	
Histological carcinoma type	Well differentiated	2	8	0.037
	Moderately differentiated	24	26	
	Poorly differentiated	15	7	

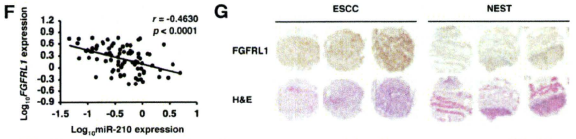


FIGURE 4. **Identification of candidate target genes of miR-210 in ESCC.** *A–D*, *FGFRL1*, *NDUFA4*, *GPR177*, and *LRP5L* expression levels in ESCCs divided into two groups on the basis of miR-210 expression levels were assessed by qRT-PCR. The values are shown relative to the value obtained for the miR-210-low group ($n = 41$; **, $p < 0.05$; *, $p < 0.01$). *E*, clinicopathologic characteristics of 82 ESCCs divided into two groups ($n = 41$) on the basis of *FGFRL1* expression levels. *F*, plot of $\log_{10}$*FGFRL1* relative expression intensity against $\log_{10}$miR-210 relative expression intensity. The *line* represents an approximated curve. The correlation coefficient (r) and the p value indicate the statistical significance of the negative correlation between the x and y variables. *G*, immunohistochemistry for FGFRL1 on ESCC and NEST. These sections were stained by anti-FGFRL1 antibody and by hematoxylin and eosin (*H&E*).

LRP5L was somewhat low (Fig. 3, *C–F*). The expression levels of FGFRL1, NDUFA4, and GPR177 proteins were also down-regulated by transfection of miR-210 (Fig. 3*G*). Meanwhile, the expression levels in LRP5L protein showed no change at 48 h after transfection of miR-210 (Fig. 3*G*). To validate these genes as targets of miR-210 further, we examined their expression in clinical ESCC samples. Of the four genes, the expression of *FGFRL1*, *GPR177*, and *LRP5L* was significantly down-regulated in the miR-210-high group compared with the miR-210-low group (Fig. 4, *A*, *C*, and *D*), whereas *NDUFA4* was not significantly different between the two groups (Fig. 4*B*). We then divided the 82 ESCC samples into two groups ($n = 41$) on the basis of their *FGFRL1*, *GPR177*, or *LRP5L* expression levels and compared the two groups in terms of their clinicopathologic characteristics. A significant association with histological type was observed for the *FGFRL1*-classified groups (Fig. 4*E*), but not for the *GPR177*- or *LRP5L*-classified ones (data not shown), which agrees well with the finding that a decrease in miR-210 expression correlates well with the poorly differentiated state of ESCC. A statistically significant negative correlation was obtained between expression levels of miR-210 and that of *FGFRL1* in ESCC (Fig. 4*F*). Furthermore, sections of ESCC and NEST were stained by immunohistochemistry for FGFRL1. The results indicated that FGFRL1 protein is expressed in ESCC and the expression levels are enhanced compared with NEST (Fig. 4*G*).

MiR-210 Targets FGFRL1 3′-UTR Directly—Five sites in the *FGFRL1* 3′-UTR are predicted to be potential target sites of miR-210 by microcosm (Fig. 5*A*), although none of them is conserved across species such as mouse, rat, and chicken. To examine whether *FGFRL1* is a direct target of miR-210, we cloned the 3′-UTR of *FGFRL1* into a pGL3-Luc vector (pGL3-Luc-*FGFRL1* 3′-UTR) to perform reporter assay. When miR-210 was transfected into the cells with this reporter construct but not the mock, luciferase activity was repressed more than 50% compared with transfection of ncRNA (Fig. 5*B*). Meanwhile, luciferase activities in cells transfected with reporter constructs containing the 3′-UTR of *WSB2 or GDI2* were not repressed by the transfection of miR-210 (Fig. 5*B*). *WSB2* and *GDI2* were not included in either predicted target genes of miR-210 or 222 genes decreased in miR-210-transfected cells (supplemental Tables 2 and 3). These results suggest that

