Abstract. Cervical cancer is one of the most common cancers in women. More than 275,100 women die from cervical cancer each year. Cervical squamous cell carcinoma (cervical SCC), one of the most frequent types of cervical cancers, is associated with high-risk human papilloma virus (HPV), although HPV infection alone may not be enough to induce malignant transformation. MicroRNAs (miRNAs), a class of small non-coding RNAs, regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner. A growing body of evidence suggests that miRNAs contribute to cervical SCC progression, development and metastasis. miRNA expression signatures in SCC (hypopharyngeal SCC and esophageal SCC) revealed that miR-218 expression was significantly reduced in cancer tissues compared with adjacent non-cancerous epithelium, suggesting that miR-218 is a candidate tumor suppressor. The aim of this study was to investigate the functional significance of miR-218 in cervical SCC and to identify novel miR-218-mediated cancer pathways in cervical SCC. Restoration of miR-218 significantly inhibited cancer cell migration and invasion in both HPV-positive and HPV-negative cervical SCC cell lines. These data indicated that miR-218 acts as a tumor suppressor in cervical SCC. Our in silico analysis showed that miR-218 appeared to be an important modulator of tumor cell processes through suppression of many targets, particularly those involved in focal adhesion signaling pathways. Gene expression data indicated that LAMB3, a laminin protein known to influence cell differentiation, migration, adhesion, proliferation and survival, was upregulated in cervical SCC clinical specimens, and silencing studies demonstrated that LAMB3 functioned as an oncogene in cervical SCC. The identification of novel tumor-suppressive miR-218-mediated molecular pathways has provided new insights into cervical SCC oncogenesis and metastasis.

Introduction

Cervical cancer is one of the most common cancers in women. It has been estimated that more than 529,800 new cases will be diagnosed each year, and approximately 275,100 women worldwide will die of cervical cancer each year (1). Cervical squamous cell carcinoma (cervical SCC) is one of the most frequent types of cervical cancers, accounting for 80-90% of cervical cancers, and the most important risk factor for cervical SCC is persistent human papilloma virus (HPV) infection (2). Epidemiological studies have indicated that more than 99% of patients with cervical SCC are positive for high-risk HPV (HPV16, HPV18 and HPV31) (3,4). The high-risk HPVs contain oncoproteins, i.e., E6 and E7, which contribute to oncogenesis of cervical SCC by silencing the tumor-suppressive p53 and Rb proteins (5-8). The molecular mechanisms of cervical SCC initiation, development and metastasis have not yet been fully elucidated. Therefore, an increased understanding of the molecular targets and pathways of cervical SCC progression and metastasis is necessary, preferably using latest approaches in genomic analysis, including non-coding RNA studies.

RNA can be divided into 2 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 the initiation, development and metastasis of these cancers (10). Some highly expressed miRNAs can function as oncogenes by repressing tumor suppressors, whereas low-level miRNAs can function as tumor suppressors by negatively regulating oncogenes (11).

We previously performed miRNA expression signature analysis of hypopharyngeal, maxillary sinus, esophageal and lung SCCs, in addition to bladder cancer and renal cell carcinoma; these studies indicated that miR-218 was significantly reduced in cancer tissues compared with adjacent non-cancerous tissues, suggesting that miR-218 is a candidate tumor-suppressive miRNA in human cancers (12-18). The results of past functional studies of miR-218 in various cancers indicated that miR-218 inhibits cancer cell proliferation and invasion through targeting oncogenic genes (19-23). Interestingly, miR-218 was underexpressed in HPV-positive cell lines, cervical lesions, and cancer tissues containing HPV16 DNA, as compared to both C-33A cells and normal cervical tissues (24).

The aim of the study was to investigate the functional significance of miR-218 in both HPV-positive and -negative cell lines and to identify the molecular pathways mediating miR-218 in cervical SCC cells. Genome-wide gene expression data for miR-218 and in silico database analyses showed that the focal adhesion pathway was a promising miR-218 target pathway. The laminins LAMB3 and LAMC1 are an important and biologically active part of the basal lamina, influencing cell differentiation, migration, adhesion, proliferation and survival. In this study, we focused on LAMB3 and investigation of the functional significance of this gene in cervical SCC. The novel tumor-suppressive miR-218-mediated cancer pathways identified herein provide new insights into the potential mechanisms of cervical SCC oncogenesis and metastasis.

