Role of miR-200c/miR-141 in the regulation of epithelial-mesenchymal transition and migration in head and neck squamous cell carcinoma

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Received October 30, 2013; Accepted December 17, 2013

DOI: 10.3892/ijmm.2014.1625

Abstract. Epithelial-mesenchymal-transition (EMT) is a critical step in tumor invasion and metastasis, while its fate is mainly defined by the balanced expression between the miR-200 family and ZEB transcription factors. In this study, we observed a reciprocal correlation between miR-200c/mir-141 and ZEB1, as well as between ZEB2 and E-cadherin expression in a panel of 13 head and neck squamous cell carcinoma (HNSCC) cell lines. We also confirmed that the enforced expression of miR-200c and miR-141 significantly reduced the migration capacity of HNSCC cells. Accordingly, the enforced expression of miR-200c and mir-141 resulted in a significant upregulation in E-cadherin expression, contrary to the significant downregulation in ZEB1 expression in 3 cell lines (UTSCC-24A, UTSCC-24B and UTSCC-6A cells). Another pair of cell lines, UTSCC-60A and UTSCC- 60B failed to show a significant change in the expression of E-cadherin or ZEB1/ZEB2 during the enforced expression of miR-200c/miR-141. To address the issue, we focused on the hypermethylation status of the ZEB1/2 promoters, which have both been shown to include wide CpG islands. We observed a marked upregulation in both ZEB1 and ZEB2 mRNA expression following treatment with a demethylating agent in both pairs of UTSCC cell lines. In conclusion, our findings confirm the existence of a reciprocal correlation between the mir-200 family and the ZEB family, and demonstrate the role of the miR-200 family in EMT, as well as in the migration and invasion ability of HNSCC cells. Furthermore, our data suggest that the promoter hypermethylation of ZEB1 and ZEB2 may play an essential role and may overshadow the effects of the miR-200 family in the regulation of EMT during carcinogenesis.

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Key words: miR-200 family, head and neck, epithelial-mesenchymal transition

Introduction

Head and neck squamous cell carcinoma (HNSCC) is comparatively frequent type of cancer worldwide and is associated with a relatively poor prognosis despite recent improvements in surgical and radiation therapy techniques. The majority of patients with HNSCC present with locally and/or regionally advanced disease which further hinders the clinical outcome, even with modern multi-disciplinary treatment approaches.

The status of cervical lymph nodes is the primary indicator of poor prognosis in patients with HNSCC, while distant metastatic disease remains a major cause of mortality. Therefore, it is critical to develop a deeper understanding of the genetic determinants of metastasis in order to develop more effective therapeutic approaches.

Epithelial-to-mesenchymal transition (EMT) is a cornerstone in the molecular steps of the metastatic process, although originally, it is a morphogenetic program of embryonic development, through which epithelial cells are converted into mesenchymal cells. During the process of EMT, the disruption of cell-to-cell junctions and the loss of apical-basal polarity results in the formation of mesenchymal cells with migratory and invasive abilities (1).

The main molecular hallmark of the EMT process is the loss of E-cadherin expression, while ZEB1 (also known as dEF1, TCF8, AREB6 or Zfhx1a) and ZEB2 (also known as SIP1 or Zfhx) are Zn finger transcription factors categorized to be master regulators of EMT by directly repressing E-cadherin (2). Currently, various transcription factors regulating E-cadherin transcription are also a matter of active investigation.

MicroRNAs (miRNAs or miRs) are endogenous small non-coding RNAs that modulate the expression of target genes through the repression of translational or mRNA degradation. They play significant roles in the regulation of biological and pathological cellular process, including differentiation, proliferation, apoptosis and metastasis. Multiple miRNAs have also been reported to be involved in the pathogenesis of HNSCC (3).

An important regulatory link between the ZEB family and various species of miRNA has been established. The miRNA-200 (miR-200) family has been identified as a major regulator of EMT through the modulation of the expression of E-cadherin (4). The miR-200 family consists of five members, which form two clusters based on the seed sequences related with binding specificities: miR-200a/141 and miR-200b/200c/429 (5). They enforce the expression of E-cadherin by regulating the transcriptional repressors, ZEB1 and ZEB2. Recent findings suggest that the balanced expression between ZEB factors and the miR-200 family based on the reciprocal control of each other regulate EMT and the progression of various types of cancer towards metastasis (6). However, the effects of the miR-200 family on EMT and the metastasis of HNSCC cells have not been fully characterized yet.

In the present study, we evaluated the role of miR-200c/mir-141 in the progression EMT and metastasis in HNSCC. Our results revealed a significant reciprocal correlation between miR-200c/mir-141 and their targets, ZEB1 and ZEB2. On the other hand, we also noted discrepancies in some of the HNSCC cell lines in response to the enforced expression of miR-200c/mir-141. Therefore, we further focused on the epigenetic control of ZEB1/ZEB2 by promoter hypermethylation, and its association with miR-200c/mir-141 in HNSCC.

