Tumor suppressive microRNA-138 contributes to cell migration and invasion through its targeting of vimentin in renal cell carcinoma

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Received March 28, 2012; Accepted May 30, 2012

DOI: 10.3892/ijo.2012.1543

Abstract. Many studies have recently suggested that microRNAs (miRNAs) contribute to the development of various types of human cancers as well as to their invasive and metastatic capacities. Previously, our miRNA expression signature of renal cell carcinoma (RCC) revealed that microRNA-138 (miR-138) was significantly reduced in cancer cells. The aim of the present study was to investigate the functional significance of miR-138 and to identify its target genes in RCC cells. Restoration of mature miR-138 in two RCC cell lines (A498 and 786-O) caused changes in the bleb-like cell morphology, characteristics of the epithelial-mesenchymal transition (EMT). Restoration also significantly inhibited migration and invasion in the two RCC cell lines, suggesting that miR-138 functions as a tumor suppressor. Genome-wide gene expression analysis (miR-138 transfectants and RCC clinical specimens) and TargetScan database studies showed that vimentin (VIM) is a promising candidate target gene of miR-138. It is well known that VIM is one of the most widely expressed mammalian intermediate filament proteins. Recent studies showed that VIM functions in cell adhesion, migration, survival and cell signaling processes via dynamic assembly/disassembly in cancer cells. We focused on VIM and investigated whether VIM was regulated by tumor suppressive miR-138 and contributed to cancer cell migration and invasion in RCC cells. Restoration of miR-138 in RCC cell lines suppressed VIM expression at both the mRNA and protein levels. Silencing studies of VIM in RCC cell lines demonstrated significant inhibition of cell migration and invasion activities in si-VIM transfectants. In clinical specimens of RCC, the expression levels of VIM were significantly upregulated in cancer tissues compared to adjacent non-cancerous tissues. Furthermore, immunohistochemistry showed that VIM expression levels in RCC specimens were significantly higher than those in normal renal tissues. These data suggest that VIM may function as an oncogene and is regulated by tumor suppressive miR-138. The existence of a tumor suppressive miR-138-mediated oncogenic pathway provides new insights into the potential mechanisms of RCC oncogenesis and metastasis.

Introduction

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney. In this disease, cancer cells form in the tubules of the kidney and approximately 80% of RCC patients are diagnosed with the clear cell RCC subtype (1). Up to 30% of RCC patients present at advanced stages, and approximately 40% of patients who undergo curative surgical resection experience recurrence during subsequent follow-up (2,3). The five-year survival rate of advanced RCC is 5-10% (4). RCC is resistant to radiotherapy and chemotherapy (5,6). Targeted therapies such as sunitinib, sorafenib, everolimus and temsirolimus have been developed and have been used widely in first- and second-line treatments, extending the period of progression-free-survival (7,8). However, these treatments are insufficient for patients who have developed relapse or metastasis. Therefore, increased understanding of the molecular mechanisms of RCC progression and metastasis is needed using the latest approaches to genomic analysis.

DNA can be divided into two categories: protein coding RNA and non-coding RNA (ncRNA). It is important to examine the functions of ncRNAs and their association with human disease, including cancer. microRNAs (miRNAs) are endogenous small ncRNA molecules (19-22 bases in length) that regulate protein coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (9). A growing body of evidence suggests that miRNAs are aberrantly expressed in many human cancers, and that they play significant roles in their initiation, development, and metastasis (10). Some highly expressed miRNAs could function as oncogenes by repressing tumor suppressors, whereas low level miRNAs could function as tumor suppressors by negatively regulating oncogenes (11).

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Key words: microRNA, miR-138, vimentin, renal cell carcinoma
We previously identified many tumor suppressive miRNAs based on our miRNA expression signatures of various types of cancer, such as RCC, bladder cancer, prostate cancer, maxillary sinus squamous cell carcinoma and hypopharyngeal squamous cell carcinoma (12-17). In oncogenic pathways, normal regulatory mechanisms are disrupted by aberrant expression of tumor suppressive or oncogenic miRNAs. Therefore, identification of miRNA-regulated pathways is important for further development in human cancer research. Thus, we have been investigating how tumor suppressive miRNA regulates novel cancer pathways. For example, the miR-1/miR-133a cluster regulates several oncogenic genes, including transgelin-2 (TAGLN2), prothymosin α (PTMA) and purine nucleoside phosphorylase (PNP) (14,15,18).

More recently, we constructed a miRNA expression signature of RCC clinical specimens and successfully identified tumor suppressive miR-1285 targeting transglutaminase 2 (TGM2) (12). Among the signatures, several miRNAs were significantly downregulated in RCC specimens as promising candidate of tumor suppressors. In this study, we focused on miR-138. This miRNA was downregulated in our previous signature, and downregulation of miR-138 has been observed in several malignancies, including anaplastic thyroid carcinoma (19) and lung cancer (20).

