Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

Cancer Research

Downregulation of MicroRNA-200 in EBV-Associated Gastric Carcinoma

Aya Shinozaki[1], Takashi Sakatani[1], Tetsuo Ushiku[1], Rumi Hino[1], Maya Isogai[1], Shunpei Ishikawa[1], Hiroshi Uozaki[1], Kenzo Takada[2], and Masashi Fukayama[1]

Abstract

EBV-associated gastric carcinoma is a distinct gastric carcinoma subtype with characteristic morphologic features similar to those of cells that undergo epithelial-to-mesenchymal transition. The effect of microRNA abnormalities in carcinogenesis was investigated by measuring the expression of the epithelial-to-mesenchymal transition–related microRNAs, miR-200a and miR-200b, in 36 surgically resected gastric carcinomas using quantitative reverse transcription-PCR analysis. MiR-200 family expression was decreased in EBV-associated gastric carcinoma, as compared with that in EBV-negative carcinoma. Downregulation of the miR-200 family was found in gastric carcinoma cell lines infected with recombinant EBV (MKN74-EBV, MKN7-EBV, and NUGC3-EBV), accompanied by the loss of cell adhesion, reduction of E-cadherin expression, and upregulation of ZEB1 and ZEB2. E-cadherin expression was partially restored by transfection of EBV-infected cells with miR-200 family precursors. Reverse transcription-PCR analysis of primary precursors of miR-200 (pri-miR-200) revealed that the transcription of pri-miR-200 was decreased in EBV-infected cells. Transfection of MKN74 cells with *BARF0, EBNA1*, and *LMP2A* resulted in a decrease of pri-miR-200, whereas transfection with EBV-encoded small RNA (*EBER*) did not. These four latent genes contributed to the downregulation of the mature miR-200 family and the subsequent upregulation of ZEB1/ZEB2, resulting in the reduction of E-cadherin expression. These findings indicate that all the latency type I genes have a synergetic effect on the downregulation of the miR-200 family that leads to reduced E-cadherin expression, which is a crucial step in the carcinogenesis of EBV-associated gastric carcinoma. *Cancer Res; 70(11); 4719–27. ©2010 AACR.*

Introduction

Gastric carcinoma is one of the most common malignancies worldwide and ranks second in terms of global carcinoma-related mortality (1). Effective therapy depends on the identification of distinct gastric carcinoma subgroups and the clarification of their pathologic features and molecular mechanisms. EBV-associated gastric carcinoma is a distinct subtype comprising 8% to 11% of all gastric carcinomas (2). Monoclonal EBV is present in nearly all neoplastic cells, strongly suggesting a causal role in gastric carcinogenesis. EBV-associated gastric carcinoma displays characteristic clinicopathologic features, including male predominance, proximal localization within the stomach, and a relatively better prognosis than EBV-negative gastric carcinoma. EBV-associated gastric carcinoma is identified by its unique morphology. It is generally referred to as a lymphoepithelioma-like carcinoma, which consists of poorly differentiated tumor cell nests intermingled with dense lymphocytic infiltration. These characteristic features reflect specific interactions between infective organisms and infected host cells, i.e., EBV and gastric epithelial cells.

Viruses represent one of the major factors that could cause excessive proliferation, migration, and prolonged survival of normal cells. EBV, originally isolated from Burkitt's lymphoma, is associated with various malignancies including Hodgkin's lymphoma, lymphoproliferative disorders in immunosuppressed patients, nasopharyngeal carcinoma, and gastric carcinoma (3–5). Several studies have investigated the role of EBV in carcinogenesis, and attempted to show that EBV gene transcripts, such as EBNA2 or latent membrane protein-1 (LMP1), disturb signal transduction in host cells, and cause immortalization or transformation (6). However, the pathologic role of latent genes in EBV latency type I neoplasms, such as EBV-associated gastric carcinoma, in which only a few viral latent genes are expressed (*BARF0, EBER, EBNA1*, and *LMP2A*), has not been fully explained.

Recently, a novel class of small, noncoding RNA molecules referred to as microRNAs (miRNA) have been identified as important posttranscriptional regulators of gene expression. MiRNAs play an important role in a wide variety of complex biological processes, including cellular development and differentiation, but investigations have only begun to clarify

Authors' Affiliations: [1]Department of Pathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan and [2]Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

Note: A. Shinozaki and T. Sakatani contributed equally to this work.

Corresponding Author: Masashi Fukayama, Department of Pathology, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81-35841-3341; Fax: 81-33815-8379; E-mail: mfukayama-tky@umin.net.

doi: 10.1158/0008-5472.CAN-09-4620

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

their significance in viral infection and carcinogenesis. In a preliminary experiment, we found that miR-200a and miR-200b were downregulated in a gastric carcinoma cell line infected with EBV. Both miRNAs belong to the miR-200 family, the downregulation of which induces epithelial-to-mesenchymal transition (EMT) in normal and neoplastic cells via dysregulation of the E-cadherin transcription repressors, ZEB1 and ZEB2. ZEB1 and ZEB2, also known as TCF8 and SIP1, respectively, are possible targets of the miR-200 family, and they were found to repress E-cadherin expression *in vitro* in the previous reports (7–10).

In the present study, we first confirmed that miR-200a and miR-200b were downregulated in surgically resected gastric carcinoma tissue, and assessed the associations between miR-200a and miR-200b downregulation and E-cadherin expression. We then verified that the miR-200 family was downregulated *in vitro* using established EBV-infected cell lines as models of EBV-associated gastric carcinoma and studied the EBV-latent gene underlying the downregulation of miR-200a and miR-200b. Our results identified a new role for miRNAs in the carcinogenesis of EBV-associated gastric carcinoma.

Materials and Methods

Tissue samples

Thirty-six cases of primary gastric carcinoma were used to investigate miR-200a and miR-200b expression in EBV-associated gastric carcinoma tissue. The cases were taken from the archives of the Department of Pathology at the University of Tokyo Hospital. The study included 18 EBV-associated gastric carcinoma cases and 18 EBV-negative gastric carcinoma cases. All aspects of the study were approved by the University of Tokyo Ethics Committee. The H&E-stained slides of the cases were reviewed. All cases of gastric carcinoma were histologically evaluated according to Lauren's classification (11). We used the tumor-node-metastasis classification of the International Union Against Cancer for tumor staging (12).

EBER *in situ* hybridization and immunohistochemistry

The presence of EBV in tumor cells was confirmed using *in situ* hybridization targeting EBV-encoded small RNA with EBER1-RNA oligonucleotide probes as previously described (13).

