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ORIGINAL

Activation of microRNA-596 induced by DNA demethylation and interferon in malignant melanoma cells

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ABSTRACT

Dysregulation of microRNA has been implicated in melanoma, although the mechanism is not fully understood. We aimed to examine the epigenetically silenced miRNAs and its involvement in the antitumor effect of DNA demethylation and interferon in melanoma. Growth suppressive effects of 5-aza-2'deoxycytidine plus interferon-β were assessed in 20 melanoma cell lines, and the highest effect was observed in TXM18 cells. A screen for miRNAs induced by 5-aza-2'deoxycytidine plus IFN-β in TXM18 cells identified a set of miRNAs including miR-7, miR-203, miR-215 and miR-596. The CpG island of the miR-596 gene was highly methylated in all melanoma cell lines tested (n = 20) whereas levels of methylation were limited in normal melanocytes. Methylation levels of miR-596 were significantly higher in clinical specimens of melanoma than in benign melanocytic nevi (40.6% vs. 30.1%, P = 0.018). Furthermore, transfection of a precursor of miR-596 into melanoma cells induced growth suppression, indicating that the effect of 5-aza-2'deoxycytidine plus interferon-β is in part due to induction of miR-596. Our data suggest that miR-596 is a novel tumor suppressor frequently silenced by DNA methylation in melanoma; that modulation of miRNAs may be involved in the antitumor effect of DNA demethylation plus interferon in melanoma.

Key words: melanoma, epigenetics, methylation, interferon, miR-596

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression by inducing degradation or translational inhibition of partially complementary target mRNAs. The approximately 1,000 miRNAs are estimated to exist in the human genome and play pivotal roles in a wide array of biological processes, including cell proliferation, differentiation and apoptosis[1]. In recent years, a number of studies have provided evidence that dysregulation of miRNA expression contributes to the initiation and progression of human cancer[2-5]. Indeed, downregulation of a subset of miRNAs is a commonly observed feature of cancers, suggesting these molecules may act as tumor suppressors. The first report of altered miRNA expression in cancer detailed the frequent chromosomal deletion and downregulated expression of miR-15 and mirR-16, two miRNAs suggested to target the antiapoptotic factor BCL2, in chronic lymphocytic leukemia[6]. Another example of tumor-associated miRNA is let-7, which negatively regulates expression of Ras oncogenes; its downregulation in tumors is thought to contribute to activation of the Ras signaling pathway[7-8].

Although the mechanisms underlying miRNA dysregulation in cancer are not yet fully understood, recent studies have shown that the silencing of several miRNAs is tightly linked to epigenetic mechanisms, including histone modification and DNA methylation[9-10]. Pharmacological or genetic unmasking through DNA demethylation and/or HDAC inhibition is a common method of identifying epigenetically silenced genes in cancer, and a number of epigenetically silenced miRNA genes have been discovered using this technique. For instance, a microarray-based screening of miRNAs in human bladder cancer cells followed by treatment with 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methyltransferase (DNMT) inhibitor, and

4-phenylbutyric acid, a histone deacetylase inhibitor, revealed upregulation of miR-127 by the drugs[11]. More recently, DNA demethylation in colorectal and gastric cancer cells revealed that downregulation of miR-34b/c is associated with hypermethylation of the neighboring CpG island[12-15].

Cutaneous malignant melanoma is the most aggressive form of skin cancer, which arises from malignant transformation of melanocytes. The annual age-adjusted incidence rates in melanoma worldwide are increasing more rapidly than that of any other type of cancer[13]. The tumors originate from melanocytes, and are associated with risk factors such as ultraviolet radiation exposure, fair skin type and predisposing gene mutations. Most of the melanomas are resistant to chemotherapy. Currently, DAV-Feron chemotherapy, consisting of dacarbazine, nimustine, vincristine and interferon (IFN)-β, is commonly carried out as an adjuvant therapy in Japan, but complete remission rate is less than 5% in the advanced stage of malignant melanoma[16]. Recent studies have shown that the DNA demethylating treatment overcomes resistance to apoptosis induction by IFNs in melanoma cells, indicating that key factors that determining the sensitivity against IFNs are epigenetically silenced in melanoma cells[17-18]. In fact, reactivation of epigenetically silenced genes such as RASSF1A or Apo2/TRAIL receptor 1 (DR4) has been shown to overcome resistance of melanoma cells to IFNs[19-20].

Dysregulated expression of miRNAs is also a common feature in melanoma[21-23]. It has been shown that miRNA expression signatures in malignant melanoma cell lines or primary melanoma samples are significantly altered compared to those in normal epithelial melanocytes or benign nevi samples. Several miRNAs including miR-34 family members are reportedly inactivated in melanoma by aberrant CpG island methylation[24]. In addition, recent studies have shown that IFN can modulate miRNA expression and that several miRNAs are involved in the antiviral or antitumor effects of IFN[25-27]. In the present study, we hypothesized that restored expression of epigenetically silenced miRNAs may be associated with the antitumor effect of DNA demethylation plus IFNs in melanoma cells. To address this question, we carried out comprehensive expression analysis of miRNAs in melanoma cells treated with a DNMT inhibitor and IFN-β, and screened for miRNAs upregulated by the combination treatment.