The aim of the study was to investigate the functional significance of miR-138 and identify its target genes in RCC cells. To identify miR-138-regulated cancer pathways, we undertook both a genome-wide gene expression analysis (miR-138 transfectants and RCC clinical specimens) and an in silico study. The results showed that vimentin (VIM) was a promising candidate target gene of miR-138. It is well known that VIM is one of the most widely expressed mammalian intermediate filament proteins. Studies have shown that VIM functions in cell adhesion, migration, survival, and cell signaling processes via dynamic assembly/disassembly in cancer cells (21). The existence of a tumor suppressive miR-138-mediated cancer pathway provides new insights into the potential mechanisms of RCC oncogenesis and metastasis.

**Materials and methods**

**Clinical specimens.** A total of 33 pairs of clear cell renal cell carcinoma (ccRCC) and adjacent non-cancerous specimens were collected from patients who had undergone radical nephrectomies at Kagoshima University Hospital. The samples were processed and stored in RNA later (Qiagen, Valencia, CA, USA) at -20°C until RNA extraction. The patient information is summarized in Table I. These samples were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer (UICC) tumour-node-metastasis classification (22). Our study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were given by the patients.

**Cell culture and RNA extraction.** We used human RCC cell lines, A498 and 786-O, obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator (5% CO₂) at 37°C. Total-RNA was extracted, as previously described (12).

| Table I. Patient characteristics of RT-PCR experiments. |
|----------------------------------------------------------|
| **No. of patients (%)**                                  |
| Total number                                             | 33                                      |
| Age (average)                                            | 36-83 (65.6)                            |
| Gender                                                  |                                         |
| Male                                                     | 22 (66.7)                               |
| Female                                                   | 11 (33.3)                               |
| Pathological tumor stage                                 |                                         |
| pT1a                                                     | 12 (36.4)                               |
| pT1b                                                     | 14 (42.4)                               |
| pT2                                                      | 2 (6.1)                                 |
| pT3a                                                     | 3 (9.1)                                 |
| pT3b                                                     | 2 (6.1)                                 |
| pT4                                                      | 0 (0.0)                                 |
| Grade                                                    |                                         |
| G1                                                       | 5 (15.2)                                |
| G2                                                       | 26 (78.8)                               |
| G3                                                       | 0 (0.0)                                 |
| Unknown                                                  | 2 (6.1)                                 |
| Infiltration                                             |                                         |
| α                                                        | 12 (36.4)                               |
| β                                                        | 21 (63.6)                               |
| γ                                                        | 0 (0.0)                                 |
| Venous invasion                                          |                                         |
| v (-)                                                    | 24 (72.7)                               |
| v (+)                                                    | 9 (27.3)                                |

**Quantitative real-time RT-PCR.** TaqMan probes and primers for VIM (P/N: Hs00185584_m1: Applied Biosystems) were assay-on-demand gene expression products. All reactions were performed in duplicate, and a negative control lacking cDNA was included. We followed the manufacturer's protocol for PCR conditions. Stem-loop RT-PCR (TaqMan MicroRNA Assays; P/N: 002284 for miR-138; Applied Biosystems) was used to quantitate miRNAs according to the earlier published conditions (23). To normalize the data for quantification of VIM mRNA and the miRNAs, we used human GUSB (P/N: Hs99999908_m1; Applied Biosystems) and RNU6B (P/N: 001973; Applied Biosystems), respectively, and we used the ΔΔCt method to calculate the fold-change. As a control RNA, we used Premium total-RNA from normal human kidney (AM 7976; Applied Biosystems).

**Mature miRNA and siRNA transfection.** As described elsewhere (23), the RCC cell lines were transfected with Lipofectamine™ RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM™ (Invitrogen) with 10 nM mature miRNA molecules. Pre-miR™ (Applied Biosystems) and negative-control miRNA (Applied Biosystems) were used in the gain-of-function experiments, whereas VIM siRNA (Cat nos. SASI_Hs01_00044033 and SASI_HS01_00044036, Sigma-Aldrich, St. Louis, MO, USA) and negative control siRNA (D-001810-10; Thermo Fisher Scientific, Waltham, MA, USA) were used in the loss-of-function experiments. Cells were seeded in 10-cm dishes for protein extraction (8x10⁵ cells per
Table II. Downregulated genes in microRNA-138 transfectants.