Immunohistochemical staining with E-cadherin (clone NCH-38, dilution 1:100; Dako), LMP1 (clone cs1-4, dilution 1:200; Dako), and EBNA2 (clone PE2, dilution 1:25; Dako) in surgically resected specimens was performed using the Ventana BenchMark automated immunostainer with the labeled streptavidin-biotin peroxidase method and visualized using 3,3′-diaminobenzidine with the appropriate positive and negative controls. The immunohistochemical results were blinded and independently evaluated by two pathologists (A. Shinozaki and T. Sakatani). The proportion of E-cadherin–positive tumor cells did not differ significantly among the cases, and E-cadherin expression was evaluated according to the staining pattern and intensity. E-cadherin expression was considered "preserved" when more than 50% of the positively stained tumor cells showed a strong and membranous staining pattern. Expression was defined as "decreased" when more than 50% of the positively stained tumor cells had a weak, discontinuous, and punctate cell membrane staining pattern. The expressions of LMP1 and EBNA2 were evaluated according to the number of cells which stained positively.

Cell culture and treatment

The gastric carcinoma cell lines used in the study were MKN7, MKN74, NUGC3, SNU719, and Raji. The former three were originally derived from gastric carcinoma with histologic features of well, moderately, and poorly differentiated adenocarcinoma without EBV infection, respectively. MKN7 and MKN74 were obtained from Riken BioResource Center Cell Bank (Tsukuba, Japan) and NUGC3 was from the Japanese Cancer Research Resource Bank (Osaka, Japan). SNU719 was derived from EBV-associated gastric carcinoma and was obtained from the Korean Cell Line Bank (Seoul, Korea). Raji was derived from Burkitt's lymphoma, and was obtained from The Global Bioresource Center, American Type Culture Collection. The cell lines were authenticated by the cell banks using short tandem repeat PCR. The cell lines were cultured in RPMI 1640 (Nacalai Tesque) supplemented with 10% FCS (MP Biomedicals), penicillin (40 units/mL), and streptomycin (50 μg/mL) at 37°C in a 5% CO_2 incubator.

EBV infection

MKN7, MKN74, and NUGC3 were infected with recombinant EBV using the cell-to-cell contact method (14). A Burkitt's lymphoma cell line, Akata, was modified to produce recombinant EBV, in which the neomycin-resistant gene is inserted into BXLF1, and was used as a source of the virus in the present study. EBV-infected cells were obtained after bulk selection with G418 (700 μg/mL; Sigma-Aldrich). EBV infection establishment was confirmed using EBER *in situ* hybridization applied to the cells grown on plastic slides after fixation with 10% formalin. The expression of EBV latent genes (*BARF0, EBER, EBNA1, EBNA2, LMP1*, and *LMP2A*) was evaluated using reverse transcription-PCR (RT-PCR) as previously described (15). EBV-infected cells were maintained in bulk in RPMI 1640 supplemented with 10% FCS and G418 (50 μg/mL), but G418 was removed from the medium 24 hours before the experiments.

RNA extraction and quantitative RT-PCR

Total RNA, including miRNA, was extracted from the paraffin-embedded tissue of surgically resected samples with a RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion). Two representative sections were selected from each case: one from gastric carcinoma tissue and the other from nonneoplastic gastric tissue. Extraction of total RNA from cultured cells was performed using the mirVana miRNA Isolation Kit (Ambion).

For miRNA analysis, mature miRNAs were reverse transcribed and quantitative PCR was performed using TaqMan

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

MiRNA assays (Applied Biosystems) according to the protocols of the manufacturer. The miR-200a and miR-200b expression data were normalized to the reference RNA, RNU6B expression, and then the expression levels relative to those of SNU719 were calculated. For the mRNA analysis of E-cadherin, ZEB1, and ZEB2, total RNA was reverse transcribed using the SuperScript II First-Strand Synthesis system (Invitrogen) with random primers. Quantitative PCR was performed using TaqMan Gene Expression assays (Applied Biosystems) according to the protocols of the manufacturer. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. All quantitative PCR analyses were performed using the 7300 Real-time PCR System (Applied Biosystems).

The miR-200 family primary precursor transcript (pri-miR-200) expression was evaluated using RT-PCR analysis, and total RNA was extracted with TRI REAGENT (Sigma-Aldrich) and subjected to reverse transcription using the Quantitect reverse transcription kit (Qiagen) according to the protocols of the manufacturer. The RT-PCR analysis was performed using primers that targeted the predicted transcript start site of miR-200b~200a~429 clusters as previously described (8).

Western blotting

Whole cell extracts were prepared using lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, and 1 mmol/L of phenylmethylsulfonyl fluoride with protease inhibitor cocktail] and 10 μg of protein per lane was loaded and fractionated on an 8% SDS polyacrylamide gel. After transfer onto a polyvinylidene difluoride membrane, probing was carried out with anti-human E-cadherin antibody (clone NCH-38, 1:100; Dako). The membranes were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare) according to the protocols of the manufacturer.

Transfection with miRNA precursors

EBV-infected MKN74 cells (MKN74-EBV) were seeded at 2×10^5 cells per well in six-well plates and transfected with synthetic precursors of miRNAs (pre-miR miRNA Precursor Molecules; Ambion) using siPORT *NeoFX* Transfection Agent (Ambion). The final concentration of each pre-miR was 20 nmol/L (pre-miR-200a), 20 nmol/L (pre-miR-200b), and 40 nmol/L (20 nmol/L pre-miR-200a and 20 nmol/L pre-miR-200b). Total RNA was collected for assay 3 days posttransfection. Cells transfected with pre-miR miRNA Precursor Negative Control no. 1 (Ambion) at a final concentration of 20 nmol/L were used as a negative control.

Transfection with EBV-latent genes

Each EBV-latent gene, *BARF0, EBER* (*EBER1* and *EBER2*), *EBNA1*, and *LMP2A* (gifts from Dr. Paul J Farrell) was cloned into a pcDNA3 vector containing FLAG-tag. MKN74 cells were transfected with each expression vector using FuGENE6 Transfection Reagent (Roche). The expression of each gene was confirmed using RT-PCR analysis as previously described (15, 16).