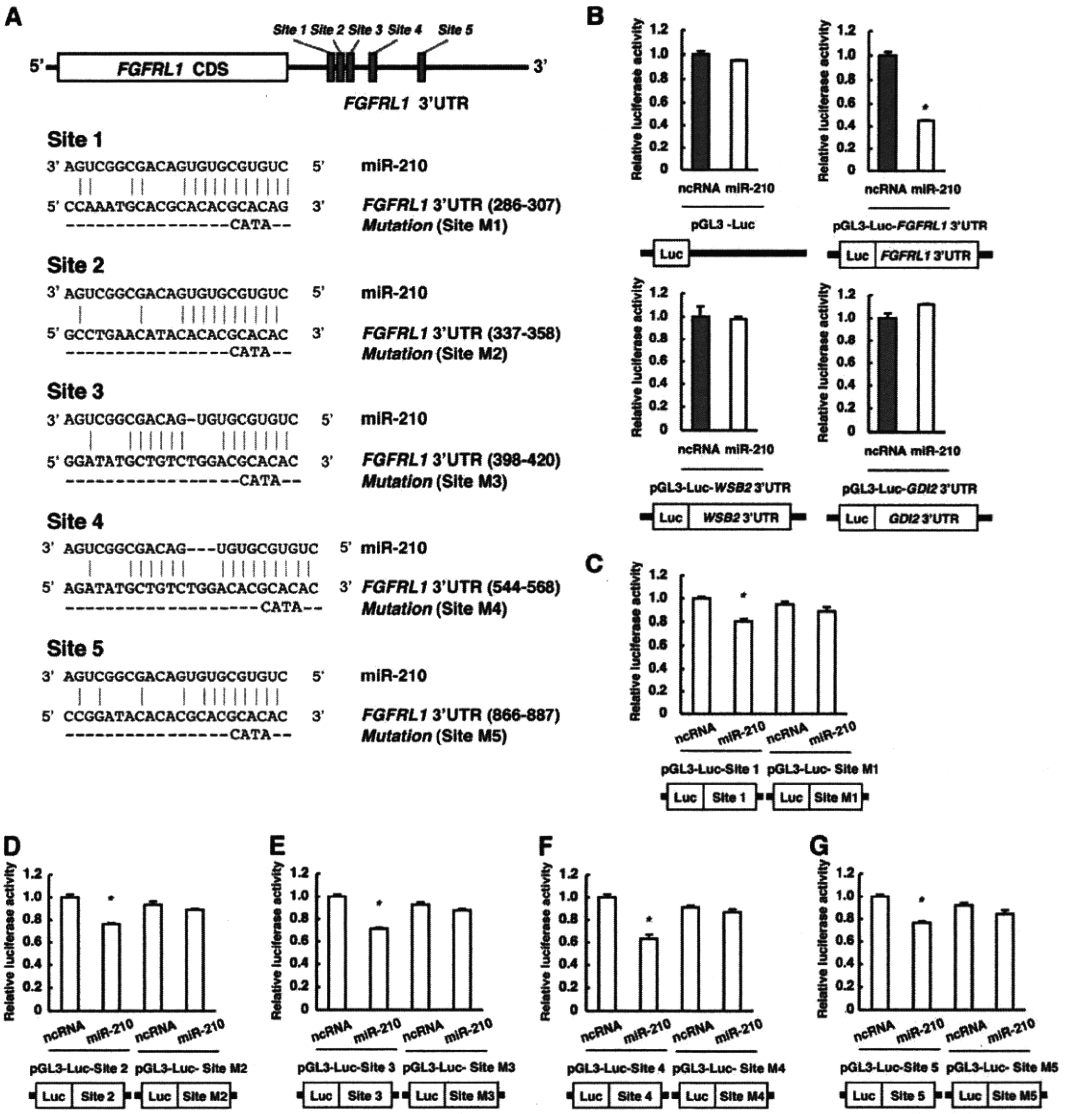
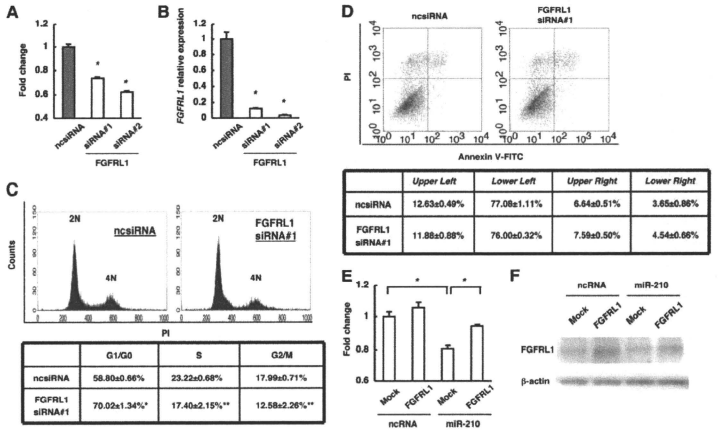