| Entrez gene ID | Symbol | Average | Target site |
|---------------|--------|---------|-------------|
| 3569          | IL6    | -5.35   | (-)         |
| 4856          | NOV    | -5.22   | (-)         |
| 84448         | ABLIM2 | -4.97   | (-)         |
| 3773          | KCNJ16 | -4.86   | (-)         |
| 6352          | CCL5   | -4.44   | (-)         |
| 4316          | MMP7   | -4.1    | (-)         |
| 3038          | HAS3   | -4.03   | (+)         |
| 91543         | RSAD2  | -3.99   | (-)         |
| 5806          | PTX3   | -3.85   | (-)         |
| 64220         | STRA6  | -3.85   | (+)         |
| 84449         | C15orf48 | -3.8   | (-)         |
| 144406        | WDR66  | -3.67   | (-)         |
| 4493          | MT1E   | -3.66   | (-)         |
| 718           | C3     | -3.65   | (-)         |
| 10964         | IFI44L | -3.64   | (-)         |
| 3990          | LIPC   | -3.64   | (-)         |
| 9121          | SLC16A5 | -3.61   | (-)         |
| 4490          | MT1B   | -3.6    | (-)         |
| 8091          | HMGA2  | -3.56   | (-)         |
| 1803          | DPP4   | -3.49   | (-)         |
| 6288          | SAA1   | -3.48   | (-)         |
| 4502          | MT2A   | -3.44   | (-)         |
| 8638          | OASL   | -3.43   | (-)         |
| 9582          | APOBEC3B | -3.39  | (-)         |
| 4500          | MT1L   | -3.32   | (-)         |
| 3437          | IFT3   | -3.3    | (-)         |
| 9076          | CLDN1  | -3.05   | (-)         |
| 8743          | TNFSF10 | -3.03    | (-)         |
| 3433          | IFT2   | -2.94   | (-)         |
| 2172          | FABP6  | -2.91   | (-)         |
| 23586         | DDX58  | -2.89   | (-)         |
| 4982          | TNFRSF11B | -2.89 | (-)         |
| 259307        | IL4I   | -2.88   | (-)         |
| 6590          | SLPI   | -2.88   | (-)         |
| 5174          | PDZK1  | -2.88   | (-)         |
| 51015         | ISOC1  | -2.86   | (+)         |
| 3434          | IFT1   | -2.84   | (-)         |
| 22822         | PHLD1A1 | -2.79    | (-)         |
| 2537          | IFI6   | -2.76   | (-)         |
| 392636        | TMEM195 | -2.76    | (-)         |
| 81610         | FAM83D | -2.73   | (+)         |
| 26154         | ABCA12 | -2.66   | (-)         |
| 4940          | OAS3   | -2.65   | (+)         |
| 5359          | PLSCR1 | -2.65   | (-)         |
| 6236          | RRAD   | -2.61   | (-)         |
| 4496          | MT1H   | -2.56   | (-)         |
| 4814          | NINJ1  | -2.54   | (+)         |
| 11309         | SLCO2B1 | -2.5    | (+)         |
| 158158        | RASEF  | -2.47   | (-)         |
| 259           | AMBP   | -2.47   | (-)         |
| 2669          | GEM    | -2.47   | (-)         |
Table II. Continued.