Materials and methods

Cell lines and cultures. UTSCC cell line series were kindly provided by Dr R. Grenman (Department of Otolaryngology, Turku University, Turku, Finland). The UTSCC cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1-μl/ml amphotericin B (Gibco™/Invitrogen, Tokyo, Japan), and all cell lines were cultured in a humidified incubator with 5% CO_2 at 37˚C. In the definition of UTSCC cell lines, 'A' represents the cell lines derived from primary tumors (e.g., UTSCC-6A cells), while 'B' represents the cells derived from metastatic tumors (e.g., UTSCC-6B cells).

mRNA processing and quantitative reverse transcription PCR (qRT-PCR). The preparation of cDNA from mRNA was performed directly from cultured cell lysates using the TaqMan® Gene Expression Cells-to-CT™ kit (Ambion, Tokyo, Japan), according to manufacturer's instructions. Briefly, cultured cells in 96-well plates were washed in PBS and lysed with lysis buffer and RNA was released into this solution. Cell lysates were reverse transcribed into cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion). Finally the cDNA was amplified by qRT-PCR using the included TaqMan Gene Expression Master Mix and the specific TaqMan primer/probe assay designed for the investigated genes: E-cadherin (Hs01023894_m1), ZEB1 (Hs00232783_m1), ZEB2 (Hs00207691_m1) and GAPDH (Hs99999905_m1) (Applied Biosystems, Tokyo, Japan).

The gene expression levels were normalized to the expression of the housekeeping gene, GAPDH, and were expressed as fold changes relative to the expression of the untreated cells. The amplification profile was initiated by 10 min of incubation at 95˚C, followed by two-step amplification of 15 sec at 95˚C and 60 sec at 60˚C for 40 cycles. All experiments were performed with non-template controls included to exclude reagent contamination. Quantification was performed with the $\Delta\Delta$Ct calculation method (7). For the determination of the absolute quantity of target genes, a standard curve was generated using ten-fold serial dilutions of a synthetic RNA duplex. Each sample was analyzed in triplicate on the 7500 Real-time PCR system in a 96-well plate (Applied Biosystems).

MicroRNA processing and qRT-PCR. Preparation of cDNA from miRNA was also performed directly from cultured cell lysates using the TaqMan MicroRNA Cells-to-CT kit (Ambion) according to the manufacturer's instructions. Briefly, the cultured cells in 96-well plates were washed in PBS and lysed with lysis buffer and RNA was released into this solution. Cell lysates were reverse transcribed into cDNA using the RT Enzyme Mix, RT buffer and appropriate RT primer (Applied Biosystems). Finally the cDNA of each mature miRNA was amplified separately by qRT-PCR using the TaqMan Universal Master Mix and the specific primer and probe mix included in pre-designed TaqMan MicroRNA Assays: hsa-miR-200c, ID: 000385; and hsa-miR-141, ID: 000385 (Applied Biosystems). The quantification of miRNA expression was performed with the same method as described above and for normalizations RNU6B RNA (RNU6B; ID: 001093, P/N: 4373381, Applied Biosystems) was used as an internal control. Each sample was analyzed in triplicate.

Cell transfection with miR-200c and miR-141 precursors. The stability-enhanced miR-200c, miR-141 precursors and the negative control, 1 ribo-oligonucleotides, were obtained from Ambion. Transfection of miRNAs was carried out using siPORT Amine in accordance with the manufacturer's instructions (Ambion). Briefly, the UTSCC cell lines were seeded ($8x10^5$ cells in 4 ml of RPMI-1640 per dish) in 96-well culture dishes and grown overnight (the day prior to transfection) in antibiotic-free medium. The transfection of miR-200c and miR-141 precursors, or each negative control (all purchased from Applied Biosystems) at the indicated concentrations was introduced into the cells using 0.3 μl siPORT Amine Transfection Agent (Applied Biosystems) in 20 μl Opti-MEM (Gibco/Invitrogen) according to the manufacturer's recommendations. The negative controls were scrambled oligonucleotides that were validated not to produce identifiable effects on known miR functions. The level of miR-200c and miR-141 expression in the transfected cells was confirmed by qRT-PCR (Taqman MicroRNA Assays) 24 h after transfection as described above.

Treatment with 5-aza-2'-deoxycytidine. Treatment with 5-aza-2'-deoxycytidine (5-Aza-CdR) (Sigma-Aldrich, Tokyo, Japan) was carried out as described in a previous study from our group (8). Briefly, the cells were incubated for 72 h with 4 μM 5-Aza-CdR, and then harvested for RNA extraction and qRT-PCR. For anti-miRNA transfection analysis, 48 h after being treated with 1 μM 5-Aza-CdR, the cells were transfected for one day and then treated again with 5-Aza-CdR for a further 24 h.

Cell proliferation assay. Cell proliferation was assessed by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-

1,3-benzene disulfonate (WST-1) tetrazolium salt assay (Roche, Japan). The UTSCC cells were plated at a density of $6x10^3$ cells/well on 96-well plates and grown overnight. The miR-200c, miR-141 precursors or the negative control were then introduced into the cell lines as described above and 24 h later, the assay was initiated by the addition of 20 μl of WST solution reagent to 100 μl of culture medium for each well. Following incubation for 3 h at 37˚C, the plates were read on a microplate autoreader (Corona MTP-450) at a wavelength of 450 nm. The results were expressed as the mean optical density for selected paradigms performed in duplicate.