2. Results

2.1. miRNA expression changes induced by demethylation and IFN-β

We first tested the effect of the treatment with 5-aza-dC plus IFN-β by using a set of malignant melanoma cell lines. Cell viability assays revealed that the combination treatment synergistically suppressed cell proliferation of a number of melanoma cell lines, among which TXM18 cells showed the most significant effect (Figure 1A, Supplementary Figure 1). Consequently, we next carried out miRNA TaqMan array analysis in TXM18 cells treated with or without 5-aza-dC and IFN-β. Of the 664 miRNAs examined, the combination treatment induced upregulation (> 5-fold) of 23 miRNAs (Figure 1B). Among them, we selected 6 miRNAs (miR-203, miR-503, miR-618, miR-886, miR-941 and miR-596) that harbored CpG islands in the proximal upstream (< 5 kb) of their coding regions, and assessed their methylation status (Supplementary Figure 2). Methylation-specific PCR (MSP) and bisulfite pyrosequencing analysis revealed hypermethylation of four miRNA genes (miR-596, miR-589, miR-886 and miR-941) in melanoma cells. However, three of the four miRNAs (miR-589, miR-886 and miR-941) were also highly methylated in benign melanocyte cells whereas miR-596 was methylated in a tumor specific manner (Supplementary Figure 2 and 3). We therefore focused on miR-596 for further analysis, and confirmed that miR-596 was induced by 5-aza-dC plus IFN-β in multiple melanoma cell lines (Figure 1C).

2.2. Analysis of miR-596 methylation in melanoma cell lines

As shown in Figure 2A, a typical CpG island is located in the proximal upstream of the pre-miR-596 coding region. We have previously carried out genome-wide chromatin signature analysis of miRNA genes in colorectal cancer cells, and showed that trimethylated histone H3 lysine 4 is a hallmark of active promoter regions of miRNA genes. Upon DNA demethylation, we observed enrichment of H3K4me3 in the CpG island of miR-596 in colorectal cancer cells, suggesting that this region is the putative promoter of miR-596 (Supplementary Figure 4). MSP analysis detected complete methylation of the CpG island in all melanoma cell lines tested, whereas normal melanocytes (HEMn-MP) exhibited only partial methylation (Figure 2B). Quantitative bisulfite pyrosequencing analysis confirmed significantly elevated methylation levels of miR-596 in all melanoma cell lines tested (n = 20; average, 88.0%),