Statistical analyses

Statistical differences between clinicopathologic variables and E-cadherin expression were determined using Fisher's exact test, Student's *t* test, or the Mann-Whitney *U* test. Associations between miRNA expression levels and clinicopathologic variables were analyzed using Student's *t* test, and ANOVA followed up with Fisher's Protected Least Significant Difference, Pearson's correlation coefficient, or Spearman's rank correlation. All analyses were performed using StatView statistical software (version 5.0, SAS Institute, Inc.). $P < 0.05$ was considered to be statistically significant.

Results

Downregulation of the miR-200 family in surgically resected EBV-associated gastric carcinoma

MiR-200a and miR-200b expression levels in the surgically resected gastric tissue are shown in Fig. 1A. Expression of both miR-200a and miR-200b was decreased in EBV-associated gastric carcinoma compared with that in EBV-negative gastric carcinoma ($P = 0.0107$ and 0.002, respectively; ANOVA, Fisher's Protected Least Significant Difference follow-up), and compared with that in nonneoplastic gastric tissue obtained from EBV-associated gastric carcinoma patients ($P = 0.003$ and 0.0001, respectively). No statistically significant differences in miR-200a or miR-200b expression were observed between the nonneoplastic tissue and tumor tissue from EBV-negative gastric carcinoma patients or between nonneoplastic tissues obtained from EBV-associated gastric carcinoma patients and those from EBV-negative gastric carcinoma patients. No statistically significant differences were observed between miR-200 family expression and age, sex, tumor size, tumor histology, stage, venous or lymphatic invasion, and lymph node metastasis.

Association between miR-200 family expression and clinicopathologic variables

E-cadherin expression was evaluated using immunohistochemistry. The typical decreased/preserved staining patterns of E-cadherin are shown in Fig. 1B. Nine out of 18 EBV-associated gastric carcinoma cases showed decreased E-cadherin expression patterns, whereas only 1 out of 18 EBV-negative cases showed decreased E-cadherin expression, and the difference was statistically significant (Fisher's exact test, $P = 0.0072$).

Tumors with decreased E-cadherin expression had lower levels of miR-200a and miR-200b than tumors with preserved E-cadherin expression, and the differences were statistically significant ($P = 0.0253$ and 0.0379, respectively, Student's *t* test; Fig. 1C).

Downregulation of the miR-200 family and E-cadherin in EBV-infected cell lines

The effect of EBV infection on downregulation of the miR-200 family and E-cadherin was further investigated *in vitro* in EBV-infected gastric carcinoma cell lines (MKN74-EBV, MKN7-EBV, and NUGC3-EBV). Western blot analysis revealed that E-cadherin expression was decreased in all three

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

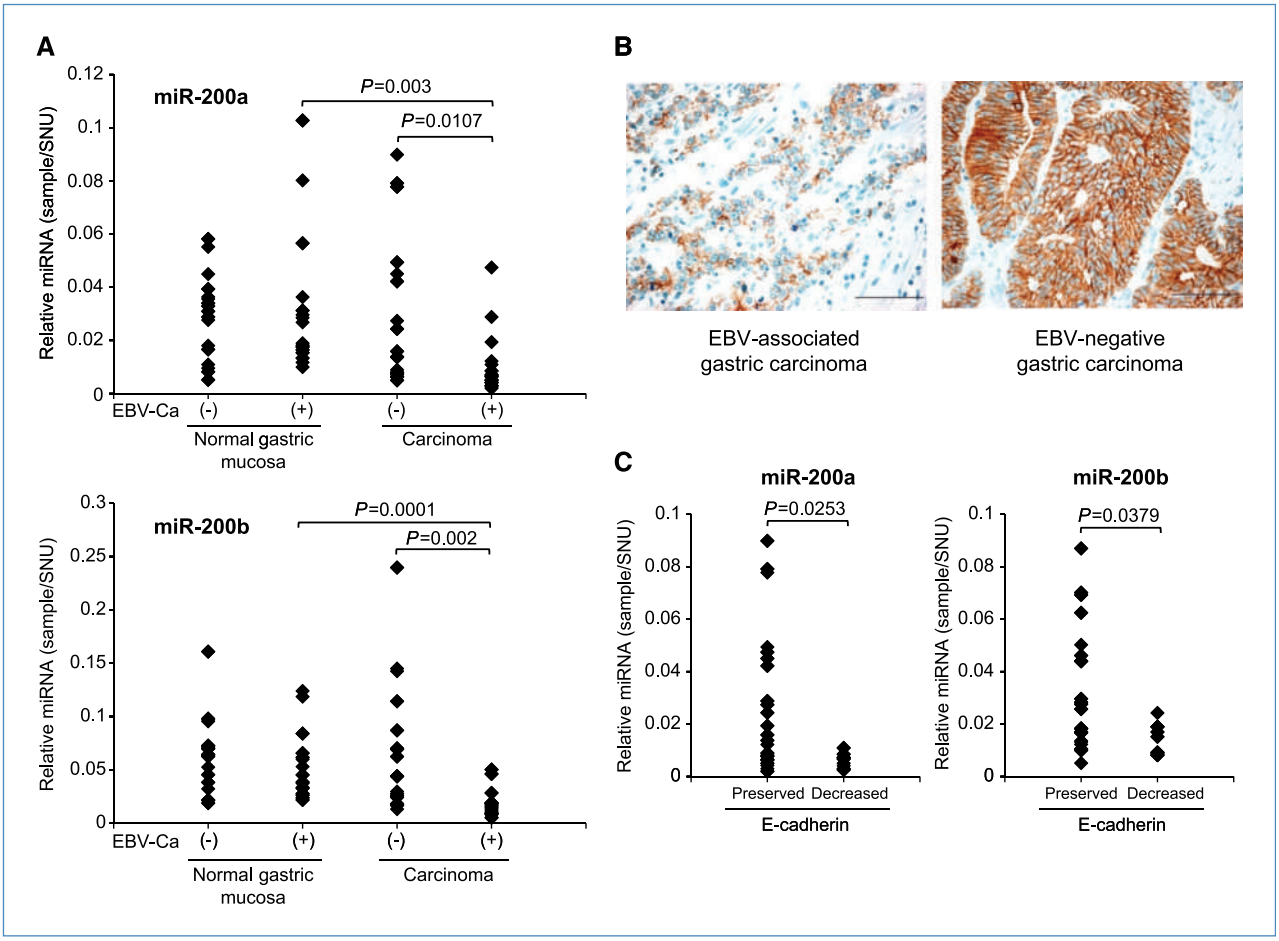

Figure 1. The expression of miR-200a, miR-200b, and E-cadherin in surgically resected gastric tissues. A, quantitative RT-PCR analysis of miR-200a and miR-200b in normal gastric mucosa and carcinoma tissue from EBV-associated and EBV-negative gastric carcinoma cases. B, decreased E-cadherin expression in EBV-associated gastric carcinoma and EBV-negative gastric carcinoma showing preserved E-cadherin expression (bar, 100 μm). C, associations between miR-200 family expression and E-cadherin expression. $P < 0.05$ were considered to be statistically significant.