FIGURE 5. **Identification of miR-210 target sites in *FGFRL1* 3′-UTR.** *A*, schematic diagram of potential miR-210-target sites in *FGFRL1* 3′-UTR. *B–G*, cells were transfected with either ncRNA or miR-210 at 24 h after transfection with the pGL3 luciferase expression vector containing 3′-UTR of each gene or candidate miR-210 target site in *FGFRL1* 3′-UTR. After 14 h, reporter luciferase activity was evaluated. The values are shown relative to the value obtained with ncRNA ($n = 3$; *, $p < 0.01$).

FGFRL1 is a direct and robust target gene of miR-210 in ESCC. For further analysis, we cloned the each putative miR-210-target site or its point mutant in sequences corresponded to "seed sequence" of miR-210 into a pGL3-Luc vector (Fig. 5*A*) and performed reporter assays (Fig. 5, *C–G*). When miR-210 was transfected into the cells, luciferase activity was significantly repressed in all five sites. By contrast, when mutation was introduced in these sites, the repressions by miR-210 were completely abolished in all cases (Fig. 5, *C–G*). These results suggested that these five sites in the *FGFRL1* 3′-UTR are target sites of miR-210.

FGFRL1 Accelerates Cancer Cell Proliferation by Preventing Cell Cycle Arrest in G_1/G_0—To investigate the roles of *FGFRL1* in cancer cell proliferation, we first examined the effect of *FGFRL1* knockdown by using two types of *FGFRL1* siRNA. With both siRNAs, knockdown of *FGFRL1* significantly de-



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ncsiRNA	12.63±0.49%	77.08±1.11%	6.64±0.51%	3.65±0.86%
FGFRL1 siRNA#1	11.88±0.88%	76.00±0.32%	7.59±0.50%	4.54±0.66%

	G1/G0	S	G2/M
ncsiRNA	58.80±0.66%	23.22±0.68%	17.99±0.71%
FGFRL1 siRNA#1	70.02±1.34%*	17.40±2.15%**	12.58±2.26%**

FIGURE 6. **Functions of FGFRL1 in KYSE-170 cells.** *A*, effect of *FGFRL1* on cell proliferation was investigated by the WST-1 assay. Cells were incubated for 48 h after transfection with ncsiRNA, *FGFRL1* siRNA1 or 2, and viability was evaluated. The values are shown relative to the value obtained with ncsiRNA ($n = 3$; *, $p < 0.01$ *versus* ncsiRNA). *B*, *FGFRL1* expression levels in *A* were assessed by qRT-PCR. The values are shown relative to the value obtained with ncsiRNA ($n = 3$; *, $p < 0.01$ *versus* ncsiRNA). *C*, effects of *FGFRL1* on the cell cycle were investigated. The PI-stained DNA content of the cells was evaluated using a FACScan flow cytometer at 48 h after transfection ($n = 3$; **, $p < 0.05$; *, $p < 0.01$ *versus* ncsiRNA). *D*, at 48 h after transfection, cells were stained with FITC-conjugated annexin V and PI, and cell death was evaluated using a FACScan flow cytometer ($n = 3$). *E*, cells were transfected with either ncRNA or miR-210 at 24 h after transfection with the *FGFRL1* expression vector or mock. After 48 h, viability was evaluated. The values are shown relative to the value obtained with ncRNA and mock transfection ($n = 3$; *, $p < 0.01$). *F*, Western blot analyses of FGFRL1 and β-actin proteins in *E* are shown.