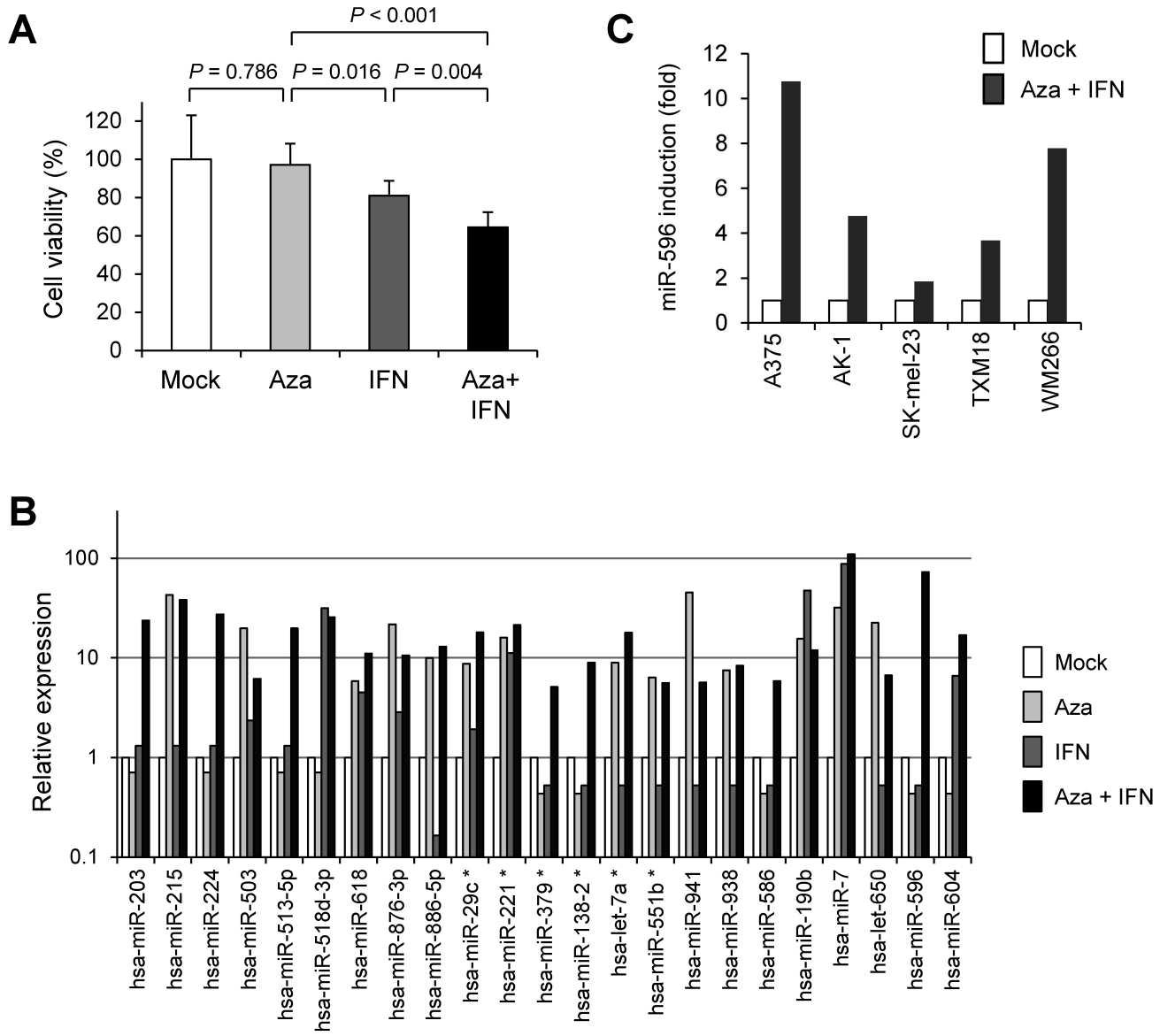

Figure 1
Screening of miRNAs upregulated by demethylation and IFN-β treatment in melanoma cells. (A) Cell viability assays with a melanoma cell line TXM18, with 5-aza-dC alone (Aza), IFN-β alone (IFN), 5-aza-dC plus IFN-β (Aza+IFN) or without treatment (Mock).
(B) TaqMan array results for 23 miRNAs upregulated by 5-aza-dC plus IFN-β in TXM18 cells. Results are normalized to internal U6 snRNA expression. (C) Quantitative RT-PCR results of miR-596 in indicated melanoma cell lines treated with or without 5-aza-dC plus IFN-β.

Supplementary Figure 1
Cell viability assays with indicated melanoma cell lines, with 5-aza-dC alone (Aza), IFN-β alone (IFN), 5-aza-dC plus IFN-β (Aza+IFN) or without treatment (Mock).

Supplementary Figure 2
The flowchart for the selection of miRNA genes in melanomas.

Supplementary Figure 3
Bisulfite pyrosequencing results of the miR-596, miR-589, miR-941 and miR-886 genes in indicated melanoma and melanocyte cell lines.

Supplementary Figure 4
Identification of miR-596 promoter region using chromatic signatures. ChIP-seq results for trimethylated histone H3 lysine 4, dimethylated histone H3 lysine 79 and trimethylated histone H3 lysine 27 in a colorectal cancer cell line HCT116 and its isogenic DNMT1□-/-; DNMT3B□-/- cell line. Note that enrichment of H3K4me3 at the miR-596 coding region and upstream CpG islands are observed in DNMTs KO cells in which DNA methylation is abrogated.

whereas methylation levels were much lower in melanocyte cell lines (n = 4; average, 17.3%) (Figure 2C). We also carried out bisulfite sequencing in selected samples, which confirmed that the CpG island was extensively methylated in melanoma cells (Figure 2D). By contrast, the majority of CpG sites were unmethylated in melanocytes (Figure 2D).