EBV-infected cell lines, as compared with their original cell lines (Fig. 2A). Furthermore, miR-200a and miR-200b expression were downregulated in these EBV-infected cell lines (Fig. 2B). Quantitative RT-PCR analysis showed ZEB1 upregulation in all EBV-infected cell lines and ZEB2 upregulation in the MKN74-EBV and NUGC3-EBV cell lines (Fig. 2B). EBV infection produced a dramatic morphologic change in the MKN74-EBV cell line (Fig. 2C). It showed significant loss of cell-to-cell adhesion, as compared with its original cell line, MKN74, which formed colonies with a cobblestone arrangement of mutually cohesive cells.

The effect of transfection with miR-200 family precursors on E-cadherin expression

The effect of transfection with miR-200 family precursors on E-cadherin expression in cells with low endogenous miR-200 family expression was investigated. We found that transfection of MKN74-EBV with miR-200a and miR-200b precursors alone or together resulted in the downregulation of ZEB1, and transfection with miR-200a resulted in the downregulation of ZEB2, as compared with their levels in MKN74-EBV transfected with negative controls (Fig. 3). Subsequently, an increase in E-cadherin at the transcriptional level was observed in MKN74-EBV cells transfected with the miR-200 family (Fig. 3).

Downregulation of pri-miR-200 in EBV-infected gastric cell lines

To investigate how EBV infection regulates miR-200a and miR-200b levels, we performed RT-PCR analysis of their primary precursor, pri-miR-200 in EBV-infected cell lines. Pri-miR-200 transcripts were detected in MKN74, MKN7, and NUGC3 cells; however, a decrease in pri-miR-200 was apparent in all of the three cell lines infected with EBV (Fig. 4A).

The effect of EBV latent gene expression on downregulation of the miR-200 family and E-cadherin

To further elucidate which viral factor underlies the downregulation of the miR-200 family, we established MKN74 cells transfected with four different EBV latent genes, *BARF0,*

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

EBERs, EBNA1, and *LMP2A*, that are expressed in EBV-associated gastric carcinoma. RT-PCR analysis confirmed the expression of these four latent genes and the absence of LMP1 and EBNA2 expression in MKN74-EBV (Fig. 4B). The lack of LMP1 and EBNA2 expression in EBV-associated gastric carcinoma was also confirmed immunohistochemically in surgically resected tissues, which defined these tumors as latency type I, as has been described previously.

RT-PCR analysis showed a decrease in pri-miR-200 transcripts in MKN74 cells transfected with *BARF0, EBNA1*, and *LMP2A*, compared with MKN74 and MKN74-Flag (Fig. 4C), although the degree of reduction of pri-miR-200 was not as prominent as in MKN74-EBV cells. In contrast, transfection of *EBER*s did not downregulate pir-miR-200.

To further evaluate the downstream effects of EBV-latent genes, the expression of miR-200a, miR-200b, ZEB1, ZEB2, and E-cadherin was investigated in MKN74 transfected with *BARF0, EBERs, EBNA1*, and *LMP2A* (Fig. 5). MKN74-BARF0, MKN74-EBERs, and MKN74-EBNA1 cells showed downregulation of mature miR-200a and miR-200b compared with MKN74 and MKN74-Flag, and MKN74-LMP2A showed downregulation of mature miR-200b compared with MKN74-Flag. Quantitative RT-PCR analyses revealed the upregulation of ZEB1 in MKN74-BARF0, MKN74-EBERs, and MKN74-LMP2A (Fig. 5A). Subsequent downregulation of E-cadherin expression was observed in all cells transfected with EBV latent genes at the transcriptional level, among which only MKN74-EBERs showed a prominent decrease in E-cadherin expression in Western blotting (Fig. 5B), together with a slight change in cell morphology and the loss of cell-to-cell adhesion (Fig. 5C).

Discussion

The role that cellular miRNA abnormalities play in the genesis of diseases such as cancer has been intensely

