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Development of a novel microRNA promoter microarray for ChIP-on-chip assay to identify epigenetically regulated microRNAs

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ABSTRACT

To gain a global view of epigenetic alterations around microRNA (miRNA) promoter regions, and to identify epigenetically regulated miRNAs, we developed a novel miRNA promoter microarray for chromatin immunoprecipitation (ChIP)-on-chip assay. We designed a custom oligo microarray covering regions spanning −10 to +2.5 kb of precursor miRNAs in the human genome. This microarray covers 541 miRNAs, each of which is covered by approximately 100 probes (60-mer) over its 12.5-kb genomic position, that includes predicted transcription start sites. Using this custom-made miRNA promoter microarray, we successfully performed ChIP-on-chip assay to identify miRNAs regulated by histone modification. Fifty-three miRNAs (9.8%) showed increased levels of both histone H3 acetylation and histone H3-K4 methylation in AGS gastric cancer cells treated with the DNA-methylation inhibitor 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor 4-phenylbutyric acid. One of these miRNAs, *miR-9*, is downregulated in gastric cancer tissues and is activated by chromatin-modifying drugs, suggesting that it may be a potential target for epigenetic therapy of gastric cancer.

1. Introduction

MicroRNAs (miRNAs) are ∼22 nucleotide (nt) non-coding RNAs that can post-transcriptionally downregulate the expression of various target genes. Currently, ∼1500 human miRNAs have been identified in the human genome, each of which potentially controls hundreds of target genes. In animals, miRNA genes are generally transcribed by RNA polymerase II (pol II) to form primary transcripts (pri-miRNAs). Pol II-transcribed pri-miRNAs are capped with 7-methylguanosine and are polyadenylated. The nuclear RNase III enzyme Drosha and its co-factor DGCR8 process pri-miRNAs into ∼60-nt precursor miRNAs (pre-miRNAs), which form an imperfect stem-loop structure. Pre-miRNAs are transported into the cytoplasm by exportin 5 and are subsequently cleaved by Dicer into mature miRNAs, which are then loaded into the RNA-induced silencing complex (RISC). The miRNA/RISC complex downregulates specific gene products by translational repression via binding to partially complementary sequences in the 3′-untranslated regions of the target mRNAs or by directing mRNA degradation via binding to perfectly complementary sequences.

MircoRNAs are expressed in a tissue-specific manner and play important roles in cell proliferation, apoptosis, and differentiation during mammalian development [1]. Links between miRNAs and the development and progression of human malignancies, including gastric cancer, are becoming increasingly apparent [2,3]. Because miRNAs can have large-scale effects through regulation of a variety of target genes during carcinogenesis, understanding the regulatory mechanisms controlling miRNA expression is important. Epigenetic alterations such as DNA methylation and histone modification play critical roles in chromatin remodeling and regulation of gene expression in mammalian development and human diseases, including cancer. We have recently reported that some miRNAs are regulated by epigenetic alterations at their CpG island promoters. Epigenetic treatment with chromatin-modifying drugs such as the DNA-demethylating agent 5-aza-2′-deoxycytidine (5-Aza-CdR) and the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (PBA) can reactivate some important tumor suppressor miRNAs, and this may be a novel therapeutic approach for human cancers [4–7]. To gain a global view of epigenetic alterations around miRNA promoter regions and to

Abbreviations: miRNA, microRNA; ChIP, chromatin immunoprecipitation; 5-Aza-CdR, 5-aza-2′-deoxycytidine; HDAC, histone deacetylase; PBA, 4-phenylbutyric acid.

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identify epigenetically regulated miRNAs, we developed a novel miRNA promoter microarray for chromatin immunoprecipitation (ChIP)-on-chip assay and used it to identify candidate miRNAs regulated by epigenetic mechanisms in human gastric cancer cells.