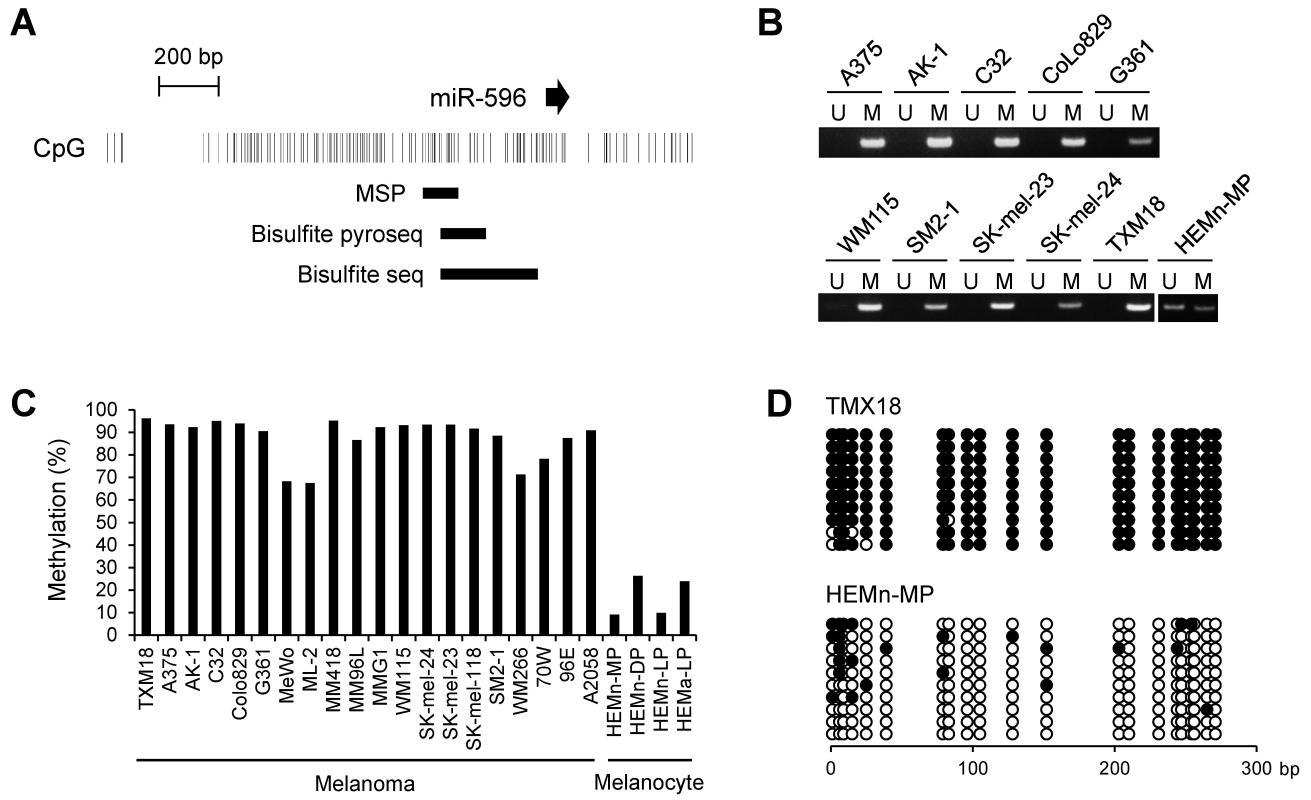

Figure 2
Methylation analysis of the miR-596 CpG island in melanoma and melanocyte cell lines. (A) Diagram of the 5' CpG island of the miR-596 gene. The pre-miR-596 coding region is indicated by an arrow. Regions analyzed by MSP, bisulfite pyrosequencing or bisulfite sequencing are indicated by bars below the CpG sites. (B) MSP analysis of the miR-596 CpG island in a set of melanoma cell lines and a melanocyte cell line HEMn-MP. (C) Bisulfite pyrosequencing results for the miR-596 CpG island in the indicated melanoma and melanocyte cell lines. (D) Representative bisulfite sequencing results for the miR-596 CpG island in the indicated melanoma and melanocyte cells. Open and filled circles represent unmethylated and methylated CpG sites, respectively.

2.3. Analysis of miR-596 methylation in primary melanoma tissues

We next analyzed the methylation of the miR-596 CpG island in a panel of malignant melanoma tissues (n = 56) and benign melanocytic nevus specimens (n = 19). Using bisulfite pyrosequencing, we detected more elevated levels of miR-596 methylation in melanoma tissues than in melanocytic nevi (40.6% vs. 30.1%). We further confirmed these results by bisulfite sequencing in selected specimens. In samples of melanocytic nevi, the majority of the alleles were unmethylated or only partially methylated, whereas malignant melanoma tissues showed more extensive methylation (representative results in Figure 3B). Receiver operator characteristic analysis suggested that miR-596 methylation was moderately discriminative between malignant melanoma and melanocytic nevi, and the most discriminating cut-off was 33.6% (sensitivity 58.9%, specificity 73.7%) (Figure 3C). In addition, elevated levels of miR-596 methylation were strongly associated with malignant melanoma when we employed this cut-off value (odds ratio, 4.02; 95% confidential interval 1.27-12.71; P = 0.018).

2.4. Cell viability assay in melanoma cell lines with ectopic expression of miR-596

To determine whether miR-596 serves as a tumor suppressor in melanoma, we transfected melanoma cell lines with a miR-596 precursor molecule or a negative control, and carried out a series of cell viability assays. Seventy-two hours after transfection, miR-596 significantly suppressed growth in TXM18, AK-1 and SK-mel-23 cells and moderately suppressed growth in C32 cells (Figure 4). These data support the hypothesis that the growth suppressive effect of DNA demethylation plus IFN-β is in part due to the induction of miR-596 in melanoma cells.