Transwell invasion assay and migration assay. The UTSCC cells were seeded at a density of $4.0x10^4$cells/well on 24-well plates, and 24 h later, the cells were transfected separately with 50 nM miR-200c precursor, miR-141 precursor or the scrambled negative control. After 24 h, the transfected cells were harvested by trypsinization, and washed twice in PBS, and $8.0x10^4$ cells were transferred to the upper chamber of a BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, Tokyo, Japan) with inserts containing an 8-μm-pore-sized membrane with a thin layer of Matrigel in the 24-well Transwell plate filled with 500 μl serum-free RPMI-1640 medium. In the lower chamber, 500 μl of the 10% FBS-containing medium containing and 10 μg/ml fibronectin were added. Following incubation for 24 h, the invaded cells were counted under a microscopic using a Diff-Quick staining kit (Sysmex, Kobe, Japan).

For the migration assay, the cells transfected with miRNA were added to the upper chamber of a Boyden chamber (BD Falcon cell culture insert 24-well 8 μm pole, no. 353097; BD Biosciences). Fibronectin (10 μg/ml) and medium with 10% FBS 750 μl are added to the lower chamber. Following incubation for 36 h at 37˚C, the invaded cells were counted under a microscopic using a Diff-Quick staining kit (Sysmex).

Wound healing assay. The UTSCC cells were transfected separately with miR-200c precursor, miR-141 precursor or the scrambled negative control. When cell confluence reached approximately 80% at 48-h post-transfection, wounds were created in confluent cells using a 200-μl pipette tip. The cells were then rinsed with medium to remove any free-floating cells and debris. Medium was then added, and the culture plates were incubated at 37˚C. Wound healing was observed at different time points within the scrape line, and representative scrape lines were photographed. Duplicate wells for each condition were examined, and each experiment was repeated three times.

Statistical analysis. Data were analyzed using GraphPad Prism 5.0 software. To evaluate significant differences between two matched pair groups or between two independent groups of samples, paired t-tests and the Mann-Whitney U test were used, respectively. Pearson's correlation coefficient (r) was used to measure correlation, and logarithmic regression was used to calculate the R^2 and to create the equation of the slope.

Results

The miR-200 family includes five members, namely miR-200a, miR-200b, miR-200c, miR-141 and miR-429. The miR-200 family can be grouped into two clusters according to seed sequences related with target specifications: miR-200a/miR-141 and miR-200b/miR-200c/miR-429. In this study, we selected miR-200c and miR-141 as representatives of each cluster. Furthermore, both miR-200c and miR-141 are located on the same genetic loci of chromosome 12 (9).

Expression status of E-cadherin, ZEB1 and ZEB2 in HNSCC. To determine the EMT status of each cell line, we evaluated the mRNA expression of E-cadherin as the key epithelial marker and that of ZEB1 and ZEB2 as mesenchymal markers in 13 head and neck cell carcinoma lines belonging to the UTSCC series originating from primary or metastatic tumors. The results of expression analysis are displayed in Fig. 1. The quantification of mRNA expression was performed by a standard curve based on the serial dilutions of the mRNA derived from the UTSCC-60A cell line. Comparatively, nine of the 13 (70%) cell lines showed a substantially increased expression of E-cadherin in contrast to the decreased expression of both ZEB1 and ZEB2. On the other hand, the remaining three (30%) cell lines showed an inverse trend of expression results. Among the three pairs of cell lines originating from primary and corresponding metastatic tumors (UTSCC-6A-B, UTSCC-24A-B and UTSCC-60A-B), the cell lines originating from metastatic tumors displayed a lower E-cadherin and higher ZEB1/ZEB2 expression in only one pair of cell lines (UTSCC-60A-B), in contrast to the two other pairs of cell lines in which converse results were obtained (Fig. 1). The origin of the cells (namely from primary tumors or metastastic tumors) was not found to be directly associated with the expression levels of E-cadherin or ZEB1/ZEB2. These results suggest that the EMT status is not constantly related with the origin of the tumor cells (from primary or metastastic tumors).

Correlation of E-cadherin, ZEB1 and ZEB2 expression with miR200c/miR-141. In order to better understand the association between the expression patterns of EMT markers and the miR-200 family, we analyzed the expression of miR-200c and miR-141 by qRT-PCR in the panel of 13 UTSCC cell lines and assessed the correlation between miR-200c/miR-141 and EMT markers, including the E-cadherin, ZEB1 and ZEB2 genes. The mRNA expression of miR-200c ($r=0.73$, $p<0.01$) and miR-141 ($r=0.64$, $p=0.02$) showed a statistically significant positive correlation with E-cadherin expression (Fig. 2A and B). Conversely, both miR-200c ($r=-0.62$, $p=0.02$) and miR-141 ($r=-0.70$, $p<0.01$) showed a significant inverse correlation with ZEB2 expression (Fig. 2C and D). Accordingly, miR-141 showed a significant inverse correlation with ZEB1 ($r=-0.61$, $p=0.02$), while the correlation between miR-200c and ZEB1 was not significant ($r=-0.52$, $p=0.07$) (Fig. 2E and F). Taken together, these data indicate that the expression of miR-200c and miR-141 is associated with the acquisition of epithelial characteristics and the simultaneous loss of mesenchymal features in the HNSCC cells, the two distinguishing features that are essential for the development of a metastatic phenotype.