Materials and methods

Clinical specimens. A total of 18 primary cervical SCC specimens and 11 non-cancerous specimens were collected from patients who had undergone surgical treatment at Chiba University Hospital. The samples were processed and stored in RNAlater (Qiagen, Valencia, CA, USA) at -20°C until RNA extraction. Patient information is summarized in Table I. Our study was approved by the Bioethics Committee of Chiba University; prior written informed consent and approval was given by each patient.

Cervical SCC cell lines. CaSkii (HPV16-positive) and ME180 (HPV39-positive) cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. HeLa (HPV18-positive) cells were grown in E-MEM medium supplemented with 10% fetal calf serum, and Yumoto (HPV-negative) cells were grown in E-MEM medium supplemented with 10% fetal bovine serum. All cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

RNA isolation. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA concentration was determined spectrophotometrically. RNA quality was confirmed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

DNA isolation and HPV status. Genomic DNA was extracted by QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands). Samples without HPV infection were determined by PCR amplification using the L1 consensus primers MY09 and MY11 as described previously (25). HPV-positive samples were analyzed to determine the presence of DNA for HPV16 and HPV18. We designed type-specific real-time PCR primers for the E6 and E7 regions of HPV16 and HPV18 (Table II),

| No. | Age | FIGO stage | Tumor size (cm) | Lymph node metastasis | HPV status |
|-----|-----|------------|----------------|----------------------|------------|
| 1   | 58  | IIB        | 1.7x1.9        | -                    | 16         |
| 2   | 64  | IIB        | ND             | -                    | 16         |
| 3   | 37  | IIB        | 3.5x3.0        | +                    | 16         |
| 4   | 41  | IB2        | 8.3x3.3        | -                    | 16         |
| 5   | 39  | IB1        | 3.5x3.4        | -                    | 16         |
| 6   | 34  | IB1        | 3.2x2.2        | -                    | 16         |
| 7   | 43  | IB2        | 4.0x8.0        | -                    | 18         |
| 8   | 56  | IIIB       | 3.0x3.1        | +                    | 16,18      |
| 9   | 77  | IIIB       | 3.0x2.7        | -                    | 16         |
| 10  | 62  | IB1        | 3.0x2.0        | -                    | 16         |
| 11  | 56  | IIIA       | 4.5x2.2        | +                    | 16         |
| 12  | 56  | IIIA       | 4.0x4.0        | -                    | 16         |
| 13  | 60  | IB1        | 4.0x4.0        | -                    | 16         |
| 14  | 32  | IIIB       | 6.0x3.0        | +                    | 16         |
| 15  | 38  | IB2        | 6.8x4.6        | +                    | 16         |
| 16  | 44  | IB1        | 3.5x2.2        | -                    | 16         |
| 17  | 40  | IB1        | 3.0x2.0        | -                    | 16         |
| 18  | 63  | IB1        | 2.7x2.4        | -                    | 16         |

| No. | Age | HPV status |
|-----|-----|------------|
| 1   | 44  | -          |
| 2   | 77  | -          |
| 3   | 75  | -          |
| 4   | 45  | -          |
| 5   | 47  | -          |
| 6   | 69  | -          |
| 7   | 40  | -          |
| 8   | 48  | -          |
| 9   | 41  | -          |
| 10  | 41  | -          |
| 11  | 34  | -          |

ND, no data.
and real-time PCR was performed using a LightCyclerNano PCR System according to the manufacturer's protocol.

Quantitative real-time RT-PCR. Stem-loop RT-PCR (TaqMan MicroRNA assays; P/N: 000521 for miR-218; Applied Biosystems, Foster City, CA, USA) was used to quantify miRNAs according to earlier published conditions (13). To normalize the data for quantification of miR-218, we used RNU48 (assay ID: 001006; Applied Biosystems). TaqMan probes and primers for LABD3 (P/N: Hs00165078_m1) and GAPDH (P/N: Hs02758991_g1) were obtained from Applied Biosystems. Primers for ACTB (P/N: ACTB 533F 37546-020, ACTB 653R 37546-021) were obtained from Sigma genetics. We used the ΔΔCt method to calculate the fold-change.