3. Discussion

The clinical effectiveness of 5-aza-dC has been shown in myelodysplastic syndrome, acute myeloid leukemia and chronic myeloid leukemia, although results of clinical trials for solid tumors including melanoma were disappointing. However, it has been shown that 5-aza-dC can sensitize cancer cells to chemotherapeutic or immunotherapeutic drugs. Importantly, recent studies have demonstrated that

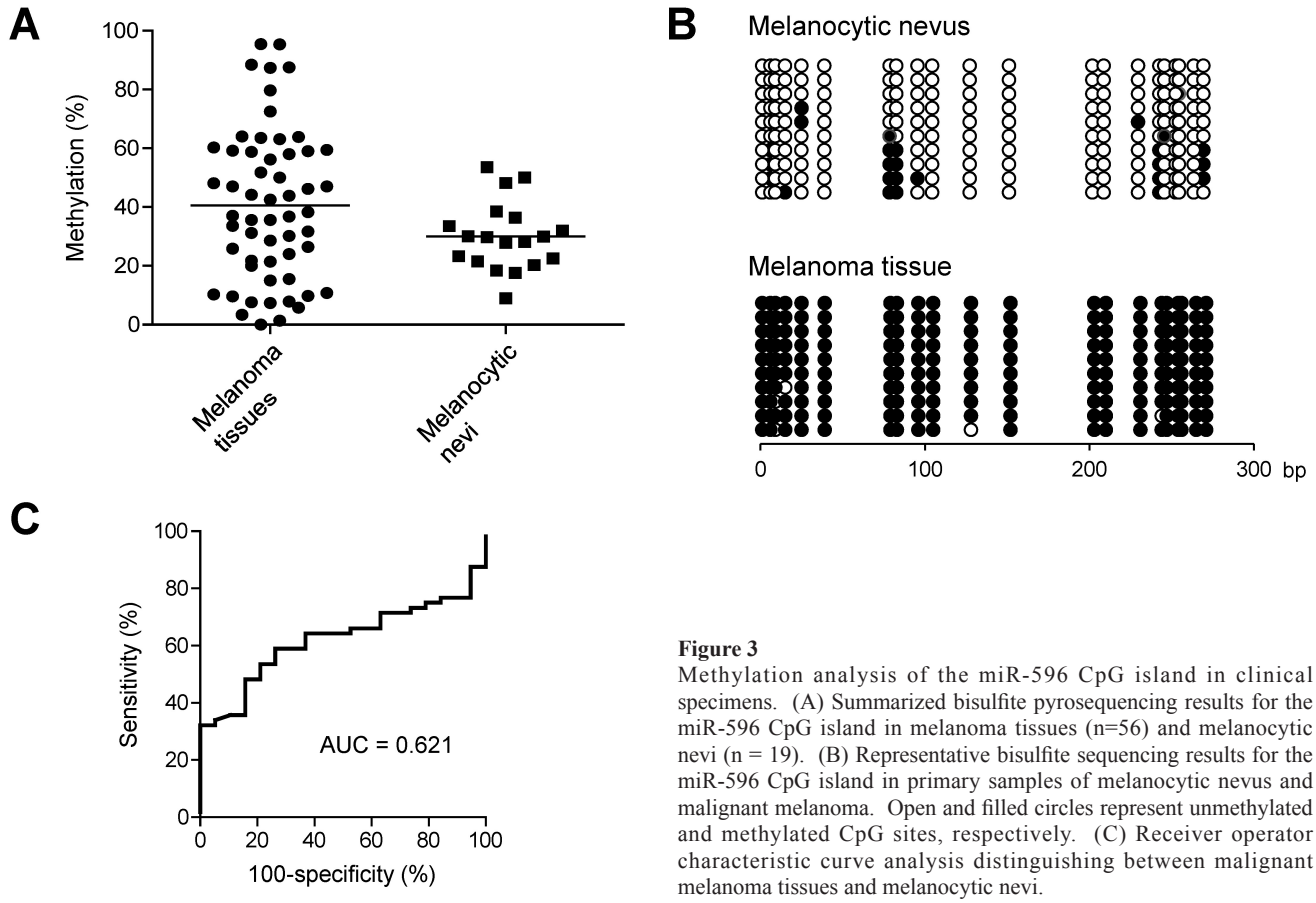

Figure 3
Methylation analysis of the miR-596 CpG island in clinical specimens. (A) Summarized bisulfite pyrosequencing results for the miR-596 CpG island in melanoma tissues (n=56) and melanocytic nevi (n = 19). (B) Representative bisulfite sequencing results for the miR-596 CpG island in primary samples of melanocytic nevus and malignant melanoma. Open and filled circles represent unmethylated and methylated CpG sites, respectively. (C) Receiver operator characteristic curve analysis distinguishing between malignant melanoma tissues and melanocytic nevi.