| Entrez gene ID | Symbol | Average | Target site |
|---------------|--------|---------|-------------|
| 3656          | IRAK2  | -2.42   | interleukin-1 receptor-associated kinase 2 (-) |
| 3880          | KRT19  | -2.41   | keratin 19 (+) |
| 1978          | EIF4EBP1 | -2.41 | eukaryotic translation initiation factor 4E binding protein 1 (+) |
| 7431          | VIM    | -2.39   | vimentin (+) |
| 57568         | SIPA1L2 | -2.38  | signal-induced proliferation-associated 1 like 2 (-) |
| 7913          | DEK    | -2.35   | DEK oncogene (+) |
| 123           | PLIN2  | -2.34   | perilipin 2 (-) |
| 4501          | MT1X   | -2.33   | metallothionein 1X (-) |
| 654346        | LGALS9C | -2.31  | lectin, galactoside-binding, soluble, 9C (+) |
| 4489          | MT1A   | -2.3    | metallothionein 1A (-) |
| 3669          | ISG20  | -2.28   | interferon stimulated exonuclease gene 20 kDa (-) |
| 2920          | CXCL2  | -2.28   | chemokine (C-X-C motif) ligand 2 (-) |
| 2274          | FHL2   | -2.27   | four and a half LIM domains 2 (-) |
| 157506        | RDH10  | -2.27   | retinol dehydrogenase 10 (all-trans) (-) |
| 25937         | WWTR1  | -2.26   | WW domain containing transcription regulator 1 (-) |
| 3690          | ITGB3  | -2.26   | integrin, β 3 (platelet glycoprotein IIIa, antigen CD61) (+) |
| 196513        | DCP1B  | -2.24   | DCP1 decapping enzyme homolog B (S. cerevisiae) (-) |
| 9518          | GDPF15 | -2.24   | growth differentiation factor 15 (-) |
| 1364          | CLDN4  | -2.23   | claudin 4 (-) |
| 23643         | LY96   | -2.2    | lymphocyte antigen 96 (-) |
| 10561         | IFI44  | -2.2    | interferon-induced protein 44 (-) |
| 84141         | FAM176A | -2.19  | family with sequence similarity 176, member A (-) |
| 6281          | S100A10 | -2.17  | S100 calcium binding protein A10 (-) |
| 7088          | TLE1   | -2.17   | transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) (-) |
| 81553         | FAM49A | -2.17   | family with sequence similarity 49, member A (-) |
| 4599          | MX1    | -2.17   | myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (-) |
| 6850          | SYK    | -2.17   | spleen tyrosine kinase (-) |
| 7364          | UGT2B7 | -2.17   | UDP glucuronosyltransferase 2 family, polypeptide B7 (-) |
| 5366          | PMAIP1 | -2.17   | phorbol-12-myristate-13-acetate-induced protein 1 (-) |
| 50515         | CHST11 | -2.16   | carbohydrate (chondroitin 4) sulfotransferase 11 (+) |
| 2982          | GUCY1A3 | -2.16  | guanylate cyclase 1, soluble, α 3 (+) |
| 6273          | S100A2 | -2.15   | S100 calcium binding protein A2 (+) |
| 54478         | FAM64A | -2.15   | family with sequence similarity 64, member A (-) |
| 3428          | IFI16  | -2.14   | interferon, γ-inducible protein 16 (-) |
| 9615          | GDA    | -2.14   | guanine deaminase (-) |
| 7849          | PAX8   | -2.13   | paired box 8 (-) |
| 10550         | ARL6IP5 | -2.11  | ADP-ribosylation-like factor 6 interacting protein 5 (+) |
| 23286         | WWC1   | -2.1    | WW and C2 domain containing 1 (+) |
| 9636          | ISG15  | -2.08   | ISG15 ubiquitin-like modifier (+) |
| 896           | CCND3  | -2.07   | cyclin D3 (+) |
| 5329          | PLAUR  | -2.07   | plasminogen activator, urokinase receptor (-) |
| 4853          | NOTCH2 | -2.06   | Notch homolog 2 (Drosophila) (+) |
| 55652         | SLC48A1 | -2.05   | solute carrier family 48 (heme transporter), member 1 (+) |
| 23476         | BRD4   | -2.04   | bromodomain containing 4 (+) |
| 2012          | EMP1   | -2.03   | epithelial membrane protein 1 (+) |
| 3429          | IFI27  | -2.02   | interferon, α-inducible protein 27 (-) |
| 79710         | MORC4  | -2.01   | MORC family CW-type zinc finger 4 (-) |
| 80820         | EEPD1  | -2.01   | endonuclease/exonuclease/phosphatase family domain containing 1 (+) |
dissected, 6-well plates for wound healing assays (2x10^4 cells per well), in 24-well plates for the mRNA extraction and Matrigel invasion assays (5x10^4 cells per well) and in 96-well plates for the XTT assays (3,000 cells per well).

**Cell morphology.** Cells were transfected with miR-138 and si-VIM for 72 h and were then examined by an inverted microscope (CK2-BIP2, Olympus).

**Cell proliferation, migration and invasion assays.** Cell proliferation was determined using an XTT assay (Roche Applied Science, Tokyo, Japan) that was performed according to the manufacturer's instructions. Cell migration activity was evaluated with a wound healing assay. Cells were plated in 6-well dishes and the cell monolayer was scraped using a P-20 micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wounding were calculated from photomicrographs. A cell invasion assay was carried out using modified Boyden Chambers consisting of Transwell-precoated Matrigel membrane filter inserts with 8-μm pores in 24-well tissue culture plates (BD Bioscience, Bedford, MA, USA). Minimum essential medium containing 10% FBS in the lower chamber served as the chemoattractant as described previously (24). All experiments were performed in triplicate.

**Screening of miR-138-regulated genes by microarray.** Oligo-microarray Human 60K (Agilent) was used for expression signature in miR-138-transfected A498 cells in comparison with the miR-negative control transfectant, as previously described (23). Briefly, hybridization and washing steps were performed in accordance with the manufacturer's instructions. The arrays were scanned using a Packard GSI Lumonics ScanArray 4000 (PerkinElmer, Boston, MA, USA). The data obtained were analyzed with DNASIS array software (Hitachi Software Engineering, Tokyo, Japan) that converted the signal intensity. Data from each microarray study were normalized by global normalization.