Table II. Primer sequences of HPV detection.

| Primer     | Sequence (5' to 3') | Location | Product size (bp) |
|------------|---------------------|----------|-------------------|
| HPV 16 E6-F | GCACCCAAAGAGAATGCAATGTT | 85-108   | 142               |
| HPV 16 E6-R | AGTCATACACCTACGTCAACATGTA | 203-226  | 81                |
| HPV 16 E7-F | CAAGTGTGAACCTCTACGTCCTGCGG | 738-759  | 79                |
| HPV 16 E7-R | GTGCCCAATACAGGTCTTCTTCAA | 796-818  | 558-581           |
| HPV 18 E6-F | CTTAGAGGGCCAGTCGCATTGCG | 503-524  | 113               |
| HPV 18 E6-R | TTATACCTTGATTTCCTCTGCGTCG | 558-581  | 113               |
| HPV 18 E7-F | TAATCATCAAACATTTACCAGGCCG | 721-744  | 810-833           |
| HPV 18 E7-R | CGTCTGACTGACCTTACTAACAAGAGAATGCAATGTT | 85-108   | 142               |

Western blot analysis. Cells were harvested and lysed 72 h after transfection. Each cell lysate (50 µg of protein) was separated using Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA), followed by subsequent transfer to PVDF membranes. Immunoblot analysis was performed with polyclonal anti-LAMB3 antibodies (HPA008069; Sigma-Aldrich, St. Louis, MO, USA). Anti-GAPDH antibodies (ab8245; Abcam, Cambridge, UK) were used as an internal control. The membrane was washed and incubated with anti-rabbit IgG, HRP-linked antibodies (#7074; Cell Signaling Technology, USA). Complexes were visualized with an Immuno-Star™WesternC Chemiluminescence Kit (Bio-Rad), and the expression levels of these proteins were evaluated by ImageJ software (ver.1.44; http://rsbweb.nih.gov/ij/index.html).

Plasmid construction and dual-luciferase reporter assays. Partial sequences of the LAMB3 3' untranslated region (3'UTR) that contain the miR-218 target site (ggcatgccattgaaactaagagctc gagatcctggcagcaaaacttaaaaaatgcgggag gagatcctggcagcaaaacttaaaaaatgcgggag) were cloned into pGL3 vector (Promega). The wild-type and mutant LAMB3 3'UTR constructs were cotransfected with miR-218 and miR-218 No into HEK293 cells for 48 h. After gentle removal of the non-migratory cells from the filter surface of the upper chamber, the cells that migrated to the lower side were fixed and stained with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of cells migrating to the lower surface was determined microscopically by counting 4 areas of constant size per well. A cell invasion assay was carried out using modified Boyden chambers containing transwell- precoated Matrigel membrane filter inserts with 8-µm pores in 24-well tissue culture plates at 1x10⁵ cells per well (BD Biosciences, USA). All experiments were performed in duplicate.

Pathway analysis and expression data of putative miR-218 target genes. To obtain putative miR-218 regulated genes, we adopted a TargetScan database searching method (http://www.targetscan.org). To identify molecular targets and signaling pathways regulated by miR-218, in silico and gene expression data were analyzed in the Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway (http://www.genome.jp/kegg/ pathway.html) categories using the GeneCodis program (http://genecodis.cnbc.csic.es/). In this study, we focused on the focal adhesion pathway, which included 48 genes. Gene expression data were applied using the GEO database (accession no. GSE6791).

Cell proliferation, migration, and invasion assays. Cell proliferation was determined using an MTT assay (Roche Applied Science, Tokyo, Japan) according to the manufacturer’s instructions. Cell migration assays were performed using modified Boyden Chambers (Transwells, Costar #3422, Corning Incorporated, Corning, NY, USA) containing uncoated Transwell polycarbonate membrane filters with 8-µm pores in 24-well tissue culture plates. Cells were transfected with 10 nM miRNA by reverse transfection and plated in 10-cm dishes at 8x10⁴ cells. After 48 h, 1x10⁴ cells were added to the upper chamber of each migration well and were allowed to migrate for 48 h. After gentle removal of the non-migratory...
atataaatctttggaagaagctgagggcttc) were inserted between the XhoI and Pmel restriction sites in the 3'UTR of the hRluc gene in the psiCHECK-2 vector (Promega, Madison, WI, USA). CaSki was then transfected with 5 ng vector, 10 nM mature miRNA. Firefly and Renilla luciferase activities in cell lysates were determined using a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the quotient of Renilla/firefly luciferase activities.

**Statistical analysis.** The relationships between 2 variables and numerical values were analyzed using the Mann-Whitney U test, and the relationships between 3 variables and the numerical values were 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 3 variables, a non-adjusted statistical level of significance of P<0.05 corresponded to the Bonferroni-adjusted level of P<0.0167.