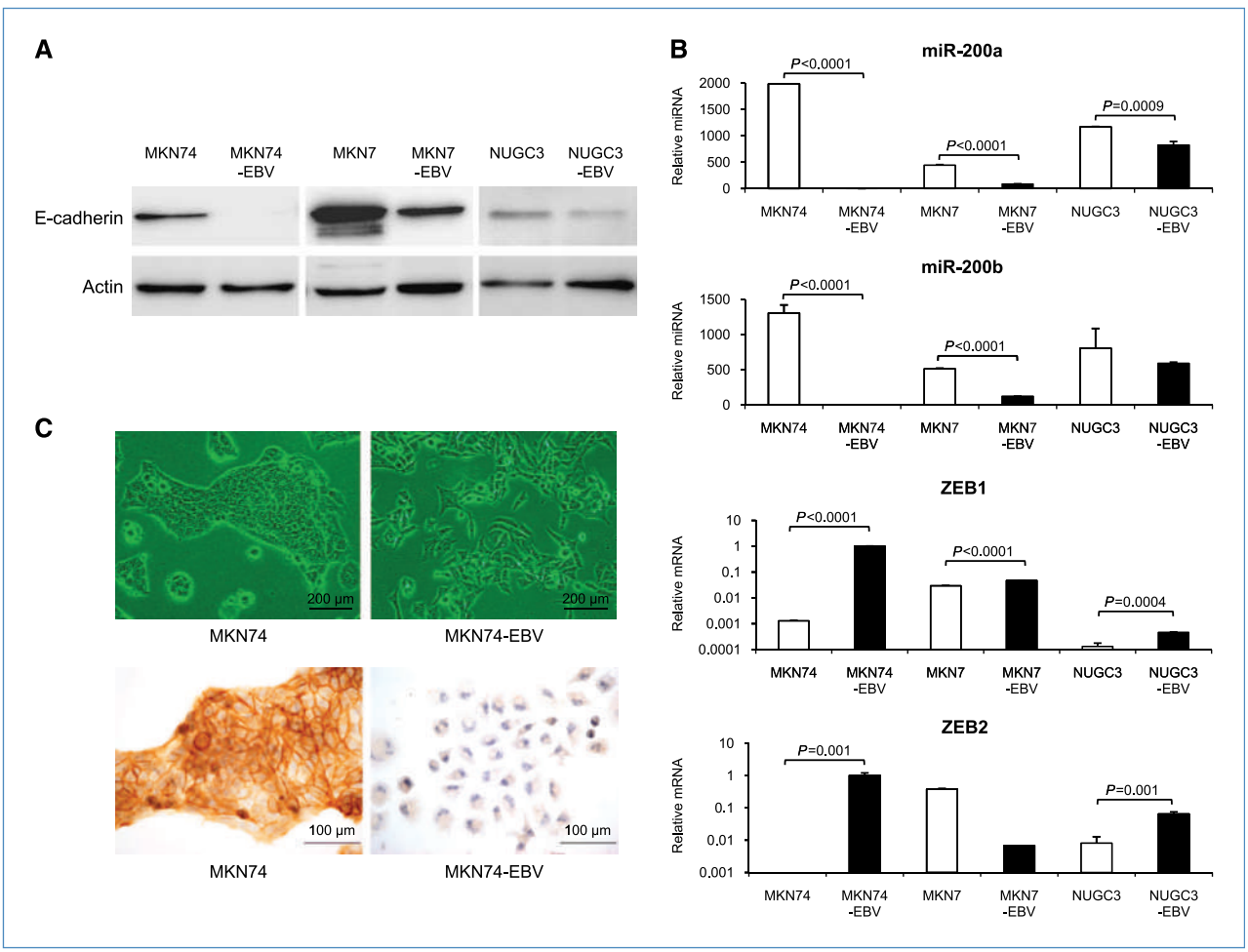

Figure 2. The expression of E-cadherin, the miR-200 family, ZEB1, and ZEB2 in the EBV-associated gastric carcinoma cell line models. A, Western blot analysis of E-cadherin in gastric carcinoma cell lines. Cells with EBV infection (MKN74-EBV, MKN7-EBV, and NUGC3-EBV) show decreased E-cadherin expression compared with their original cell lines. B, quantitative RT-PCR analysis of the miR-200 family, ZEB1, and ZEB2 in EBV-infected cell lines. Columns, averages of measurements done in triplicate; bars, SD ($P < 0.05$ was considered to be statistically significant). C, a significant loss of cell-to-cell adhesion observed in EBV-infected MKN74 cells. Immunohistochemical staining shows decreased E-cadherin expression in MKN74-EBV cells.

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

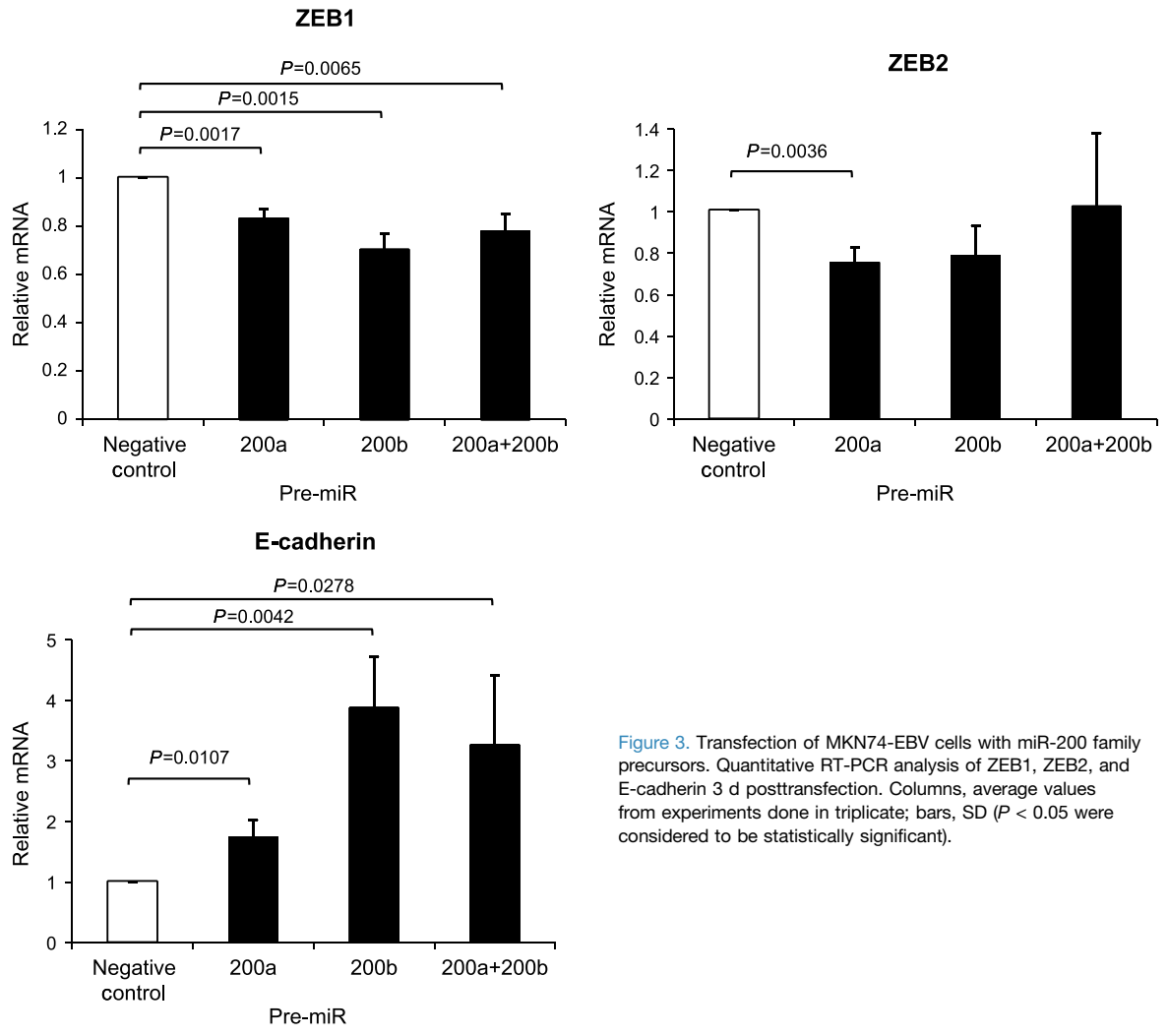

Figure 3. Transfection of MKN74-EBV cells with miR-200 family precursors. Quantitative RT-PCR analysis of ZEB1, ZEB2, and E-cadherin 3 d posttransfection. Columns, average values from experiments done in triplicate; bars, SD ($P < 0.05$ were considered to be statistically significant).