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**Table III. Patient characteristics of immunohistochemistry.**

| Total number | 67 |
| Age (average) | 30-80 (54.4) |
| Gender | Male 45 (67.2) / Female 22 (32.8) |
| Pathological tumor stage | pT1 15 (22.4) / pT2 28 (41.8) / pT3 22 (32.8) / pT4 2 (3.0) |
| Grade | G1 52 (77.6) / G2 14 (20.9) / G3 1 (1.5) |
| Normal tissue | 10 |

**Expression signature of RCC clinical specimens by microarray.** Oligo-microarray Human 60K (Agilent) was used for expression signature in 5 pairs of RCC clinical specimens compared with adjacent non-cancerous tissues. Their age ranged from 42 to 77 years; 3 were G1 and 2 were G2 in their tumor grading; and all were pT1N0M0 tumors.

**Western blot analysis.** After three days of transfection, protein lysates (40 μg) were separated by NuPAGE on 4-12% bis-tris gels (Invitrogen) and transferred onto polyvinylidene fluoride membranes. Immunoblotting was done with diluted (1:500) polyclonal VIM antibody (HPA001762; Sigma-Aldrich) and GAPDH antibody (MAB374; Chemicon, Temecula, CA, USA). The membrane was washed and then incubated with goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualized with an echocleumluminescence (ECL) detection system (GE Healthcare, Little Chalfont, UK), and the expression levels of these genes were evaluated by ImageJ software (ver. 1.43; http://rsweb.nih.gov/ij/index.html).

**Immunohistochemistry.** A tissue microarray of 67 RCC samples and 10 normal kidney samples was obtained from US Biomax Inc. (KD806; Rockville, MD, USA). Detailed information on all tumor specimens can be found at http://www.biomax.us/index.php. Patient characteristics are summarized in Table III. Immunostaining was done on the tissue microarray following the manufacturer's protocol by UltraVision Detection System (Thermo Scientific). The primary rabbit polyclonal antibodies against VIM (Sigma-Aldrich) were diluted 1:500. The slides were treated with biotinylated goat anti-rabbit. Diaminobenzidine hydrogen peroxidase was the chromogen, and the counterstaining was done with 0.5% hematoxylin. Immunostaining was evaluated according to a scoring method described previously (14). Each case was scored on the basis of the intensity and area of staining. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, 30-60% stained positive; 3+, >60% stained positive. A combined staining score (intensity + extent) of <2 was low expression, a score between 3 and 4 was moderate expression, and a score between 5 and 6 was high expression.

**Statistical analysis.** The relationships between two variables and numerical values were analyzed using the Mann-Whitney U test, and the relationship between three variables and the numerical values was analyzed using the Bonferroni-adjusted Mann-Whitney U test. Expert Stat View analysis software (ver. 4; SAS institute Inc., Cary, NC, USA) was used in both analyses. In the comparison of three variables, a non-adjusted statistical level of significance of P<0.05 corresponded to the Bonferroni-adjusted level of P<0.0167.

**Results**

**Effect of miR-138 transfection on cell proliferation, migration, and invasion activity of RCC cell lines.** In this study, we firstly observed that restoration of miR-138 in RCC cell lines (A498 and 786-O) changed the bleb-like cell morphology characteristic of the epithelial-mesenchymal transition (EMT) (Fig. 1A). A morphological change of cancer cells by miRNA transfection is an important discovery and it suggested that miR-138 functions...
Figure 1. Effect of miR-138 transfection on RCC cell lines. (A) The change of morphology of miR-138 transfectants. A498 and 786-O cells were transfected with miR-138 for 72 h and were then examined by an inverted microscope. (B) miR-138 expression in A498 and 786-O cell lines and in normal kidney. miR-138 expression levels in A498 and 786-O were significantly lower than those in normal human kidney RNA. RNU6B was used as an internal control. (C-E) Effect of miR-138 transfection of A498 and 786-O cells. (C) Cell proliferation determined by the XTT assay; (D) cell migration activity determined by wound healing assay; and (E) cell invasion activity determined by the Matrigel invasion assay. *P<0.001, **P<0.0001.
miR-138 regulates molecular targets in RCC cells. (A) Heatmap derived from five RCC samples. A total of 99 genes were downregulated less than -2.0-fold in miR-138 transfectants. We checked their mRNA expression levels in RCC by using our previous gene expression analysis of five RCC specimens. Ninety-nine gene expression levels are shown in the heat map diagram. VIM was the top upregulated gene among the genes which have miR-138 target sites in the heat map diagram. (B) VIM mRNA expression after 24 h transfection with 10 nM miR-138. (C) VIM protein expression after 72 h transfection of miRNAs. GAPDH was used as a loading control. The mRNA and protein levels of VIM were repressed in the transfectants. *P<0.01, **P<0.0001, (D) miR-138 binding sites in the 3'UTR of VIM mRNA.
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as a tumor suppressor in RCC cells. To explore that possibility, the following experiments were conducted.