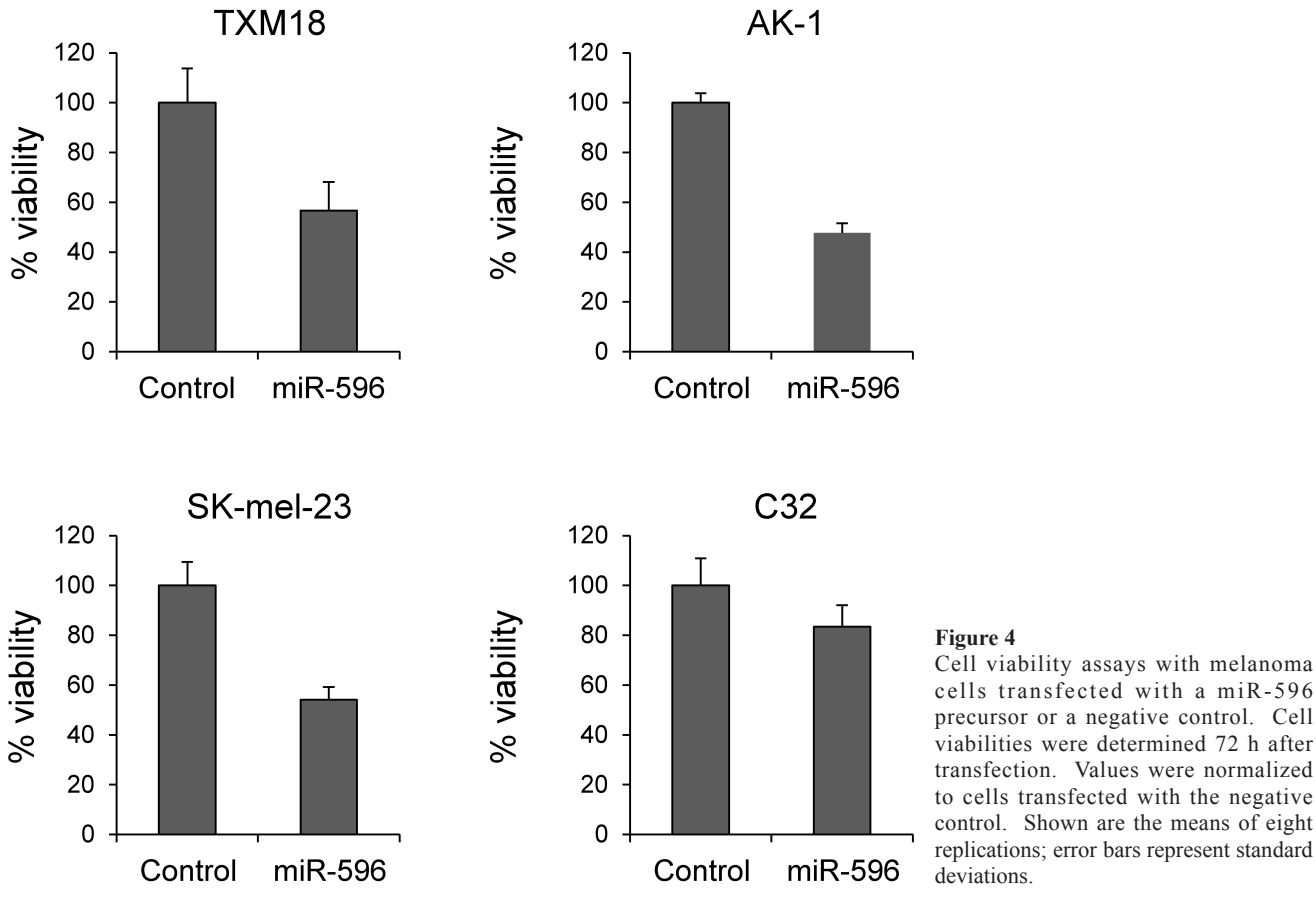

Figure 4
Cell viability assays with melanoma cells transfected with a miR-596 precursor or a negative control. Cell viabilities were determined 72 h after transfection. Values were normalized to cells transfected with the negative control. Shown are the means of eight replications; error bars represent standard deviations.

DNA demethylating treatment can sensitize melanoma cells to IFN treatment[18]. IFNs are commonly used in the treatment of melanoma, and their antitumor activities are dependent on induction of gene expression in cancer cells, immune cells and cells regulating angiogenesis[28]. Unsatisfactory response rates of melanoma to IFNs are in part due to epigenetic silencing of tumor suppressor genes that are frequently silenced in melanoma. However, the involvement of miRNA in the combination therapy has not yet been tested. In the current study, we identified a set of miRNAs which are upregulated by 5-aza-dC plus IFN-β in melanoma cells, although their responses to either 5-aza-dC or IFN-β alone were various among miRNAs.

A number of miRNAs upregulated by 5-aza-dC plus INF-β have been implicated in human malignancies. For instance, miR-203 is reportedly downregulated in prostate cancer, and its reexpression attenuates proliferation and metastasis by prostate cancer cells[29-30]. miR-215 is a downstream effector of the tumor suppressor p53, and it induces p21 accumulation and cell cycle arrest[31-33]. The miR-29 family is known to target DNMT 3A and DNMT 3B, and miR-29c is reportedly downregulated in various malignancies including melanoma. miR-7 targets p21-activated kinase 1 (Pak1), and ectopic miR-7 expression in breast cancer cells inhibited motility, invasiveness and anchorage-independent growth[17,34]. In addition, miR-7 serves as a tumor suppressor in gastric cancer, and a mice model suggested that its downregulation in gastric mucosa was associated with inflammatory responses induced by Helicobacter infection. These results indicate that the modulation of miRNA expression by 5-aza-dC plus IFN may contribute to the antitumor effect in melanoma cells.