**Results**

**Expression levels of miR-218 in cervical SCC clinical specimens and cell lines.** The expression of miR-218 was significantly lower in clinical cervical SCC specimens (n=18; 0.043±0.077) than in non-cancerous specimens (n=11; 0.153±0.110, P=0.0026; Fig. 1). We also evaluated the expression of miR-218 in cervical cancer cell lines. miR-218 expression levels in CaSki (HPV16-positive), ME180 (HPV39-positive), HeLa (HPV18-positive), and Yumoto (HPV-negative) were significantly lower than that in non-cancerous cervical epithelium (P<0.0001; Fig. 1).

**Effects of miR-218 restoration on cell proliferation, migration and invasion in cervical SCC cell lines.** To investigate the functional role of miR-218, we performed gain-of-function studies using cells transfected with a precursor of miR-218. The XTT assay revealed that cell proliferation was significantly inhibited in miR-218 transfectants in comparison with non-transfectants (mock) and miRNA-control transfectants (control) in ME180 cells (78.1±3.7%, 100.0±2.5% and 96.0±3.5%, respectively; P<0.0001), while no significant inhibition was seen in CaSki cells (94.7±3.5%, 100.0±2.3% and 97.6±4.1%, respectively; P>0.0167), HeLa cells (93.0±5.0%, 100.0±7.5% and 97.6±4.1%, respectively; P>0.0167), and Yumoto cells (106.3±11.1%, 100.0±3.6% and 110.8±11.8%; P>0.0167; Fig. 2A).

![Figure 1. Expression of miR-218 in cervical-SCC clinical specimens and cell lines.](image)

![Figure 2. (A) Effects of miR-218 restoration on proliferation of cervical-SCC cell lines.](image)
Migration and Matrigel invasion assays demonstrated that the number of invading cells significantly decreased in miR-218 transfectants in comparison with mock and miR-control transfectants in all cell lines tested. In fact, migration in miR-218 transfectants was reduced to only 11.5±3.4% in CaSki cells (mock, 100.0±20.8; control, 115.4±12.2; P<0.0001), 20.3±3.8% in ME180 cells (mock, 100.0±14.8%; control, 77.2±13.8%; P<0.0001) 49.0±5.9% in HeLa cells (mock, 100.0±6.8%; control, 114.1±16.8%; P<0.0001), and 24.9±8.8% in Yumoto cells (mock, 100.0±18.2%; control, 102.2±6.8%; P<0.0001; Fig. 2B).

Similarly, in the Matrigel invasion assay, the number of invading cells was significantly decreased in miR-218 transfec-tants in comparison with mock and miR-control transfectants in all cell lines. Cell invasion was reduced to 3.9±1.6% in CaSki cells (mock, 100.0±8.5%; control, 107.0±16.3%; P<0.0001), 37.1±9.2% in ME180 cells (mock, 100.0±14.8%;
Identification of miR-218-mediated molecular pathways and putative miR-218 target genes. We first obtained putative miR-218 target genes by searching the TargetScan database. According to the database, 4,946 conserved targets, with a total of 1,865 conserved sites and 4,372 poorly conserved sites, were deposited in this database. These genes were analyzed and characterized in KEGG pathway categories using the GeneCodis program. This analysis revealed 105 signaling pathways (Table III). In these pathways, we focused on the focal adhesion pathway and the 48 genes contained within this pathway (Table IV). To search for genes regulated by tumor-suppressive miR-218 in cervical SCC, we applied gene expression profiles in the GEO database (accession no. GSE6791). Among 48 genes, 24 genes were upregulated in cervical SCC compared to adjacent non-cancerous tissues. The expression levels of up- or downregulated genes in clinical specimens are shown in Table IV. As a result of our expression data, we identified LAMB3 as one of the most highly upregulated genes in clinical specimens; this gene has 1 putative miR-218 binding site. LAMB3 is a laminin that is an important and biologically active part of the basal lamina, functioning in a variety of pathways, such as cell differentiation, migration, adhesion, proliferation and survival. Thus, we focused on LAMB3 as a promising target gene of miR-218 in cervical SCC.