We evaluated the expression levels of miR‑138 in two RCC cell lines, A498 and 786-O. RNA was extracted and miRNA expression levels of miR‑138 were determined by real-time RT-PCR. The expression levels of miR‑138 were significantly lower in both RCC cell lines compared with normal kidney RNA (relative to normal kidney RNA, 0.090±0.008 and 0.102±0.009, respectively) (Fig. 1B).

The XTT assay revealed that cell proliferation was significantly inhibited in miR‑138 transfectants in comparison with the transfectant reagent only (mock) and the miR-control transfectants. The percentages of cell proliferation for A498 were 94.6±0.9, 100.0±0.8 and 100.0±1.0, respectively, each P=0.0008. For 786-O, the percentages were 83.5±1.1, 100.0±0.4 and 100.3±0.6, respectively, P<0.0001 (Fig. 1C).

The wound healing assay demonstrated that significant inhibition of cell migration occurred in the miR‑138 transfectants in comparison with mock and the miR-control transfectants. The percentages of wound closure for A498 were 6.5±2.3, 100.0±2.7 and 104.8±4.9, respectively, each P<0.0001. For 786-O, the percentages were 30.7±3.8, 100.0±4.4 and 95.9±5.4, respectively, each P<0.0001 (Fig. 1D).

The Matrigel invasion assay demonstrated that the number of invading cells significantly decreased in the miR‑138 transfectants in comparison with mock and the miR-control transfectants. The percentages of cell invasion for A498 were 0.9±0.4, 100.0±8.6 and 83.1±7.4, respectively, each P<0.0001. For 786-O, the values were 10.9±1.1, 100.0±4.4 and 75.3±6.2, respectively, each P<0.0001 (Fig. 1E).

miR‑138 regulation of molecular targets assessed by genome‑wide gene expression analysis. To confirm that miR‑138 regulated molecular targets in RCC cells, we performed genome-wide gene expression analysis using miR‑138 transfectants compared with miRNA-control transfectants in A498 cells. A total of 99 genes were downregulated in miR‑138 transfectants. Among them, 24 genes had putative target site(s) in their 3' untranslated region (3'UTR) according to the TargetScan miRNA program (Table II).

Furthermore, we performed gene expression analysis using RCC clinical specimens (5 pairs of RCC and adjacent non-cancerous tissues). Several protein-coding genes were differentially expressed in this signature (data not shown). We selected 99 genes that were downregulated in miR‑138 transfectants and demonstrated their expression levels in a heatmap diagram (Fig. 2A). Entries from the microarray data were approved by the Gene Expression Omnibus (GEO), and were assigned GEO accession numbers GSE 36951 (RCC clinical specimens) and GSE 37119 (miR‑138 transfectants).

The two expression signatures in this study (miR‑138 transfectants and RCC clinical specimens) revealed that VIM was a promising putative target gene in miR‑138 in RCC. Thus, we focused on the VIM gene and investigated the functional significance of VIM in RCC cells.

VIM as a direct target of repression by miR‑138 in RCC cells. The mRNA and protein expression levels of VIM were markedly downregulated in miR‑138 transfectants (A498 and 768-O) in comparison with the mock and miRNA-control transfectants (Fig. 2B and C). The predicted target site of miR‑138 in VIM in the 3'UTR is shown in Fig. 2D.

Silencing of VIM in RCC cell lines and the effect on cell proliferation, migration and invasion. First, we assessed the expression level of VIM in cells to be used for functional analysis of VIM. VIM mRNA expression levels in A498 and 786-O were significantly higher than those in normal human kidney RNA (relative to normal kidney RNA, 4.879±0.131 and 9.298±0.255, respectively, each P<0.0001) (Fig. 3A).
Figure 4. Response to VIM silencing by si-VIM transfection of RCC cell lines. (A) The change of morphology of si-VIM transfectants. A498 and 786-O cells were transfected with si-VIM for 72 h and were then examined by an inverted microscope. (B-D) VIM-knockdown effects on A498 and 786-O cell lines transfected with si-VIM-1 and si-VIM-2. (B) Cell proliferation determined by the XTT assay; (C) cell migration activity determined by the wound healing assay; and (D) cell invasion activity determined by the Matrigel invasion assay. *P<0.005, **P<0.0001.
To examine the functional role of VIM, we performed loss-of-function studies using two different siRNAs, si-VIM-1 and si-VIM-2 transfected into A498 and 768-O cell lines. The mRNA and protein expression levels of VIM were markedly downregulated in both si-VIM-1 and si-VIM-2 transfectants (A498 and 768-O) in comparison with the siRNA-control transfectants (Fig. 3B and C). This result shows that two siRNA were useful for loss-of-function assays in this study.