2. Materials and methods

2.1. MicroRNA promoter microarray

As shown in Fig. 1, we designed a custom oligo microarray covering regions −10 to +2.5 kb surrounding the genomic positions of pre-miRNAs in the human genome (NCBI36/hg18). Briefly, we first downloaded genomic coordinates of pre-miRNAs from the Manchester (previously Sanger) miRBase v10.1. The set of genomic coordinates at the 5′ end of the pre-miRNAs was positioned at zero, and *in silico* pre-designed probes were searched to fit a 4 × 44 K microarray from the high-definition ChIP probe database in eArray provided by Agilent Technologies (Tokyo, Japan). During the probe search, the Tm filter was applied and no homology filter was applied. This microarray covers 541 miRNAs, and each miRNA, spanning an estimated 12.5 kb of genomic sequence (including predicted transcription start sites (TSSs)), is covered by approximately 100 probes (60-mer).

2.2. Cell line and epigenetic treatment

The human gastric cancer cell line AGS was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. They were seeded at 1×10^5 cells per 100 mm dish 24 h before treatment with 5-Aza-CdR (3 μM, Sigma–Aldrich, St. Louis, MO) and PBA (3 mM, Sigma–Aldrich). After 24 h, 5-Aza-CdR was removed, while the cells were continuously exposed to PBA for 96 h.

2.3. ChIP-on-chip assay

The ChIP assay was performed as described previously [4] using 10 μl of anti-dimethylated histone H3-K4 (Upstate Biochemistry, Lake Placid, NY) and 10 μl of anti-acetylated histone H3 antibodies (Upstate Biochemistry). After ChIP assay, immunoprecipitated DNA was amplified and labeled using Agilent genomic DNA labeling kit

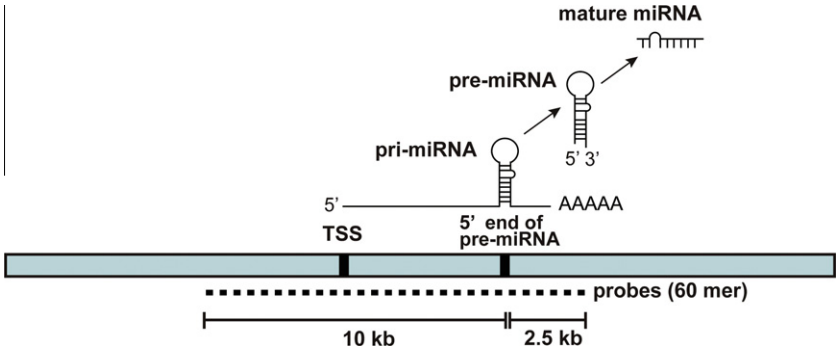

Fig. 1. A design of a custom oligo microarray covering from −10 to +2.5 kb surrounding the genomic positions of pre-miRNAs in the human genome. This microarray covers 541 miRNAs with 125 base spacing between the probes on average, and each miRNA is covered with approximately 100 probes (60-mer) on its 12.5-kb genomic position that includes predicted TSSs.