investigated. In EBV-related tumors, the EBV-latent membrane protein, LMP1, was recently found to activate several miRNAs, including miR-146a and miR-155, through NF-κB pathways, and this mechanism might underlie the development of B-cell malignancies (17, 18). However, the role of miRNAs in carcinogenesis has not been investigated in EBV-associated gastric carcinoma. The abnormal expression of several miRNAs in gastric carcinoma has been reported *in vitro* and *in vivo*. Zhang and colleagues reported that miR-21 was overexpressed in gastric cancer and that it accelerated cell proliferation and had antiapoptotic effects (19). Recently, Du and colleagues showed that miR-141, a member of the miR-200 family, had an inhibitory effect on cell proliferation and was downregulated in 80% of gastric carcinomas; however, the authors did not investigate the association between miR-141 and E-cadherin or epithelial-to-mesenchymal transition in their study (20).

Abnormal E-cadherin expression plays a key role in gastric carcinogenesis. In our previous study, hypermethylation of the *E-cadherin* gene promoter was more frequent in EBV-associated gastric carcinoma than in EBV-negative gastric carcinoma. This finding is consistent with the abnormal expression of E-cadherin in cancer tissues (21). In the present study, we observed that the miR-200 family was specifically downregulated in surgically resected EBV-associated gastric carcinoma tissue, leading to the reduction of E-cadherin expression. This phenomenon was also confirmed *in vitro* in the EBV-infected gastric cancer cell lines; EBV infection induced downregulation of the miR-200 family and the subsequent upregulation of ZEB1 and ZEB2, resulting in the inhibition of E-cadherin expression and the corresponding morphologic change, especially in MKN74 cells. These results are consistent with previous studies that showed a role for the miR-200 family in the induction of

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

epithelial-to-mesenchymal transition in normal cells and various cancer cells (7–10). It is noteworthy that EBV-associated gastric carcinoma has a unique expression profile among other cell adhesion molecules, including major tight junction proteins such as claudins (22). E-cadherin dysregulation induced by EBV infection further contributes to the disruption of cell-to-cell adhesion and promotion of cell migration. These abnormalities might be closely associated with the development, progression, and unique histologic features of EBV-associated gastric carcinoma.

The results of the present study showed that EBV infection downregulates pri-miR-200 at the transcriptional level. Considering that MKN74-EBV showed the most prominent morphologic change that well reflected the marked loss of E-cadherin expression, we selected MKN74-EBV as the most suitable model of EBV-associated gastric carcinoma for further experimentation to investigate which EBV latent gene contributed to the regulation of pri-miR-200. Among various kinds of EBV-associated malignancies, EBV-associated gastric carcinoma is distinctive owing to the limited number of EBV latent genes expressed in tumor cells. It is classified as latency type I; expressed latent genes are restricted to *BARF0, EBERs, EBNA1*, and *LMP2A*, excluding *LMP1* or *EBNA2* which have been considered to be essential for transforming ability (3, 5, 6). We recently revealed that LMP2A plays an important role in the dysregulation of intracellular signaling pathways, such as phosphorylation of signal transducers and activators of transcription 3, leading to the promotion of DNMT1-mediated CpG island methylation (16). In contrast, Iwakiri and colleagues showed that EBERs upregulate insulin-like growth factor I to function as an autocrine growth factor (23, 24). In the present study, transfection of MKN74 with *BARF0, EBNA1*, and *LMP2A* downregulated the pri-miR-200 transcript, whereas transfection of *EBER*s did not. However, all four of these genes played a role in the downregulation of mature miR-200 and the subsequent decrease in E-cadherin expression. The results of our study revealed that all the latency type I genes have a synergetic effect on the downregulation of miR-200 family in EBV-associated gastric carcinoma; the repression of pri-miR-200 transcription by BARF0, EBNA1, and LMP2A, and the inhibition of mature miR-200 at the posttranscriptional step by EBERs. The subsequent decrease in E-cadherin expression was attributed to the upregulation of ZEB1 and/or ZEB2 in most of the cell lines transfected with EBV latent genes, whereas MKN74-EBNA1 was not associated with the upregulation of ZEB1 or ZEB2. This implies the presence of some other mechanisms which do not involve ZEB1 or ZEB2. Recently, Tryndyak and colleagues showed in their study that miR-200 family–mediated transcriptional upregulation of E-cadherin was associated indirectly with increased