Transfection of si-VIM-1 and si-VIM-2 in the RCC cell lines (A498 and 768-O) caused EMT-like changes in cell morphology, as that observed when cells were transfected with miR-138 (Fig. 4A).

The XTT assay revealed that cell proliferation was inhibited in both si-VIM-transfectants in comparison with the si-control transfectants. The percentages of cell proliferation for A498 were 76.1±2.6, 87.5±1.9 and 100.0±3.1, respectively, P<0.0001 and P =0.0038. For 786-O, the values were 43.1±1.0, 89.9±0.7 and 100.0±1.1, respectively, P<0.0001 (Fig. 4B).

The wound healing assay demonstrated that significant inhibition of cell migration occurred in the si-VIM-transfectants in comparison with the si-control transfectants. The percentage of wound closure for A498 were 26.0±10.6, 44.7±8.3 and 100.0±2.6, respectively, each P<0.0001. For 786-O, the values were 60.8±7.3, 73.0±1.7 and 100.0±7.1, respectively, P<0.0001 and P=0.0002 (Fig. 4C).

The Matrigel invasion assay demonstrated that the number of invading cells significantly decreased in the si-VIM-transfectants in comparison with the si-control transfectants. For A498, the percentages of cells invading were 26.5±4.7, 54.8±8.3 and 100.0±12.4, respectively, P<0.0001 and P =0.0019. For 786-O, the values were 43.6±8.3, 61.6±3.3 and 100.0±11.1, respectively, P<0.0001 and P=0.0034 (Fig. 4D).

Expression levels of miR-138 and VIM mRNA in RCC clinical specimens. Quantitative stem-loop RT-PCR demonstrated that the expression levels of miR-138 were significantly reduced in 33 RCC samples (Table I) in comparison with adjacent non-cancerous specimens (clinical RCC specimens, 0.346±0.201 versus adjacent normal tissues, 2.983±0.715, P<0.0001) (Fig. 5A).

On the other hand, the mRNA expression level of VIM was significantly higher in RCC than adjacent non-cancerous specimens (clinical RCC specimens, 6.017±0.622, adjacent normal tissues; 1.316±0.224, P<0.0001) (Fig. 5B).

The mRNA expression of ≥T2 specimens (n=7) was significantly higher than that of T1 (n=26) (T1, 5.230±0.607; ≥T2, 8.773±1.349, P=0.0277) (Fig. 5C).

Immunohistochemistry of VIM in tissue microarray. VIM was detected by immunohistochemical staining. Fig. 6A-D shows representative results of immunohistochemical staining of VIM. VIM was strongly expressed in tumor lesions (Fig. 6A-C), whereas no expression was observed in normal tissue (Fig. 6D). The expression score of VIM was significantly higher in 67 RCC specimens in comparison with ten normal kidney specimens (Fig. 6E). The VIM expression of ≥T2 specimens (n=52) was higher than that of T1 (n=15). There was a trend but no significant difference in the expression score of VIM between T1 and ≥T2 specimens (P=0.053, Fig. 6F). While there was a trend between VIM expression and grade, the difference was not significant. Patient characteristics are summarized in Table III.

Discussion

The incidence of RCC has increased over the last few decades, and it currently represents approximately 2% of all cancer-related deaths (25). Although two-thirds of RCC patients have clinically localized disease and will undergo curative surgery, up to 40% of patients develop distant metastasis and their outcomes are poor (2-4). Many studies have indicated that cell adhesion and extra-cellular matrix proteins contribute to the cells’ acquired abilities for invasion, migration and metastasis (26).

EMT is an embryologically conserved genetic program that is an essential step enabling cancer cell invasion and metastasis. For example, epithelial cells lose intercellular
Recent data indicate that the microRNA-200 family (miR-200a, -200b, -200c, -141 and -429) is downregulated in aggressive human cancers. Moreover, it plays critical roles in the inhibition of key regulators of EMT and β-catenin/Wnt signaling (28,29). Interestingly, our miRNA expression signature of RCC showed that all miR-200 family members were reduced in clinical specimens and that restoration of the miR-200 family inhibited cancer cell migration and invasion (data not shown).

Our previous study showed that miR-138 was reduced in RCC miRNA expression signature (12), we validated the downregulation of miR-138 in RCC clinical specimens in this study.