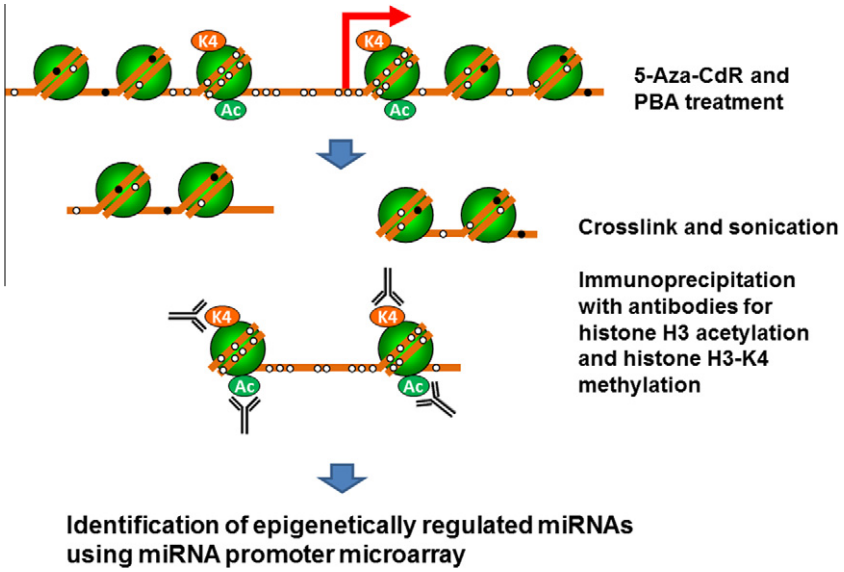

Fig. 2. A scheme of the experimental procedure for ChIP-on-chip assay with miRNA promoter microarray. AGS cells were treated with the DNA-methylation inhibitor 5-Aza-CdR and the HDAC inhibitor PBA. After crosslinking and sonication, chromatin was immunoprecipitated using antibodies for histone H3 acetylation and histone H3-K4 methylation, and immunoprecipitated DNA was hybridized on the miRNA promoter microarray. Open circle, unmethylated DNA; filled circle, methylated DNA; Ac, histone H3 acetylation; K4, histone H3-K4 methylation.

(Agilent Technologies) according to the manufacturer's instructions. Subsequently, labeled DNA was hybridized on the miRNA promoter microarray (Fig. 2).

2.4. ChIP-PCR

Quantitative analysis of ChIP products was performed by real-time PCR with the CYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan) using the Thermal Cycler Dice Real-Time System (Takara Bio). The sequences of the primers used were as follows: *miR-9–1* Forward: 5′-CTCAAGGAGAGAAGGAAACAGC-3′, *miR-9–1* Reverse: 5′-TCACAACCCTGGGTGATCTC -3′; *miR-9–3* Forward: 5′-GCTAGAT CTACTGCAAGTGCTG-3′, *miR-9–3* Reverse: 5′-GGACCATCAGAGTTT GG GAG-3′.

The fraction of immunoprecipitated DNA was calculated as follows: (immunoprecipitated DNA with each antibody – nonspecific antibody control (NAC))/(input DNA – NAC).

2.5. Tissue specimens of gastric cancers

Tissue specimens from advanced gastric cancers and the surrounding non-tumor gastric mucosae were obtained from materials surgically resected from 13 patients at the National Cancer Center Hospital (Tokyo, Japan). This study was approved by the Ethics Committee of the National Cancer Center and was performed in accordance with the 1964 Declaration of Helsinki. Written informed consent was obtained from all patients.

2.6. Quantitative RT-PCR of miR-9

Levels of miRNA expression were analyzed by quantitative RT-PCR using the TaqMan microRNA assay for *miR-9* (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Expression levels were normalized to those of U6 RNA.

3. Results

3.1. Identification of candidates of epigenetically regulated miRNAs by ChIP-on-chip assay with a novel miRNA promoter array

We designed a custom oligo microarray covering from −10 to +2.5 kb surrounding the genomic positions of pre-miRNAs in the human genome (Fig. 1). This microarray covers 541 miRNAs with 125 base spacing between the probes on average, and each miRNA is covered with approximate 100 probes (60 mer) over its 12.5-kb genomic position, that includes predicted TSSs.

To investigate miRNAs, which are regulated by epigenetic alterations, we treated AGS cells with the DNA-methylation inhibitor 5-Aza-CdR and the HDAC inhibitor PBA. Histone H3 acetylation and histone H3-K4 methylation are enriched at transcriptionally active gene promoters. Fig. 2 shows a schematic of the experimental procedure for ChIP-on-chip assay using the miRNA promoter microarray. After crosslink and sonication, chromatin was immunoprecipitated using antibodies for histone H3 acetylation and histone H3-K4 methylation, and immunoprecipitated DNA was hybridized on the miRNA promoter microarray. We considered miRNAs to be candidates for epigenetic regulation when five or more of their probes showed increased level ($>2^{0.5}$) of both histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment. Table 1 summarizes the results of our miRNA ChIP-on-chip assay using the miRNA promoter array. Fifty-three miRNAs (9.8%; 53 of 541 miRNAs) showed increased levels of both histone H3 acetylation and histone H3-K4 methylation. Among these 53 miRNAs, 19 miRNAs were located in the intronic regions of their host genes, and 12 miRNAs were located near neighboring genes. Twenty-one miRNAs were located near CpG islands. We suggest that these miRNAs may be regulated by epigenetic alterations, such as acetylation and methylation of histone H3, in gastric cancer cells.