creased proliferation of ESCC cells, whereas a ncsiRNA had no inhibitory effect (Fig. 6*A*). The level of *FGFRL1* expression was reduced to ~10% of the original level with both siRNAs (Fig. 6*B*). Next, we examined the effects of *FGFRL1* knockdown on the cell cycle (Fig. 6*C*). Knockdown of *FGFRL1* resulted in a significant increase in the proportion of cells in G_1/G_0 phase and a decrease in that in S and G_2/M phases. These results indicated that down-regulation of *FGFRL1* induced cell cycle arrest in G_1/G_0. Down-regulation of *FGFRL1* did not significantly change the proportions of FITC-annexin V-positive cells, FITC-annexin V-negative/PI-positive cells, and FITC-annexin V-negative/PI-negative cells, which indicated that *FGFRL1* is not involved in apoptosis and necrosis (Fig. 6*D*). In contrast, overexpression of *FGFRL1* with no 3′-UTR significantly reduced the inhibitory effect of miR-210 on cell proliferation (Fig. 6*E*). Then, expression levels of FGFRL1 proteins are actually increased in cells transfected with a FGFRL1 expression vector without the 3′-UTR of *FGFRL1* (Fig. 6*F*). These findings suggest that *FGFRL1* increases the proliferation of ESCC cells by inhibiting cell cycle arrest in G_1/G_0 phase.

Endogenous MiR-210 Regulates Expression Levels of FGFRL1 and Cancer Cell Proliferation—To examine whether endogenous miR-210 regulates the expression levels of endogenous *FGFRL1*, we examined the effect of anti-miR-210 in KYSE-590 cells, which relatively highly expresses miR-210. The treatment by 2′-*O*-methylated antisense RNA of miR-210 significantly enhanced the expression levels of FGFRL1 mRNA and protein (Fig. 7*A*). Then, cancer cell proliferation was significantly increased (Fig. 7*B*). These results may indicate that miR-210 endogenously regulates the expression levels of *FGFRL1* and cancer cell proliferation.

DISCUSSION

In the present study, we showed for the first time that expression of miR-210 is down-regulated in ESCC cell lines as well as in clinical samples and that miR-210 induces cell cycle arrest and apoptosis *in vitro*, thus inhibiting the proliferation of cancer cells. Furthermore, we found that not only miR-210 expression was decreased in ESCC, but the degree of tumor differentiation corresponded well with the levels of miR-210 in clinical ESCC samples; thus, more poorly differentiated ESCCs exhibited less miR-210 expression. The results indicate that the level of miR-210 expression may serve as a clinical maker for the degree of tumor differentiation *in vivo*. We also examined whether the lower levels of miR-210 expression in poorly differentiated ESCC were related to the enhanced

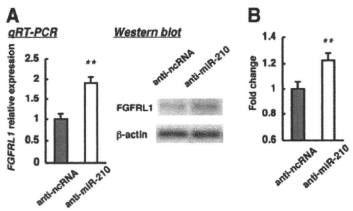

FIGURE 7. **Effects of functional inhibition of endogenous miR-210.** *A*, effect of inhibition of endogenous miR-210 on *FGFRL1* expression levels was assessed by qRT-PCR and Western blot analyses using KYSE-590 cells. Cells were incubated for 48 h after transfection with anti-ncRNA or anti-miR-210. The values are shown relative to the value obtained with anti-ncRNA ($n = 3$; **, $p < 0.05$ *versus* anti-ncRNA). *B*, cell viability in *A* was evaluated by the WST-1 assay. The values are shown relative to the value obtained with anti-ncRNA ($n = 3$; **, $p < 0.05$ *versus* anti-ncRNA).

proliferation of such carcinomas, and we found that miR-210 acts as a tumor-suppressive miRNA in ESCC.