Table III. Significantly enriched annotations regulated by miR-218 (top 20 pathways).

| No. of genes | P-value | Annotations          |
|--------------|---------|----------------------|
| 59           | 1.55E-16| Endocytosis          |
| 40           | 4.29E-12| Glutamatergic synapse|
| 69           | 4.30E-11| Pathways in cancer   |
| 58           | 4.21E-10| MAPK signaling pathway|
| 38           | 4.87E-10| Insulin signaling pathway|
| 48           | 4.98E-10| Focal adhesion       |
| 29           | 1.53E-09| ErbB signaling pathway|
| 25           | 3.70E-09| Long-term depression |
| 35           | 6.58E-09| Axon guidance        |
| 43           | 1.99E-08| Chemokine signaling pathway|
| 24           | 6.10E-08| Chronic myeloid leukemia|
| 23           | 6.35E-08| Long-term potentiation|
| 36           | 1.00E-07| Wnt signaling pathway|
| 33           | 1.06E-07| Tight junction       |
| 26           | 1.21E-07| Prostate cancer      |
| 26           | 1.21E-07| Gap junction         |
| 21           | 2.87E-07| Glioma               |
| 26           | 2.98E-07| FcγR-mediated phagocytosis |
| 22           | 5.37E-07| Adherens junction    |
| 38           | 5.54E-07| Calcium signaling pathway |

Identification of miR-218-mediated molecular pathways and putative miR-218 target genes. We first obtained putative miR-218 target genes by searching the TargetScan database. According to the database, 4,946 conserved targets, with a total of 1,865 conserved sites and 4,372 poorly conserved sites, were deposited in this database. These genes were analyzed and characterized in KEGG pathway categories using the GeneCodis program. This analysis revealed 105 signaling pathways (Table III). In these pathways, we focused on the focal adhesion pathway and the 48 genes contained within this pathway (Table IV). To search for genes regulated by tumor-suppressive miR-218 in cervical SCC, we applied gene expression profiles in the GEO database (accession no. GSE6791). Among 48 genes, 24 genes were upregulated in cervical SCC compared to adjacent non-cancerous tissues. The expression levels of up- or downregulated genes in clinical specimens are shown in Table IV. As a result of our expression data, we identified LAMB3 as one of the most highly upregulated genes in clinical specimens; this gene has 1 putative miR-218 binding site. LAMB3 is a laminin that is an important and biologically active part of the basal lamina, functioning in a variety of pathways, such as cell differentiation, migration, adhesion, proliferation and survival. Thus, we focused on LAMB3 as a promising target gene of miR-218 in cervical SCC.

Table IV. Expression of target genes by miR-218 involved in focal adhesion pathways.

| Entrez gene | Gene symbol | FC | Regulation | P-value |
|-------------|-------------|----|------------|---------|
| 3265        | HRAS        | 4.54 | Up         | 4.19E-02 |
| 3914        | LAMB3       | 3.54 | Up         | 4.40E-03 |
| 3676        | ITGA4       | 3.21 | Up         | 6.03E-03 |
| 5062        | PAK2        | 2.40 | Up         | 7.29E-05 |
| 1282        | COL4A1      | 2.33 | Up         | 2.21E-02 |
| 6646        | SHC1        | 2.15 | Up         | 3.05E-04 |
| 87          | ACTN1       | 2.14 | Up         | 3.18E-03 |
| 1399        | CRKL        | 2.10 | Up         | 4.50E-04 |
| 2932        | GSK3B       | 1.83 | Up         | 1.36E-03 |
| 394         | ARH GAP5    | 1.75 | Up         | 9.48E-04 |
| 3915        | LAMC1       | 1.75 | Up         | 8.18E-03 |
| 3371        | TNC         | 1.51 | Up         | 3.09E-01 |
| 5501        | PPP1CC      | 1.50 | Up         | 6.03E-03 |
| 387         | RHOA        | 1.45 | Up         | 2.04E-01 |
| 1301        | COL11A1     | 1.44 | Up         | 3.87E-01 |
| 5829        | PXN         | 1.33 | Up         | 1.27E-01 |
| 858         | CAV2        | 1.27 | Up         | 3.90E-01 |
| 3480        | IGF1R       | 1.26 | Up         | 2.42E-01 |
| 100291393   | LOC100291393| 1.09| Up         | 8.39E-01 |
| 25759       | LOC100291393| 1.09| Up         | 8.39E-01 |
| 2321        | FLT1        | 1.05 | Up         | 7.22E-01 |
| 5601        | MAPK9       | 1.03 | Up         | 8.39E-01 |
| 896         | CCND3       | 1.02 | Up         | 8.79E-01 |
| 5293        | PIK3CD      | 1.02 | Up         | 8.39E-01 |
| 3691        | ITGB4       | -1.02| Down       | 9.59E-01 |
| 5500        | PPP1CB      | -1.07| Down       | 4.76E-01 |
| 63923       | TNN         | -1.17| Down       | 4.46E-01 |
| 1289        | COL5A1      | -1.17| Down       | 6.84E-01 |
| 5170        | PDK1        | -1.19| Down       | 7.51E-02 |
| 5058        | PKA1        | -1.23| Down       | 1.40E-01 |
| 7057        | THBS1       | -1.23| Down       | 4.46E-01 |
| 10451       | VAV3        | -1.23| Down       | 7.60E-01 |
| 5156        | PDGFRα      | -1.41| Down       | 2.63E-01 |
| 10000       | AKT3        | -1.46| Down       | 6.71E-02 |
| 1956        | EGFR        | -1.51| Down       | 5.99E-02 |
| 399694      | SHC4        | -1.57| Down       | 9.33E-02 |
| 55742       | PARVA       | -1.58| Down       | 1.68E-02 |
| 5579        | PRKCB       | -1.71| Down       | 9.50E-03 |
| 53358       | SHC3        | -1.80| Down       | 5.33E-02 |
| 5649        | RELN        | -1.81| Down       | 2.52E-02 |
| 2318        | FLNC        | -1.81| Down       | 2.21E-02 |
| 5295        | PIK3R1      | -1.88| Down       | 4.19E-02 |
| 1277        | COL1A1      | -2.38| Down       | 8.38E-02 |
| 8515        | ITGA10      | -2.61| Down       | 9.01E-05 |
| 6714        | SRC         | -2.85| Down       | 1.36E-03 |
| 81          | ACTN4       | -2.86| Down       | 3.05E-04 |
| 3479        | IGF1        | -2.97| Down       | 8.18E-03 |
| 595         | CCND1       | -5.52| Down       | 1.14E-03 |
Expression of LAMB3 in cervical SCC clinical specimens. The expression of LAMB3 was significantly lower in clinical cervical SCC specimens (n=18; 0.053±0.049) than in non-cancerous specimens (n=11; 0.017±0.014, P=0.0104; Fig. 3A). Moreover, LAMB3 expression was significantly inversely correlated with miR-218 expression (r=-0.377; P=0.0461; Fig. 3B).