Aberrant expression of miR-138 has been observed in several types of cancer such as head and neck squamous cell carcinoma (HNSCC) (30), anaplastic thyroid carcinoma (19) and lung cancer (20). Two miR-138 precursor genes, miR-138-1 and miR-138-2, have identical sequences in the mature miRNA and map to human chromosomes 3p21.33 and 16q13, respectively. Although it is believed that genomic deletion or epigenetic silencing of miRNA in cancer cells, the molecular mechanism of downregulated miRNAs in RCC is not clear. In the human chromosomal region 3p, LOH is frequently observed in many cancers including RCC (31,32). This problem can be solved by genome-based high throughput analysis in each clinical case.
Importantly, we found significant morphologic change in RCC cell lines (A498 and 786-O) by miR-138 transfection. Furthermore, restoration of miR-138 significantly inhibited cancer cell migration and invasion in RCC cells. These data suggested that miR-138 functions as a tumor suppressor that inhibits RCC invasion and metastasis. miRNAs are unique in their ability to regulate many protein-coding genes. A single miRNA is capable of targeting a number of genes to regulate biological processes globally. Bioinformatic predictions suggest that miRNAs regulate more than 30% of protein-coding genes (9). The elucidation of new molecular pathways regulated by tumor suppressive miR-138 is important for our understanding of human RCC invasion and metastasis. Based on this view, we performed molecular target searches for miR-138 in cancer cells by combining two genome-wide gene expression studies (miR-138 transfectants and RCC mRNA clinical signature) and in silico analysis.

In this study, we focused on VIM as a putative candidate of miR-138 in RCC cells. We chose VIM for the following reasons. First, downregulation of VIM was recognized in the expression signature of miR-138 transfectants. Second, overexpression of VIM was observed in RCC clinical specimens. Third, VIM has a putative miR-138 target site in its 3' untranslated region. Our data demonstrated that restoration of miR-138 significantly inhibited both mRNA and protein expression levels of VIM in RCC cells, suggesting VIM was regulated by tumor suppressive miR-138. It is well known that VIM is an essential constituent of cytoskeletal proteins of mesenchymal cells and VIM is a marker of EMT (21). During EMT, cytoskeletal proteins are changed from keratin-rich networks to VIM-rich networks connected to focal adhesions. Morphological changes of RCC cells and accelerated cell migration and invasion is caused by the reduction of miR-138 and the upregulation of VIM pathways. Interestingly, it has been shown that miR-138 regulated cell migration and invasion by targeting Rhoc, ROCK, ZEB2, EZH2 and VIM in HNSCC cells (33,34). Importantly, restoration of miR-138 in an HNSCC cell line changed the EMT-like cell morphology and suppressed cell migration and invasion (34). This result is in accord with the data obtained in our study of RCC. Furthermore, overexpression of miR-138 reduced cell viability and colony formation in HCC cell lines targeting CCND3 (35). The report also showed that protein expression of CCND3 was negatively correlated with miR-138 expression in HCC tissues. Our data of miR-138 transfectants in RCC cell lines demonstrated that CCND3 is a putative target of miR-138 in RCC, suggesting that miR-138 regulation of the CCND3 pathway is important for RCC oncogenesis.

The results of this study and previous data indicate that VIM is a functional target of tumor suppressive miR-138, and this pathway contributes to cancer cell migration, invasion, and metastasis. In this study, we also demonstrated overexpression of VIM in clinical specimens of RCC. Previous studies indicated that VIM was a sensitive and specific marker for conventional RCCs (36,37). The combination of VIM and CD9 staining was found to distinguish clear cell RCC and chromophobe RCC (37). Our tissue microarray data showed a positive correlation between VIM expression and tumor grade in RCC specimens. In this analysis, we were not able to obtain a correlation of VIM expression and metastasis in RCC patients. Silencing of VIM in RCC cell lines changed cell morphology and significantly inhibited cell migration and invasion in this study. Thus, we propose that overexpression of VIM participates in metastasis of RCC. Studies of a large number of samples with balanced pathological backgrounds are needed to elucidate the precise correlation between VIM and/or miR-138 expression and clinicopathological parameters.

In summary, the reduction of miR-138 and the increased expression of VIM are frequent events in RCC clinical specimens. Restoration of miR-138 in RCC cells changed the EMT-like morphology and suppressed cell migration and invasion. The tumor suppressive miR-138-mediated cancer pathway provides new insights into the potential mechanisms of RCC oncogenesis and metastasis.

Acknowledgements

This research was supported by the Ministry of Education, Science, Sports and Culture Grant-in-Aid for Scientific Research (C), 20591861 and 21592187.

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