In contrast to our study, miR-210 was reported to be up-regulated in breast cancer, pancreatic tumors, and head and neck cancer and to be correlated with their poor outcome (21–24). Moreover, previous studies have shown that miR-210 inhibits apoptosis and bypasses cell cycle arrest in cancer cell lines such as MCF-7 and HCT116 $Dicer^{ex5}$ cells (25, 26). Thus, our finding appears to be different from the previous reports that miR-210 is up-regulated in cancer tissues and enhances cell survival. The reason for this apparent discrepancy is uncertain. A likely explanation in the function of miR-210 is that it is possible that the expression of miR-210 might only reflect the hypoxia status in these cases as a surrogate marker for tumor hypoxia because miR-210 is the most robustly induced miRNA under hypoxia (9, 25, 26). Additionally, the apparently discrepant results in cancer cell lines might be explained by cell type-specific differences in the expression levels of target genes of miR-210. The results of our transcriptome analysis on ESCC cells that expressed miR-210 ectopically supported this possibility because induction of miR-210 did not affect the expressions of *CASP8AP2* and *MNT* (supplemental Table 2), which are target genes of miR-210 and induce apoptosis and cell cycle arrest, respectively (26, 27). Similar to our findings, Huang *et al.* have reported recently that miR-210 represses the initiation of tumor growth when human head and neck or pancreatic tumor cell lines that are expressing miR-210 ectopically are implanted into immunodeficient mice (10).

In the present study, we found that miR-210 is targeted to the *FGFRL1* 3′-UTR and suppresses *FGFRL1* expression in ESCC. Five sites in the 3′-UTR of *FGFRL1* were identified as target sites of miR-210. The repression in the *FGFRL1* 3′-UTR containing these five sites was considerably larger than that in each miR-210 target sites. These five miR-210 target sites in the *FGFRL1* 3′-UTR may function synergistically in the decrease in the *FGFRL1* expression level. It has been actually reported that closely located multiple miRNA target sites in the 3′-UTR of a gene may function synergistically (28, 29). As suggested by the observation that overexpression of miR-210 in ESCC cells down-regulated expression of *FGFRL1* more than that of the other predicted target genes, we found that *FGFRL1* mRNA expression was down-regulated in the miR-210-high group of clinical samples and that the level of expression was correlated inversely with the degree of differentiation. Moreover, knockdown of *FGFRL1* by siRNA inhibited ESCC cell proliferation, whereas overexpression of *FGFRL1* effectively rescued the miR-210-induced suppression of ESCC cell proliferation. Together, these findings show that miR-210 might exert its tumor-suppressive effect in ESCC mainly by targeting *FGFRL1*. This conclusion was supported by a recent study showing that *FGFRL1* could partially rescue the phenotype (suppression of tumor growth) caused by ectopic expression of miR-210 in tumor xenografts (10). However, targeting of *FGFRL1* by miR-210 appears to explain only part of the action of miR-210: down-regulation of *FGFRL1* arrested the cell cycle in G_1/G_0 phase, but not in G_2/M phase, although miR-210 induced cell cycle arrest in both phases. Hence, miR-210-induced cell cycle arrest in G_2/M phase and apoptosis might be regulated by other targets of miR-210. In fact, our transcriptome analysis identified several genes whose expression was regulated differentially by miR-210. Further studies are clearly required to understand fully the molecular mechanism of tumor suppression by miR-210.

In conclusion, the results of our present study show that miR-210 inhibits the proliferation of ESCC cells by inducing cell cycle arrest and apoptosis and that the effects of miR-210 are mediated mainly by the targeting of *FGFRL1*. Our data suggest that down-regulation of miR-210 might play an important role in the proliferation of ESCC and that miR-210 and *FGFRL1* might serve as clinical markers for tumor differentiation and therapeutic targets of ESCC.

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