LAMB3 is a direct target of miR-218. We performed quantitative real-time RT-PCR and western blot analysis to investigate whether LAMB3 mRNA and protein were downregulated by restoration of miR-218. Importantly, both LAMB3 mRNA and protein levels were significantly repressed in miR-218-transfectants in comparison with mock transfectants (Fig. 4A and B).
To determine whether the 3’UTR of LAMB3 had an actual target site for miR-218, we performed a luciferase reporter assay by using a vector encoding the 3’UTR of LAMB3 mRNA. We found that the luminescence intensity was significantly reduced in miR-218 transfectants as compared to mock and miRNA-control transfectants (P<0.0001; Fig. 4C).

Silencing of LAMB3 mRNA and protein in a cervical SCC cell line. Next, we examined the impact of si-LAMB3 transfection in CaSki cells. The expression of LAMB3 mRNA was reduced in 2 si-LAMB3 transfectants in comparison with mock and si-control transfectants (P<0.0001; Fig. 4C). Additionally, the expression of LAMB3 protein was reduced in si-LAMB3-1 and si-LAMB3-2 transfectants in comparison with mock and si-control transfectants (P<0.0001 and P=0.0002, respectively; Fig. 5B). These results showed that the 2 siRNAs were useful for loss-of-function assays in this study.

Effects of LAMB3 silencing on cell proliferation, migration and invasion in cervical SCC cell lines. To investigate the functional role of LAMB3, we performed loss-of-function studies using si-LAMB3 transfectants. The XTT assay revealed that cell proliferation was not inhibited in the 2 si-LAMB3 transfectants as compared with mock and si-control transfectants in CaSki cells (82.4±10.7%, 100.2±6.1%, 100.0±14.6% and 95.6±11.2%; P>0.0083; Fig. 6A).

Additionally, the number of migrating cells was significantly decreased in both si-LAMB3 transfectants as compared with
mock and si-control transfectants in CaSki cells (10.6±2.7%, 72.2±10.7%, 100.0±6.5% and 91.8±11.0%, respectively; P<0.0001; Fig. 6B).

The number of invading cells was also significantly decreased in si-LAMB3 transfectants as compared with mock and si-control transfectants in CaSki cells (13.5±4.9%, 73.1±16.2%, 100.0±13.7% and 88.4±18.0%, respectively; P<0.0001; Fig. 6C).