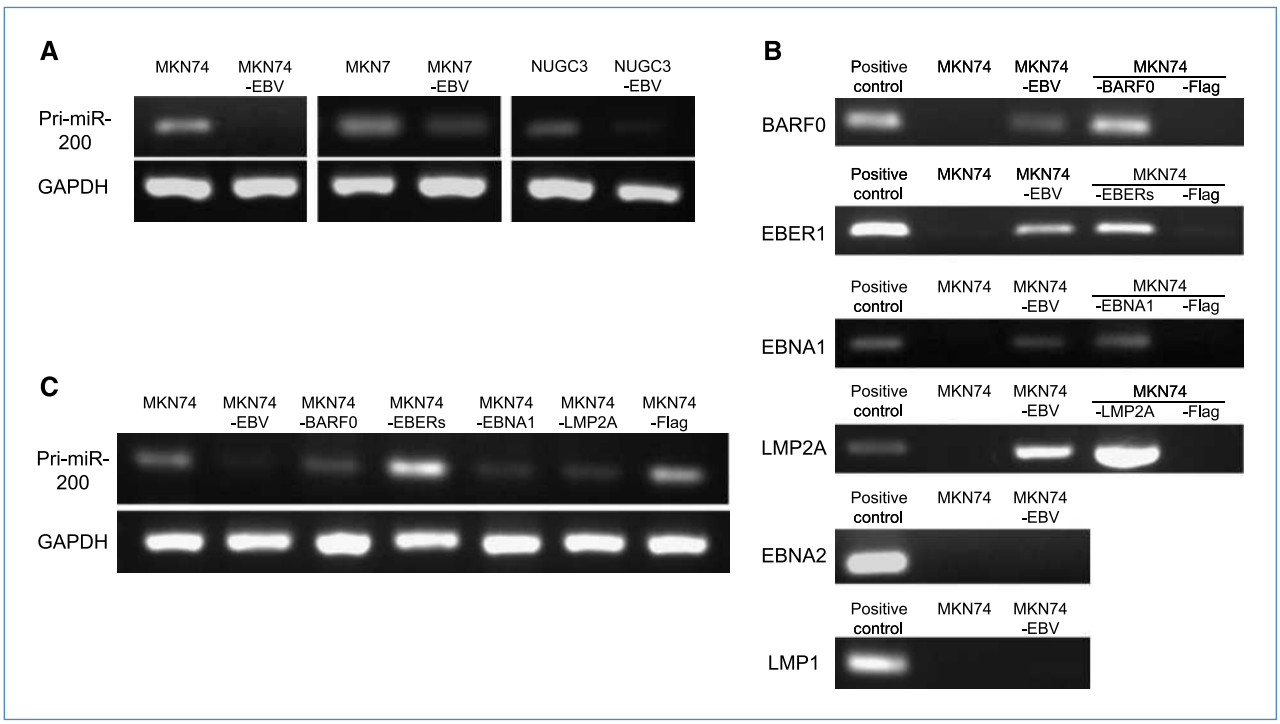

Figure 4. The effect of EBV and its latent genes on downregulation of primary precursor of miR-200 (pri-miR-200). A, RT-PCR analysis of pri-miR-200 in gastric carcinoma cell lines. B, transfection of MKN74 cells with EBV latent genes. RT-PCR analysis of EBV latent genes in MKN74-EBV cells and MKN74 cells transfected with vectors containing each latent gene or Flag tag (Flag). SNU719 was used as a positive control for latency type I genes *BARF0, EBER*s, *EBNA1*, and *LMP2A*, and Raji was used as a positive control for *EBNA2* and *LMP1*. C, RT-PCR analysis of pri-miR-200 in MKN74 cells transfected with EBV latency type I genes.

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

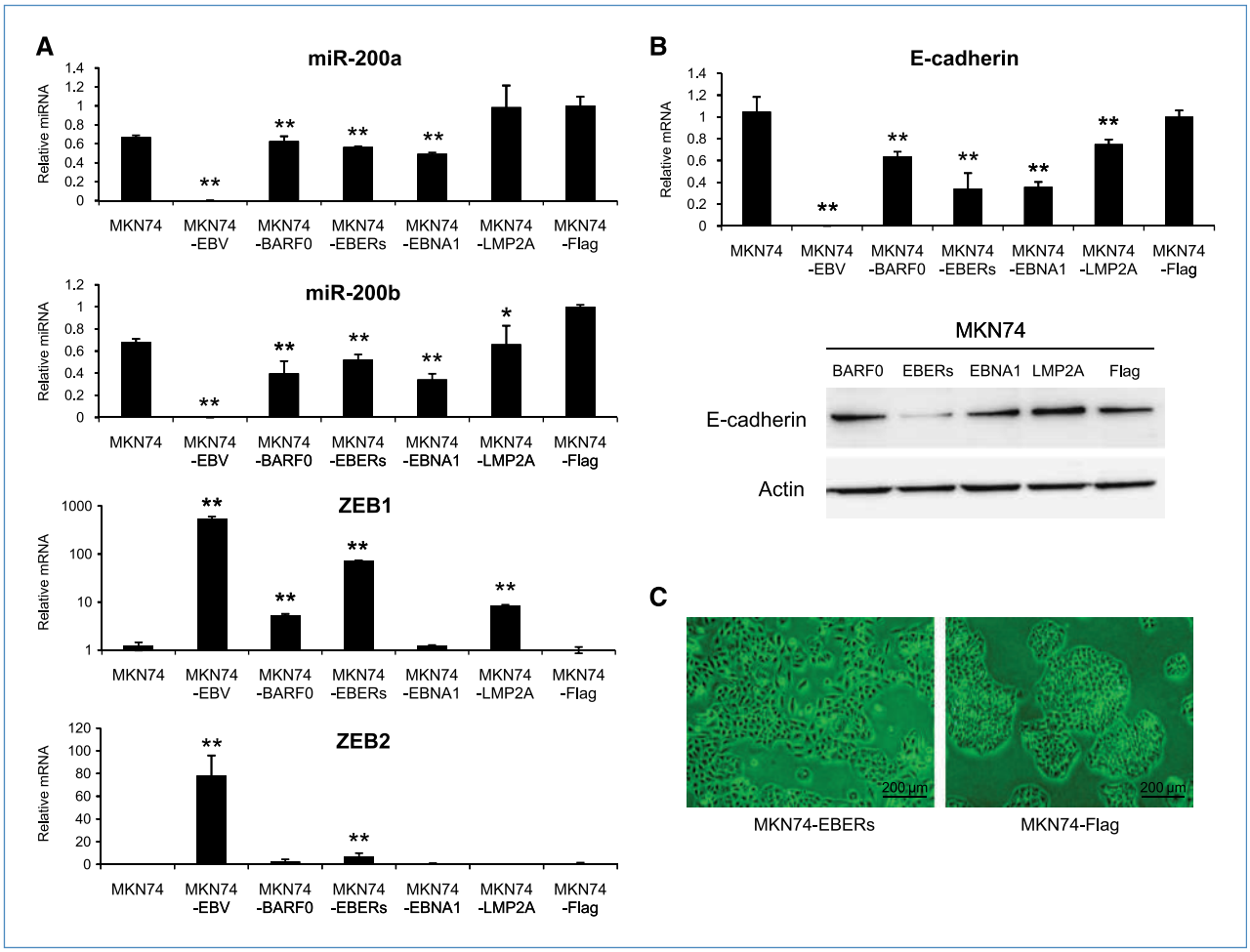

Figure 5. The effect of EBV latent genes on downregulation of miR-200 family, ZEB1/ZEB2, and E-cadherin. A, quantitative RT-PCR analysis of the miR-200 family, ZEB1, and ZEB2 in MKN74 cells transfected with EBV latency type I genes. Columns, average values of measurements done in triplicate; bars, SD (**, $P < 0.05$, statistically significant compared with MKN74 and MKN74-Flag; *, $P < 0.05$, statistically significant compared with MKN74-Flag). B, quantitative RT-PCR analysis and Western blot analysis of E-cadherin expression in MKN74 cells transfected with EBV latent genes. Columns, average values of quantitative RT-PCR analyses done in triplicate; bars, SD (**, $P < 0.05$, statistically significant compared with MKN74 and MKN74-Flag). C, morphologic changes observed in MKN74-EBER cells with a slight decrease in cell-to-cell adhesion.

acetylation of histone H3 at the E-cadherin promoter, the mechanism independent of the expression level of ZEB1 (25). The effect of histone acetylation status should be investigated in future studies to clarify the role of miR-200 family in the regulation of E-cadherin expression in EBV-associated gastric carcinoma.