Table 1
miRNAs immunoprecipitated with antibodies for both histone H3 acetylation and histone H3-K4 methylation.

miRNAs	Host genes	Genes near miRNAs	CpG islands
let-7c	C21orf34		
miR-1–1		FLJ30313	■
miR-7–3	PGSF1		
miR-9–1	C1orf61		■
miR-9–3			■
miR-29b-2			
miR-30a			
miR-30b			
miR-33b	SREBF1		■
miR-34a			■
miR-99a	C21orf34		
miR-100			
miR-124a-1			
miR-125b-2	C21orf34		
miR-129–1			
miR-129–2			■
miR-135b			
miR-138–2		NUP93	
miR-142		BZRAP1	
miR-143			
miR-147b	C15orf48		■
miR-148b	COPZ1		
miR-149	GPC1		■
miR-183			
miR-195		BCL6B	
miR-196a-1			■
miR-196a-2		HOXC10	
miR-196b		HOXA10	
miR-202		ADAM8	
miR-205			
miR-296			
miR-328		LRRC29	
miR-335	MEST		■
miR-337			
miR-365–2			
miR-370			
miR-375		CRYBA2	■
miR-424			■
miR-455	COL27A1		
miR-483	IGF2		■
miR-497		BCL6B	
miR-511–2	MRC1		
miR-532			■
miR-548c	RASSF3		■
miR-549	KIAA1199		■
miR-566	SEMA3F		■
miR-612			
miR-636		MFSD11	■
miR-769		PGLYRP1	
miR-770			■
miR-933	ATF2		■
miR-1233	GOLGA8A		
miR-1237	RPS6KA4		■

3.2. Downregulation of miR-9 in gastric cancer tissues and miR-9 activation by chromatin-modifying drugs

Our ChIP-on-chip assay identified *miR-9–1* (chr1q22) and *miR-9–3* (chr15q26) as candidates for epigenetic regulation in gastric cancer cells. Recent studies have shown that *miR-9* is regulated by epigenetic alterations in human cancer metastasis [8], and that *miR-9–1* is epigenetically inactivated in human breast cancer [9]. We therefore selected *miR-9* for validation as a candidate epigenetically controlled miRNA in human gastric cancer cells. ChIP-PCR for