Overexpression of miR200c/miR-141 inhibits the expression of ZEB1, while inducing that of E-cadherin. To further investigate the association between the miR-200 family and EMT markers, we analyzed the effects of the enforced expression of

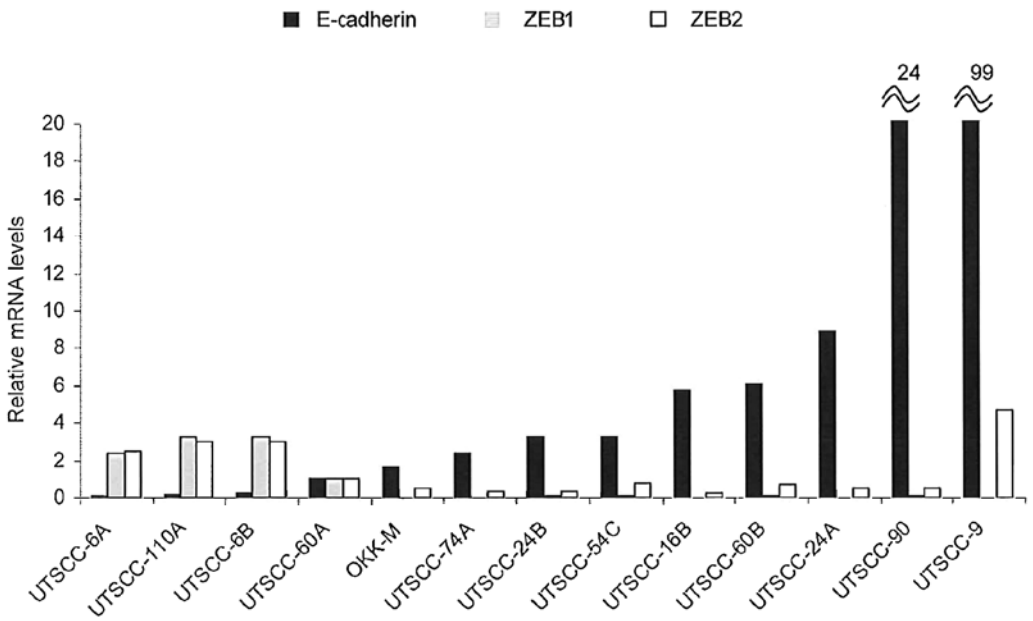

Figure 1. mRNA expression of E-cadherin, ZEB1 and ZEB2 by qRT-PCR in 13 head and neck squamous cell carcinoma cell lines belonging to the UTSCC series. The quantification of mRNA expression was performed by a standard curve based on the serial dilutions of the mRNA derived from the UTSCC-60A cell line. The mRNA levels of UTSCC-90 and UTSCC-9 exceed the maximum value of the y-axis. The curved lines indicate this excess; 24 and 99 represent the mRNA levels of UTSCC-90 and UTSCC-9, respectively. GAPDH was used as a housekeeping gene in normalization. Each sample was run in triplicate. (Error bars represent the means ± SD).

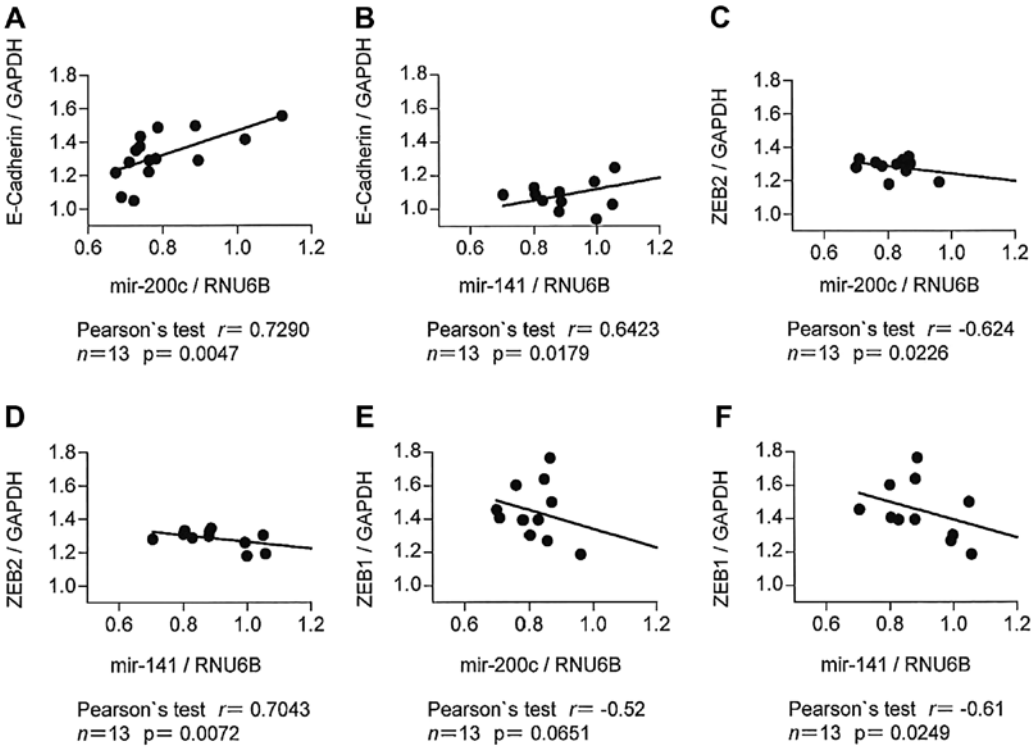

Figure 2. Analysis of the correlation between the mRNA expression of the miR-200 family and EMT-related genes in the panel of 13 UTSCC cell lines by qRT-PCR. (A and B) Analysis of the correlation between E-cadherin expression with miR-200c and miR-141. (C and D) Analysis of the correlation between ZEB2 expression with miR-200c and miR-141. (E and F) Analysis of the correlation between ZEB1 expression with miR-200c and miR-141. Pearson's correlation coefficient was calculated for each analysis.