Discussion

The discovery of non-coding RNA during the human genome sequencing project had a significant impact in cancer research (26). The reconstructing of genome-wide studies to include non-coding RNA is therefore necessary for cancer research. miRNAs are a class of small non-coding RNAs, and a growing body of evidence has suggested that miRNAs also contribute to cancer initiation, development and metastasis in many types of cancers, including cervical cancer (10).

It is believed that normal regulatory mechanisms can be disrupted by aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. Therefore, identification of aberrantly expressed miRNAs is the first step toward elucidating miRNA-mediated oncogenic pathways. Based on this, we identified the miRNA expression signatures in several human squamous cell carcinomas, including esophageal SCC, hypopharyngeal SCC, maxillary sinus SCC and lung SCC, allowing the elucidation of multiple tumor-suppressive miRNAs (12-15). Our previous studies showed that miR-218 is a frequently downregulated miRNA and that restoration of this miRNA inhibited cancer cell migration and invasion in head and neck SCC (HNSCC) cells (19). We also searched for downregulated miRNAs in cervical SCC by examining expression signatures published in public databases (27-31). These data indicate that miR-218 is frequently downregulated miRNA in cervical SCC. Thus, we focused on miR-218 and investigated the functional significance of this miRNA in mediating cancer pathways.

In the human genome, 2 miR-218 precursor genes, miR-218-1 and miR-218-2, have identical sequences in the mature miRNA and map to human chromosomes 4p15.31 and 6q35.1, respectively. Interestingly, the genomic regions of miR-218-1 and miR-218-2 are located in the introns of the SLIT2 and SLIT3 genes, respectively. Downregulation of miR-218 in cancer cells has been shown to be caused by promoter hypermethylation of SLIT2 and SLIT3 genes (20). Silencing of miR-218 by DNA hypermethylation has also been reported in oral SCC using a function-based screening approach (21). On the other hand, several reports have indicated that silencing of miR-218 in cervical SCC was caused by HPV infection (24,32). Our expression data showed that miR-218 expression was significantly reduced in both HPV-positive cells (CaSki, HeLa and ME180) and HPV-negative cells (Yumoto), in addition to clinical specimens. Since the molecular mechanisms of miR-218 silencing in cervical SCC are still unclear, further study is necessary to solve this problem.

In the current study, we found significant inhibition of cancer cell migration and invasion in cervical SCC cell lines (CaSki, HeLa, ME180 and Yumoto) by miR-218 restoration, suggesting that miR-218 is a tumor-suppressive miRNA in cervical SCC. Our previous reports in HNSCC also showed that miR-218 contributes to cancer cell migration and invasion (19). The tumor-suppressive function of miR-218 has also been reported in several types of cancers, and miR-218 has been shown to target several oncogenic genes, such as Rictor (oral cancer), survivin and ROBO1 receptor (nasopharyngeal cancer and gastric cancer) (20-22). Our recent report also indicated that restoration of miR-218 inhibited cancer cell proliferation, migration, and invasion in bladder cancer (23,33). These data suggested that miR-218 is an important tumor-suppressive miRNA that is deeply involved in human cancers.

miRNAs are unique in their ability to regulate many protein-coding genes. Bioinformatic predictions have indicated that miRNAs regulate more than 30% of protein-coding genes (34). A single miRNA is capable of targeting a number of genes to globally regulate biological processes. The identification of novel cancer pathways and responsible genes regulated by tumor-suppressive miR-218 in cervical SCC is the next step for our understanding of cervical SCC oncogenesis. Thus, we pursued GeneCodis analysis to reveal the functional significance of these genes potentially regulated by miR-218 in cervical SCC. The GeneCodis analysis applies many genes to known pathways in the KEGG Pathway Database, and these data facilitate our understanding of tumor-suppressive miRNA-mediated molecular pathways in human cancer. This method of analysis has previously been used to efficiently identify tumor-suppressive miRNA-mediated cancer pathways in our laboratory (19). In the current study, the GeneCodis analysis revealed 105 signaling pathways, as highlighted in Table II. In these pathways, we focused on the focal adhesion pathway and the 48 genes contained within this pathway.

To search for genes regulated by tumor-suppressive miR-218 in cervical SCC, we used gene expression profiling in this study (deposited in the GEO database as accession no. GSE6791). Among the 48 genes in the focal adhesion pathway, 10 were upregulated in cervical SCC clinical specimens, indicating that these genes were candidates for regulation by tumor-suppressive miR-218 in cervical SCC. From these genes, we focused on LAMB3, a component of laminin-332, and investigated the functional significance of this gene.