Epigenetic alterations, including aberrant DNA methylation and histone modifications are common in cancer cells as well as in melanoma. Hypermethylation of promoter CpG islands is associated with silencing of multiple genes including tumor suppressors, apoptotic factors, DNA repair enzymes, adhesion molecules and immune system modulators. Recent evidence has demonstrated that, in addition to protein coding genes, miRNAs are important targets of epigenetic silencing in cancer. The list of miRNA genes silenced in association with DNA methylation in cancer is rapidly growing, suggesting the possible roles of these miRNAs in tumorigenesis. For instance, members of the miR-34 family (miR-34a, miR-34b and miR-34c) are downstream effectors of p53, and their expression induces cell cycle arrest and apoptosis in cancer cells[33,35-36]. Recent studies have shown aberrant DNA methylation of miR-34a and miR-34b in melanoma and reexpression of miR-34b inhibited melanoma cell invasion and motility. In addition, screening of miRNAs in melanoma cells followed by DNA demethylation and histone deacetylase inhibition identified miR-375 as a target of epigenetic silencing.

In this study, we identified that the CpG island of miR-596 is frequently methylated in melanoma cell lines and primary melanoma samples. Although physiological and pathological roles of miR-596 remain largely unknown, recent evidence suggests its involvement in human malignancies[2,37]. The miR-596 gene is located at 8p23, a region which is frequently deleted in many types of cancer[38]. High-resolution copy number analyses in multiple cancer cell lines have identified heterozygous or homozygous loss of miR-596 and a neighboring gene ARHGEF10, suggesting that they may be candidate tumor suppressor genes. Recently, a high-throughput methylation analysis with deep sequencing has identified the miR-596 locus as a target of DNA methylation in hepatocellular carcinoma. Analysis of miRNA expression profiles in ependymoma revealed that expression of three miRNAs including miR-596 is strongly associated with overall survival. Furthermore, we observed that ectopic expression of miR-596 in melanoma cells suppressed cellular growth. These results are indicative of a possible tumor suppressor role of miR-596, although further study is needed to unravel the molecular function and target genes of miR-596.

The molecular mechanism by which 5-aza-dC and INF-β induces miR-596 expression remains unknown. Interestingly, 5-aza-dC without IFN failed to induce miR-596 expression in melanoma cells, suggesting that DNA demethylation alone is insufficient to modulate its expression. It is known that IFN-inducible genes contain an IFN-stimulated response element in their promoter regions. By using a transcription factor search program, we identified several putative IFN-stimulated response elements in the upstream regions of miR-596, suggesting that it could be a direct target of type I IFN (data not shown). Further study will be necessary to determine precisely how interferon induces miR-596.

In summary, we have shown that a novel miRNA gene is epigenetically silenced in melanoma. Taken together, the high rate of miR-596 methylation and the results of our functional study suggest it is a candidate tumor suppressor gene in melanoma. Our results also suggest a novel involvement of miRNA in the antitumor effect of DNA demethylation plus IFN treatment in melanoma cells.

4. Materials and methods

4.1. Cell lines and tissue samples

Malignant melanoma cell lines SM2-1, MMG1 and ML-2 were kindly provided by M. Takada (Shinshu University School of Medicine, Japan), Y. Kawakami (Keio University School of Medicine, Japan) and A. Yamamoto (Saitama Medical University International Medical Center, Japan), respectively. AK-1 was provided by T. Moriuchi (Institute for Genetic Medicine, Hokkaido University, Japan). G361, SK-mel-23, AK-mel-118 and 70W were kindly provided by A. N. Houghton (Memorial Sloan-Kettering Cancer Center, USA). MM418, MM96L and MM96E were kindly provided by P. G. Parson (Queensland Institute of Medical Research, Australia). A2058, C32, Colo 829, SK-mel-24, TXM18, WM115 and WM266 were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). A375 and MeWo were purchased from European Collection of Cell Cultures (ECACC; Salisbury, UK). Melanoma cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. In addition, human epidermal melanocyte cell lines HEMn-MP, HEMn-DP, HEMn-LP and HEMa-LP were purchased from Cascade Biologics Inc (Portland, Oregon, USA) and cultured in Medium 254 (M-254-500; Cascade Biologics, Portland, Oregon, USA) supplemented with human melanocyte growth supplement. A set of 56 malignant melanoma specimens (10 fresh frozen specimens and 46 formalin-fixed paraffin embedded sections) and 19 benign nevus specimens (1 fresh frozen specimen and 18 from formalin-fixed paraffin embedded sections) were surgically resected from 75 patients at Sapporo Medical University Hospital. Informed consent was obtained from all patients before collection of the specimens. Genomic DNA was extracted using the standard phenol-chloroform procedure. Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with a DNA-free kit (Ambion Inc, Austin, TX, USA).