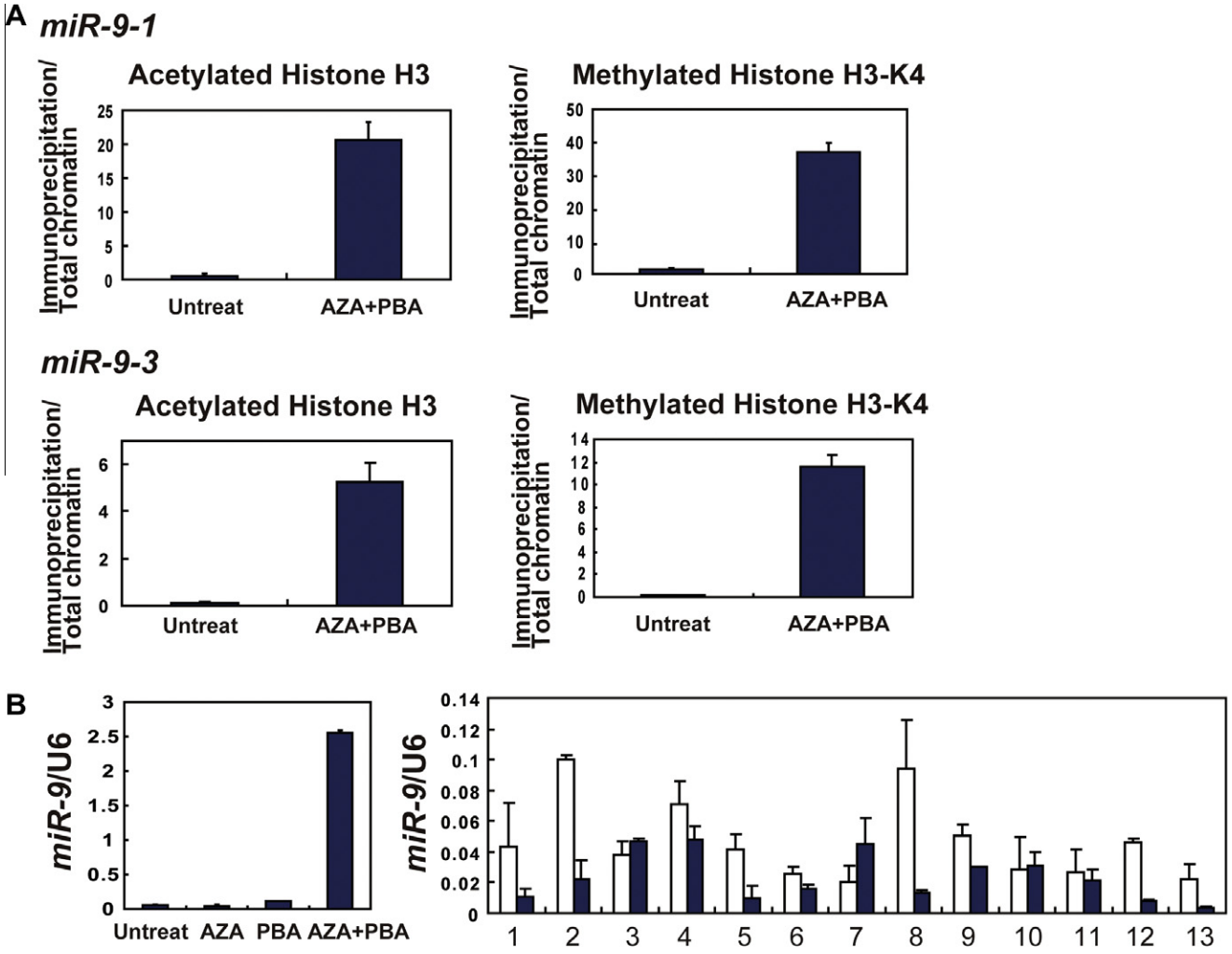

Fig. 3. Confirmation of the results of ChIP-on-chip assay with the miRNA promoter microarray. (A) ChIP-PCR for *miR-9–1* and *miR-9–3* in AGS cells treated with 5-aza-CdR and PBA. The levels of histone H3 acetylation and histone H3-K4 methylation around the promoter regions of *miR-9–1* and *miR-9–3* significantly increased after epigenetic treatment of AGS cells. (B) Quantitative RT-PCR for *miR-9* expression in AGS cells and human gastric cancer samples. The expression level of *miR-9* significantly increased in AGS cells after treatment with 5-aza-CdR and PBA. Expression levels of *miR-9* reduced in 77% (10 of 13 cases) of gastric cancer tissues (filled bar) compared with the levels in the corresponding non-tumor gastric mucosae (open bar). The average levels of *miR-9* expression were significantly lower in gastric cancer tissues than in the corresponding non-tumor gastric mucosae ($p < 0.05$).

miR-9–1 and *miR-9–3* (Fig. 3A) showed that the levels of histone H3 acetylation and histone H3-K4 methylation around the promoter regions of *miR-9–1* and *miR-9–3* significantly increased after epigenetic treatment of AGS cells. Because histone H3 acetylation and histone H3-K4 methylation are active chromatin marks associated with increased level of gene expression, we examined expression levels of *miR-9* in AGS cells after treatment with 5-aza-CdR and PBA. The expression level of *miR-9* significantly increased after combination treatment with 5-aza-CdR and PBA, whereas *miR-9* expression was not induced by either 5-aza-CdR alone or PBA. We next examined *miR-9* expression levels by quantitative RT-PCR in tissue specimens of gastric cancers. Expression levels of *miR-9* reduced in 77% (10 of 13 cases) of gastric cancer samples compared with the levels in the corresponding non-tumor gastric mucosae (Fig. 3B). The average levels of *miR-9* expression were significantly lower in gastric cancer tissues than in the corresponding non-tumor gastric mucosae ($p < 0.05$).