Laminin-332, a heterotrimer composed of 3 chains (LAMA3, LAMB3 and LAMC2), is an adhesion substrate for epithelial cells and regulates epithelial cell migration during epithelial regeneration and repair processes (35,36). Several immunohistochemical studies have shown that laminin-332 or its subunit LAMC2 is expressed in tumor cells at the invasion front or in budding tumor cells in many types of human cancers, such as adenocarcinomas of the colon, breast, pancreas and lung, SCC of the esophagus and melanoma (35). Our data demonstrated that LAMB3 was directly regulated by miR-218 and functioned as an oncogene, contributing to cancer cell migration and invasion in cervical SCC. Many studies have indicated that laminin-332 binds to several cell-surface receptors, such as integrins, epidermal growth factor receptor and syndecan-1 (37-39). Among these binding partners, integrins are cell surface transmembrane proteins that mediate extracellular signals and intracellular pathways.
leading to control of the cell cycle, cell migration, and invasion in cancer cells (40). It will be necessary to analyze the signal pathways associated with the interactions of integrins and laminin-332 in cervical SCC in the future.

Acknowledgements

This study was supported by KAKENHI (C), 24592590.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Walboomers JM, Jacobs MV, Manos MM, et al: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189: 12-19, 1999.
3. Muñoz N, Bosch FX, De Sanjosé S, et al: Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348: 518-527, 2003.
4. Clifford GM, Smith JS, Plummer M, Muñoz N and Franceschi S: Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 88: 63-73, 2003.
5. Band V, Dalal S, Delmolino L and Androphy EJ: Enhanced degradation of p53 protein in HPV-6 and BPV-1 E6-immortalized human mammary epithelial cells. EMBO J 12: 1847-1852, 1993.
6. Lechnerl MS, Mackl DH, Finicle AB, Crook T, Vousden KH: Enhanced degradation of p53 protein in HPV-6 and BPV-1 E6-immortalized human mammary epithelial cells. EMBO J 12: 1847-1852, 1993.
7. Thomas M, Pim D and Banks L: The role of the E6-p53 interaction in the molecular pathogenesis of HPV. Oncogene 18: 7690-7700, 1999.
8. Münger K and Howley PM: Human papillomavirus immortalization and transformation functions. Virus Res 89: 213-228, 2002.
9. Filipowicz W, Bhattacharyya SN and Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? Nat Rev Genet 9: 102-114, 2008.
10. Nelson KM and Weiss GJ: MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7: 3655-3660, 2008.
11. Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with tumour-suppressive activity. Nat Rev Cancer 6: 259-269, 2006.
12. Lechnerl MS, Mackl DH, Finicle AB, Crook T, Vousden KH: Enhanced degradation of p53 protein in HPV-6 and BPV-1 E6-immortalized human mammary epithelial cells. EMBO J 12: 1847-1852, 1993.
13. Thomas M, Pim D and Banks L: The role of the E6-p53 interaction in the molecular pathogenesis of HPV. Oncogene 18: 7690-7700, 1999.
14. Münger K and Howley PM: Human papillomavirus immortalization and transformation functions. Virus Res 89: 213-228, 2002.
15. Filipowicz W, Bhattacharyya SN and Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? Nat Rev Genet 9: 102-114, 2008.
16. Nelson KM and Weiss GJ: MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7: 3655-3660, 2008.
17. Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.
18. Kikkawa N, Hanazawa T, Fujimura L, et al: miR-489 is a tumour-suppressive miRNA target PTEN1 in hypopharyngeal squamous cell carcinoma (HSCC). Br J Cancer 103: 877-884, 2010.
19. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
20. Kikkawa N, Hanazawa T, Fujimura L, et al: miR-489 is a tumour-suppressive miRNA target PTEN1 in hypopharyngeal squamous cell carcinoma (HSCC). Br J Cancer 103: 877-884, 2010.
21. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
22. Kikkawa N, Hanazawa T, Fujimura L, et al: miR-489 is a tumour-suppressive miRNA target PTEN1 in hypopharyngeal squamous cell carcinoma (HSCC). Br J Cancer 103: 877-884, 2010.
23. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
24. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
25. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
26. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
27. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
28. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
29. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
30. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
31. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
32. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
33. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
34. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
35. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
36. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
37. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
38. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
39. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.
40. Nohata N, Hanazawa T, Nohata N, et al: Tumour suppressive microRNA-218 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 103: 877-884, 2010.