miR-200c and miR-141 on E-cadherin, ZEB1 and ZEB2 gene epxression by transfecting the UTSCC-24A and UTSCC-24B cells with precursor miRNAs. Following transfection with miRNA precursors, the enforced expression of miR-200c/miR-141 was confirmed by qRT-PCR. The UTSCC-24B cells had substantially lower levels of endogenous miR-200c and

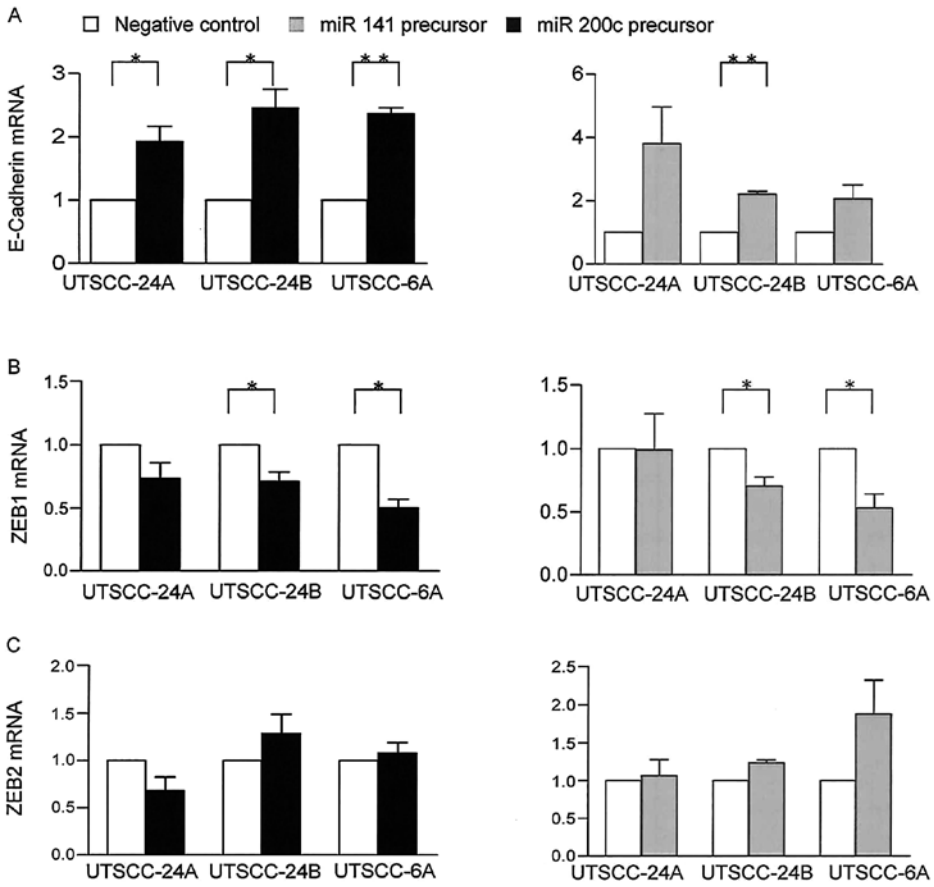

Figure 3. Changes in (A) E-cadherin, (B) ZEB1 and (C) ZEB2 mRNA expression following transfection with the negative control, miR-200c or miR-141 precursors in UTSCC-24A, UTSCC-24B and UTSCC-6A cells. The horizontal bars represent the mean expression levels; $^{*}p<0.05$, $^{**}p<0.01$, from paired t-test.

mir-141 compared with the UTSCC-24A cells. The enforced expression of miR-200c and mir-141 resulted in the significant upregulation of E-cadherin expression compared with the control cells (p=0.03 and p=0.10 in UTSCC-24A cells) and (p=0.02 and p<0.01 in UTSCC-24B cells, paired t-test) (Fig. 3A). By contrast, ZEB1 showed a significant downregulation under the same conditions (p=0.01 and p=0.44 in UTSCC-24A cells) and (p=0.03 and p=0.03 in UTSCC-24B cells, paired t-test) by qRT-PCR (Fig. 3B). On the other hand, the expression of ZEB2 remained almost unaltered (p>0.05), (Fig. 3C). These findings suggest that ZEB1 plays a prominent role as opposed to ZEB2, in the regulation of E-cadherin by the mir-200 family in HNSCC.

To confirm these results, we also examined another pair of cells (UTSCC-60A and UTSCC-60B), using same experimental conditions. In contrast to the UTSCC-24A and UTSCC-24B cells, the UTSCC-60A and UTSCC-60B cells did not show a significant change in the expression of E-cadherin or ZEB1/ZEB2 in response to the enforced expression of miR-200c and miR-141. These results suggest that the effects of the miR-200 family on EMT-related target gene expression may be hindered by another epigenetic mechanism rather than the one involving miRNAs.