4. Discussion

Using a custom miRNA promoter microarray, we performed comprehensive ChIP-on-chip analysis of histone modifications in predicted human miRNA promoter regions. Because recent studies have shown that the majority of TSSs occur within 10 kb upstream of the 5′-end of pre-miRNAs [10,11], we designed microarray probes from 10 kb upstream to 2.5 kb downstream (relative to the TSS) of each pre-miRNAs to comprehensively analyze histone modifications. We identified miRNAs as candidates for epigenetic regulation when five or more of their probes showed increased levels ($>2^{0.5}$) of both histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment. Our microarray platform contains approximately 100 probes per miRNA with an average spacing of 125 bases. Enrichment of five probes therefore monitors histone modification events over at least 500 bp of sequence, which is considered sufficient to change chromatin structure.

Our assay identified miRNAs regulated by histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment of AGS cells. Because DNA-methylation inhibitors and HDAC inhibitors synergistically induce open chromatin structure associated with active gene expression [12], we treated AGS cells with the DNA-methylation inhibitor 5-Aza-CdR and the HDAC inhibitor PBA. Our results indicate that chromatin structural changes resulting from acetylation and methylation of histone H3 by epigenetic treatment can affect the expression of a substantial number of

miRNAs. Approximately 10% of miRNAs on the array showed increased levels of both histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment, indicating that these miRNAs are regulated by histone modification.

Gastric cancer is the second most common cause of cancer-related death worldwide, and systemic chemotherapy is the only treatment available for advanced gastric cancer. Because epigenetic alterations due to *Helicobacter pylori* infection or various exogenous antigen exposures are frequently observed in the stomach, chromatin-modifying drugs such as DNA-methylation inhibitors and HDAC inhibitors may have an inhibitory effect on gastric cancer growth. In our ChIP-on-chip assay using AGS cells with epigenetic treatment, *miR-9* was identified as a miRNA showing increased levels of both histone H3 acetylation and histone H3-K4 methylation at 2 different genomic positions corresponding to *miR-9–1* and *miR-9–3*. Expression levels of *miR-9* in gastric cancer tissues were significantly decreased compared with the levels in the corresponding non-tumor gastric mucosae, and combination treatment with 5-Aza-CdR and PBA markedly activated *miR-9* expression. These findings indicate that *miR-9* is a potential tumor suppressor miRNA in gastric cancer and that its expression is regulated by chromatin-modifying drugs, suggesting that *miR-9* may be a potential target for epigenetic therapy of gastric cancer. Recent studies have also shown that *miR-9* is a potential tumor suppressor miRNA that is inactivated by epigenetic mechanisms in human cancers [8,9]. Besides *miR-9*, a number of other important miRNAs have been identified, such as *miR-34a*, which has been identified as a target of p53, and which induces G(1) cell cycle arrest, senescence and apoptosis [13,14]. Recent studies have shown that *miR-34a* is a tumor suppressor miRNA that is silenced in several types of cancer because of aberrant CpG methylation in its promoter region [15].

Our novel miRNA promoter array can be used to carry out ChIP-on-chip assays to identify miRNAs, which are regulated by other epigenetic marks, such as histone H3-K27 methylation, as well as to determine the specific transcription factors, which bind to miRNA promoters. Further studies are necessary to gain comprehensive understanding of the regulatory mechanism of miRNA expression and to identify critical miRNAs as therapeutic targets for epigenetic therapy of human cancer.

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