In mammalian miRNA biogenesis, pri-miRNAs are cleaved into pre-miRNAs by the nuclear RNase III Drosha and further processed into mature miRNAs by cytosolic Dicer. The decrease in mature miRNAs in human cancers may be caused by genomic or epigenetic alterations, as well as by the impairment of miRNA processing steps (26). The exact mechanisms of how BARF0, EBNA1, and LMP2A repress pri-miR-200 transcription remain to be elucidated in future studies, and how EBERs reduce mature miR-200 family expression must be clarified as well. One possible explanation might be the hypermethylation of promoter regions of these miRNA genes, an interaction between viral and cellular noncoding RNAs, or a decrease in the half-life of mature forms of the miR-200 family; however, these hypotheses need further elucidation (27).

In conclusion, the present study showed that EBV infection of epithelial cells causes downregulation of the miR-200 family by repressing transcription of pri-miRNAs and by posttranscriptional dysregulation of the miRNA, and all the latency type I genes have a synergetic effect on these processes. Downregulation of the miR-200 family causes a reduction in E-cadherin expression through the upregulation of the E-cadherin repressors, ZEB1 and ZEB2. The loss of cell-to-cell adhesion is essential for tumor progression, and our findings provide some clues for clarifying viral carcinogenesis in EBV-associated gastric carcinoma.

Published OnlineFirst May 18, 2010; DOI: 10.1158/0008-5472.CAN-09-4620

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Paul J. Farrell (Tumor Virology, Division of Investigative Science, Imperial College, London, United Kingdom) for supplying the LMP2A cDNA.

Grant Support

Grants-in Aid for Scientific Research 19790253 (T. Sakatani) and 20249022 (M. Fukayama).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/19/2009; revised 03/22/2010; accepted 04/01/2010; published OnlineFirst 05/18/2010.

References

1. Ohgaki H, Matsukura N. Stomach cancer. In: Stewart BW, Kleihues P, editors. World Cancer Report. Lyon: IARC Press; 2003, p. 194–7.
2. Fukayama M, Hino R, Uozaki H. Epstein-Barr virus and gastric carcinoma: virus-host interactions leading to carcinoma. Cancer Sci 2008;99:1726–33.
3. Kutok JL, Wang F. Spectrum of Epstein-Barr virus-associated diseases. Annu Rev Pathol 2006;1:375–404.
4. Fukayama M, Ibuka T, Hayashi Y, Ooba T, Koike M, Mizutani S. Epstein-Barr virus in pyothorax-associated pleural lymphoma. Am J Pathol 1993;143:1044–9.
5. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757–68.
6. Young LS, Murray PG. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene 2003;22:5108–21.
7. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 2008;283:14910–4.
8. Bracken CP, Gregory PA, Kolesnikoff N, et al. A double-negative feedback loop between ZEB1-1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res 2008; 68:7846–54.
9. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 2008;10:593–601.
10. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 2008; 22:894–907.
11. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965; 64:31–49.
12. Sobin L, Wittekind C. TNM classification of malignant tumours. 6th ed. New Jersey: John Wiley & Sons; 2002.
13. Fukayama M, Hayashi Y, Iwasaki Y, et al. Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus infection of the stomach. Lab Invest 1994;71:73–81.
14. Imai S, Nishikawa J, Takada K. Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells. J Virol 1998;72:4371–8.
15. Iwasaki Y, Chong JM, Hayashi Y, et al. Establishment and characterization of a human Epstein-Barr virus-associated gastric carcinoma in SCID mice. J Virol 1998;72:8321–6.
16. Hino R, Uozaki H, Murakami N, et al. Activation of DNA methyltransferase 1 by EBV latent membrane protein 2A leads to promoter hypermethylation of PTEN gene in gastric carcinoma. Cancer Res 2009;69:2766–74.
17. Cameron JE, Yin Q, Fewell C, et al. Epstein-Barr virus latent membrane protein 1 induces cellular microRNA miR-146a, a modulator of lymphocyte signaling pathways. J Virol 2008;82:1946–58.
18. Gatto G, Rossi A, Rossi D, Kroening S, Bonatti S, Mallardo M. Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-κB pathway. Nucleic Acids Res 2008;36:6608–19.
19. Zhang Z, Li Z, Gao C, et al. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab Invest 2008;88:1358–66.
20. Du Y, Xu Y, Ding L, et al. Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. J Gastroenterol 2009; 44:556–61.
21. Sudo M, Chong JM, Sakuma K, et al. Promoter hypermethylation of E-cadherin and its abnormal expression in Epstein-Barr virus-associated gastric carcinoma. Int J Cancer 2004;109:194–9.
22. Shinozaki A, Ushiku T, Morikawa T, et al. Epstein-Barr virus-associated gastric carcinoma: a distinct carcinoma of gastric phenotype by claudin expression profiling. J Histochem Cytochem 2009;57:775–85.
23. Iwakiri D, Eizuru Y, Tokunaga M, Takada K. Autocrine growth of Epstein-Barr virus-positive gastric carcinoma cells mediated by an Epstein-Barr virus-encoded small RNA. Cancer Res 2003;63:7062–7.
24. Nanbo A, Yoshiyama H, Takada K. Epstein-Barr virus-encoded poly (A)- RNA confers resistance to apoptosis mediated through Fas by blocking the PKR pathway in human epithelial intestine 407 cells. J Virol 2005;79:12280–5.
25. Tryndyak VP, Beland FA, Pogribny IP. E-cadherin transcriptional down-regulation by epigenetic and microRNA-200 family alterations is related to mesenchymal and drug-resistant phenotypes in human breast cancer cells. Int J Cancer 2010;126:2575–83.
26. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of microRNA processing by p53. Nature 2009;460: 529–33.
27. Swaminathan S. Noncoding RNAs produced by oncogenic human herpesviruses. J Cell Physiol 2008;216:321–6.

AACR American Association for Cancer Research

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Downregulation of MicroRNA-200 in EBV-Associated Gastric Carcinoma

Aya Shinozaki, Takashi Sakatani, Tetsuo Ushiku, et al.

Cancer Res 2010;70:4719-4727. Published OnlineFirst May 18, 2010.