Hypermethylation of ZEB1 and ZEB2 blocks the modulation by miR-200c/miR-141 in HNSCC. The expression of ZEB2 in both UTSCC pairs of cell lines and ZEB1 in one pair of cell lines failed to be suppressed by the enforced expression of miR-200c and miR-141. To address this issue, we aimed to identify the effects of other epigenetic mechanisms in the regulation of ZEB1/ZEB2. Both ZEB1 and ZEB2 genes have wide CpG islands in their promoter regions. To clarify whether the CpG methylation of ZEB1 and ZEB2 gene promoters is involved in their transcriptional repression, we examined the expression mRNA before and after treatment with 5-Aza-CdR, a DNA demethylating agent, by qRT-PCR in the UTSCC-60A and UTSCC-60B cells. We observed a marked upregulation in both ZEB1 and ZEB2 mRNA expression following treatment with 5-Aza-CdR in both cell lines, while the expression of E-cadherin was also increased, although to a lesser degree (Fig. 4A-C). On the other hand, mir-200c and mir-141 expression was decreased in those cells (Fig. 4D and E). These results suggest that the effects of the miR-200 family on EMT-related gene expression are blocked by the methylation of target gene promoters.

Overexpression of miR200c/miR-141 impairs HNSCC cell migration but not cell invasion and proliferation. To determine the role of the microRNA-200 family in the metastatic process, we assessed the effects of the induced expression of miR-200c and miR-141 on cell motility and invasion by transfecting the UTSCC-24A and UTSCC-24B cells with precursor microRNAs. Cell migration assays demonstrated that the overexpression of miR-200c and miR-141 significantly reduced the migration capacity of both cell lines compared to the controls

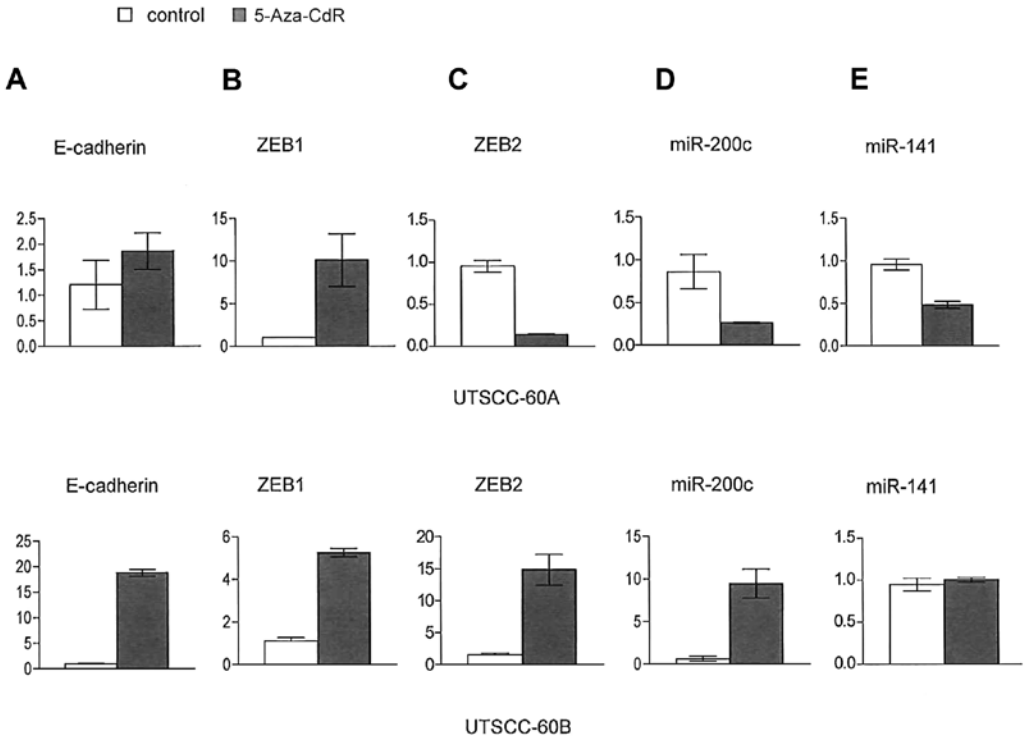

Figure 4. mRNA expression of E-cadherin, ZEB1, ZEB2, miR-200c and miR-141 before and after 5-Aza-CdR treatment in UTSCC-60A and UTSCC-60B cells. (A) E-cadherin, (B) ZEB1, (C) ZEB2, (D) miR-200c and (E) miR-141. In normalization, GAPDH was used as a housekeeping gene for ZEB1, ZEB2 and E-cadherin, and RNU6B was used for miR-200c and miR141. Data are shown as the means ± SD of triplicates and are representative of two independent experiments.

($p<0.01$; Fig. 5A). Similarly, a wound healing assay confirmed these results in 48-h time course of transfection compared to the controls (Fig. 5B). On the other hand, Matrigel invasion assay revealed no significant change in the invasion abilities of the cells in response to the overexpression of miR-200c and miR-141 (Fig. 5C).

To identify the role of the microRNA-200 family in the progression of HNSCC, we determined whether miR-200c and miR-141 may also affect the proliferation of HNSCC cells. In the WST assay, the proliferation rates of the UTSCC-24A and UTSCC-24B cells transfected with precursor miRNAs were not altered significantly compared to the control cells ($p>0.2$) (Fig. 5D). These results indicate that the miR-200 family inhibits the migration of HNSCC cells, independent of cell invasion and proliferation *in vitro*.