4.2. Drug treatment and cell viability assay

Melanoma cells ($2x10^3$ cells per well) were seeded in 96-well plates 24 h prior to drug treatment. Cells were treated with 5-aza-dC (Sigma-Aldrich, St Louis, MO, USA) alone, IFN-β (DAIICHI SANKYO, Japan) alone or the combination of 5-aza-dC plus IFN-β as follows. Cells were treated with 1 μM of 5-aza-dC or mock for 72 h, with the drug and medium replaced every 24 h. Alternatively, cells were treated with mock for 48 h and then treated with 1,000 U/ml of IFN-β for 24 h. For the combination treatment, cells were treated with 1 μM of 5-aza-dC for 48 h and then treated with 1 μM of 5-aza-dC plus 1,000 U/ml of IFN-β for 24 h. In all cases, cells were further incubated for an additional 24 h. The cell viability was analyzed utilizing water-soluble tetrazolium salt assays using a Cell Counting kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions.

4.3. miRNA expression profiling

Expression of 664 miRNAs was analyzed using a TaqMan MicroRNA Array v2.0 (Applied Biosystems, Foster City, CA, USA). Briefly, 1 μg of total RNA was reverse transcribed using a Megaplex Pools kit (Applied Biosystems), after which miRNAs were amplified and detected using PCR with specific primers and TaqMan probes. The PCR was run on a 7900HT Fast Real-Time PCR System (Applied Biosystems), and SDS2.2.2 software (Applied Biosystems) was used for comparative delta Ct analysis. U6 snRNA (Applied Biosystems) served as an endogenous control.

4.4. Quantitative RT-PCR of miRNA

Expression of selected miRNAs was analyzed using TaqMan microRNA Assays (Applied Biosystems). Briefly, 5 ng of total RNA were reverse transcribed using specific stem-loop RT primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control.

4.5. Methylation analysis

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). MSP, bisulfite sequencing and bisulfite pyrosequencing were then carried out as described previously. For bisulfate sequencing, amplified PCR products were cloned into a pCR2.1-TOPO vector (Invitrogen), and 10 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). For bisulfite pyrosequencing, a biotinylated PCR product was purified, made single-stranded and used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The PCR products were bound to streptavidin sepharose beads HP (Amersham Biosciences, Piscataway, NJ), after which beads containing the immobilized PCR product were purified, washed and denatured using a 0.2 M NaOH solution. After the addition of 0.3 μM sequencing primer

to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotage, Uppsala, Sweden) and Pyro Q-CpG software (Biotage). Primer sequences and PCR product sizes are listed in Table 1.

4.6. Transfection and cell viability assay

Melanoma cells ($3x10^5$ cells in 6-well plates) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Ambion) or Pre-miR miRNA Molecules Negative Control #1 using Lipofectamine 2000 (Invitrogen). After incubation for 24 h, the transfectants were seeded into 96-well plates to a density of $5x10^3$ cells per well. After incubation for an additional 48 h, cell viability assays were carried out as described above.

4.7. Statistical analysis

To compare differences in continuous variables between groups, t tests or ANOVA with post hoc Tukey's tests were performed. Fisher's exact test was used for analysis of categorical data. Receiver operator characteristic curves were constructed based on the levels of methylation. Values of $P < 0.05$ (two-sided) were considered statistically significant. Statistical analyses were carried out using GraphPad Prism ver. 5.0.2 (GraphPad Software, La Jolla, CA, USA).

5. Conflicts of interest

The authors declare that they have no competing interests.

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悪性黒色腫においてDNA脱メチル化剤とインターフェロンにより誘発したmicroRNA-596の活性化

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microRNAの調節異常は悪性黒色腫に関連があるがそのメカニズムはよく理解されておりません．私たちは悪性黒色腫においてエピジェネティックに抑制されたmiRNAとDNA脱メチル化とIFNの抗腫瘍効果におけるその関連性を調べることを目的としました．5-aza-2‘deoxycytidineおよびIFN-βの増殖抑制効果を20の悪性黒色腫細胞株で調べ，最も高い効果がTMX18という細胞株で観察されました．TXM18細胞に5-aza-2‘deoxycytidineおよびIFN-βによって誘導されるmiRNAを網羅的に調べ，miR-7，miR-203，miR-596を含んだmiRNAが誘導されることが分かりました．miR-596遺伝子は正常メラノサイトではメチル化レベルが制限されているにもかかわらず，今回調べた20個全ての悪性黒色腫細胞株で高くメチル化されていることが分かりました．miR-596のメチル化レベルは色素性母斑よりも悪性黒色腫検体において特に高くメチル化されておりました（40.6% vs 30.1%, P=0.018）．更にmiR-596前駆物質の悪性黒色腫細胞株への導入は増殖抑制を起こしました．このことは5-aza-2‘deoxycytidineおよびIFN-βの効果は一部，miR-596の産生によるものであることを示唆します．我々のデータはmiR-596が悪性黒色腫においてDNAメチル化によって抑制された新しい腫瘍抑制因子であり，miRNAの調節はメラノーマにおいてDNAメチル化およびIFNの抗腫瘍効果に関与している可能性があることが示唆されました．