Discussion

In this study, we demonstrated that the miR-200 family plays an essential role both in the suppression of the EMT process and in the metastatic ability of HNSCC cells. Our results highlight the importance of the upregulation of the miR-200c/miR-141 cluster in the prevention of the metastatic ability of the HNSCC cells. On the other hand, we also observed that other mechanisms of epigenetic silencing, such as the promoter hypermethylation of target genes may disrupt the role of the miR-200 family in the regulation of EMT-related gene expression.

We observed that ZEB2 mRNA expression was not suppressed by the enforced expression of mir-200c/miR-141 in all four HNSCC cell lines examined. Similarly, ZEB1 mRNA expression was not inhibited in one group of HNSCC cells by mir-200c/miR-141. Since wide CpG islands exist in the promoter regions of both genes, the suppression of expression by hypermethylation seems to be a possible mechanism to explain this condition. Our expression analysis after inhibiting DNA methylation by 5-Aza-CdR revealed a marked upregulation in the mRNA expression of ZEB1 and ZEB2. To our knowledge, this is the first study to demonstrate that both ZEB1 and ZEB2 genes have already been suppressed by hypermethylation, and are able to block the regulatory role of the miR-200 family. Although a number of studies have defined the status of hypermethylation in the promoter region of the miR-200 family itself (10-12), the role of methylation in the promoter regions of their target genes has not yet been fully elucidated. To date, only one study investigated the association between miR-200a/miR-200b and the hypermethylation of ZEB2 in pancreatic carcinomas. Li *et al* found that the majority of pancreatic cancers highly express miR-200a and miR-200b; however, this expression does not affect ZEB2 expression, since the promoter of ZEB2 has already been silenced by hypermethylation (13). Similarly, the promoter methylation of ZEB2 was also identified in two other types of epithelial cancers, including breast carcinoma (14) and hepatocellular carcinoma (15). However, the methylation status of the ZEB1 promoter was not investigated in these studies. Previously, Hidaka *et al* observed the activation of ZEB1 expression in adult T-cell leukemia/lymphoma (ATLL) cell lines follwoing treatment with the DNA methylation agent, 5-Aza-CdR (16); however, the association between the miR-200 family and ZEB1 was not investigated.

DNA methylation (transcriptional level) and miRNA modulation (post-transcriptional level) are two major epigenetic regulatory mechanisms, which affect opposite sites of

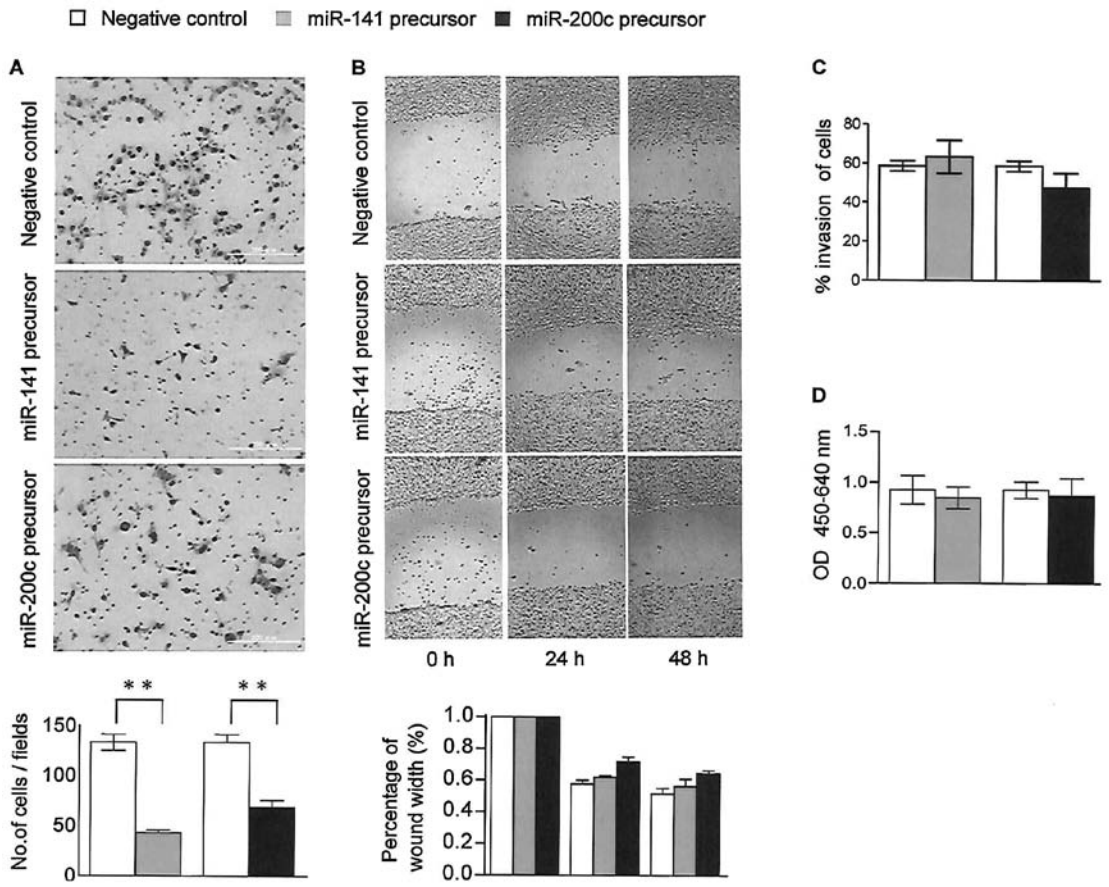

Figure 5. Functional analysis of UTSCC-24B cells following transfection with negative control, miR-200c or miR-141 precursors. (A) Cell migration assays of UTSCC-24B cells toward serum-containing medium using Transwell (upper panel, representative images of migration chambers; bottom panel, average counts from four random microscopic fields). The data are the average number of cells that migrated in triplicate experiments and are presented as the means ± SE. (B) Wound healing assay. Upper panel, representative images of cell monolayers, which were scratched with a pipette tip. Images were taken 0, 24 and 48 h after wound formation; bottom panel, percentage of wound widths during 0, 24 and 48 h. (C) Cell invasion assays using Matrigel-coated Transwell membranes: percentage of average counts from 4 random microscopic fields. (D) WST proliferation assays. $^{**}p<0.01$.

genes at the 5' promoter and 3' untranslated regions, respectively. A number of studies have investigated each epigenetic mechanism separately (10-12,14); however, the combined effects of both mechanisms have not yet been elucidated. A recent bioinformatics analysis demonstrated that there is a complementary regulation between these two mechanisms at the genome level, while cancer-related genes have a low methylation status and more miRNA target sites (17). Thus, the role of promoter methylation of target genes in the modulation by the miR-200 family in the EMT process in HNSCC and other types of cancer requires further investigation.

In EMT, E-cadherin is a representative marker of epithelial transition, while ZEB1 and ZEB2 inhibit E-cadherin expression and induce mesenchymal transition in cancer progression. Our preliminary survey revealed that there was a broad range in the expression patterns of E-cadherin as opposed to those of ZEB1/ZEB2; there was an inverse correlation between the expression of E-cadherin and ZEB1/ZEB2 in the HNSCC cell lines. Our results suggested that the EMT status differs between cancer cells, occurring at different time points. Of note, the origin of the cell lines (from primary or metastatic tumors) did not demonstrate a clear association with the expression range of E-cadherin or ZEB1/ZEB2. Consistent with these findings, Wiklund *et al* did not identify any specific changes in miRNA expression profiles, including the miR-200 family between non-metastatic and metastatic tumors of oral squamous cell carcinoma (18). By contrast, Lo *et al* demonstrated that miR-200c expression in the regional metastatic lymph node of HNSCC tissues was significantly decreased compared with primary tumors (19). In literature, there are also controversial results concerning the expression status of the miR-200 family and other EMT-related factors between primary and metastatic tumors. To explain these discrepancies, a recent study on breast carcinomas suggested a dynamic dual role for the miR-200 family in the progression of metastasis, hindering the entry of tumor cells into the circulation, as opposed to promoting colonization in distant organs of those cells that enter into the intravascular circulation (20).

In this study, we demonstrated that miR-200c/miR-141 expression correlated with the increased E-cadherin and reduced ZEB1/ZEB2 expression, suggesting that during cancer progression, HNSCC cells initially lose epithelial features, while simultaneously gaining the mesenchymal characteristics required for EMT. We also showed that miR-200c/miR-141 suppressed the migration ability of HNSCC cells. These results are consistent with those of previous studies on other epithelial cancers (21). However, studies which systematically analyze the correlation between the miR-200 family and EMT in HNSCC

are limited. Lo *et al* examined the role of the miR-200 family in HNSCC cancer stem cells specifically and revealed that the overexpression of miR-200c in those cells downregulated ZEB1/ZEB2 expression and blocked the ability of the cells to invade and metastasize (19). Another study compared the miR-200 family and desmosomal cadherins between spindle cell carcinoma and squamous cell carcinoma of the head and neck region. They found that the expression of the miR-200 family was significantly downregulated in spindle cell carcinoma, while it was unaltered in squamous cell carcinoma tumor tissues compared to normal mucosa (22). Desmosomal cadherins, including E-cadherin and N-cadherin also did not show a significant change in expression in HNSCC. These findings suggest that only a small part of the HNSCC cell population with a low miR-200 status has the potential to undergo the EMT process.

One limitation of our study was that the experiments were performed *in vitro* by using cell lines only. Cell lines derived from human solid tumors may not adequately represent the tumors of origin. Therefore, the results may need to be validated by subsequent *in vivo* studies using primary tumor cells from tissue samples of human tumors.

In conclusion, our molecular pathological findings indicate that the expression of the miR-200 family positively correlates with the epithelial marker, E-cadherin, while inversely correlates with the mesenchymal markers, ZEB1 and ZEB2. Furthermore, we found that the miR-200 family negatively regulates the expression of ZEB1. These results confirm the reciprocal balance between the miR-200 family and EMT in HNSCC. Notably, we revealed that the promoter hypermethylation of ZEB1 and ZEB2 plays an essential role and may overshadow the effects of the miR-200 family in the regulation of EMT during carcinogenesis. Thus, the role of epigenetic mechanisms and their regulation concerning the miR-200 family-ZEB1/ZEB2 feedback loop in EMT requires further investigation.

Acknowledgements

We thank Dr Hirohashi for his comments and suggestions regarding the manuscript. This study was partially presented at the 8th International Conference on Head and Neck Cancer. This study was partially supported by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to S.T. and L.B.B.).

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