Identification of non-coding RNAs embracing microRNA-143/145 cluster

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Abstract
In a variety of cancers, altered patterns of microRNA (miRNA) expression are reported and may affect the cell cycle and cell survival. Recent studies suggest that the expression level of miRNAs that act as tumor suppressors is frequently reduced in cancers because of chromosome deletions, epigenetical changes, aberrant transcription and disturbances in miRNA processing. miR-143 and -145, which are located approximately 1.3 kb from each other at chromosome 5q33, are highly expressed in several tissues, but down-regulated in most cancers. However, the mechanism of this down-regulation has not been investigated in detail. Here, we show that both miRNAs were expressed well under the same control program in human tissues, but were down-regulated equally in the most of the cancer cell lines tested. Then we identified the host gene encoding both miRNAs. The transcripts of this gene were approximately 11, 7.5, and 5.5 kb long, and the expression of these transcripts was coordinated with that of its resident miRNAs and down-regulated in the cancer cell lines tested as well as in colorectal cancer tissue samples. These data demonstrate that the host gene can function as a primary miRNA transcript and suggest that the down-regulation of host gene expression caused the low-expression of its encoded microRNAs-143 and -145 in human cancer cell lines and in cancer tissues.

Findings
MicroRNAs (miRNAs) are tiny, endogenously expressed noncoding RNAs (18-25 nucleotides in length) that act as crucial posttranscriptional regulators of gene expression [1-3]. For several miRNAs, their participation in essential biological processes has been proved, such as in cell proliferation control, cell lineage fate decision, cell survival, tissue patterning for development, and cell metabolism [4]. Cancer is a very complex genetic disease characterized by alterations in genes encoding oncogenic and tumor-suppressor proteins [5]. Recently, it has been noted that the expression profiles of miRNAs can be used for classification, diagnosis, and prognosis of human malignancies; and the deletion or amplification of the locus encoding an miRNA in a variety of cancers has been reported. Altered patterns of miRNA expression may affect cell-cycle and survival programs and be involved in tumor initiation and progression. We previously found that microRNA-143 (miR-143) and -145 (miR-145) were down-regulated in colon cancers [6,7], gastric cancers [8], chronic lymphocytic leukemias, and B cell lymphomas [9], and in several human cancer cell lines [7]. Several groups also reported the down-regulation of both of these miRNAs in many other types of cancers, such as bladder cancers and their cell lines [10,11], cervical cancers and their cell lines [12], colorectal cancers [13-16], nasopharyngeal carcinoma [17], and prostate cancer [18]. Furthermore, such abnormal expression was found not only in malignant cells but also in cells in premalignant stages such as colon adenoma cells [13,19]. The introduction of the mature type of either miR-143 or -145 into colon cancer cells [6,7,20], B cell lymphoma [9], and gastric cancer cells [8,21] results in a significant growth inhibition that occurs in a dose-dependent manner; and the target genes, ERK5 [22] and KRAS [20] for miR-143 and IRS-1 [23] and c-myc [21] for miR-145, were posttranscriptionally down-regulated. Taken together, these findings suggest that miR-143 and -145 act as tumor suppressors and provide an important clue in the study of the mechanism of tumor initiation and progression involving miRNAs.

In the present study, we identified non-coding RNAs carrying an miR-143 and -145 cluster (NCR143/145: Non-coding RNA encoding miR-143/145) and investigated the expression of NCR143/145 in all cancer cell lines tested. Importantly, the down-regulation of this host

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gene expression caused the low expression of both miRNAs in human cancer cell lines, which could lead to tumor development and progression.

Expression of miR-143 and -145
We examined the expression levels of mature miR-143 and -145 in human normal tissues by performing TaqMan microRNA assays (Fig. 1). In human normal tissues, miR-143 and -145 showed good expression in stomach, intestine, cervix, uterus, colon, and prostate (Fig. 1A, B). Whereas, in cancer cell lines, they were expressed at an extremely low level compared with that in human normal cell lines (Additional file 1 - Figure S1). Compared with their expression in corresponding normal tissues, the expression levels of both miRNAs were obviously down-regulated in all cancer cell lines and cancer tissue samples tested, just as many groups had previously reported [6-18]. Such a similar expression pattern of them indicates that the expression of both miRNAs may be regulated by a similar mechanism. Additionally, the DNA loci of both miRNAs are very close to each other, within 1.3 kb, which led us to speculate that both precursors may originate from the same primary transcript. Genomic PCR spanning this region demonstrates the fragment in most of the cancer cell lines tested [6, 7, 9]. Therefore, we decided to isolate the gene that carried both miRNAs in a cluster.

Identification of non-coding RNA carrying the miR-143/145 cluster
First, we carried out RT-PCR and inverted PCR cloning method using human placenta and uterus cDNA, and a placental cDNA library, and isolated each of the cDNA clones designated in Figure 2. The 143-145 clone was 2.2 kb long and detected in human tissues such as uterus, prostate, and testis by RT-PCR (data not shown). The iPCR145 clone, which encoded miR-145, was 1.7 kb long and corresponded to the transcriptional unit for only miR-145 identified by Sachdeva [21] and Xu [24]. Clone 41 was 373 bp long (Fig. 2B) and amplified at a high level in human normal tissues by semi-qRT-PCR, but hardly amplified in several cancer cell lines examined (data not shown). Clone AK126481 was 3.8 kb long and identical to AK126481 in GeneBank, and clone AKF1-10 was 1.8 kb long and overlapped with AK126481. Clone 4-35 was 129 bp long and contained a part of the predicted first exon and novel second exon (Fig. 2A). At the upstream of this predicted first exon, hypothetical transcriptional start site was localized, which was shown by Fujita [25]. Also, the homolog of this gene (IE 1071) and promoter region were cloned in the mouse by Ebisuya [26] and shown to be comparatively conserved between human and mouse. This indicates that the predicted transcriptional start site near the sequence of clone 4-35 is a putative promoter region of the miR-143 and -145-encoding gene.

Next, we performed Northern blot analysis to look for the transcripts that originated from the host gene encoding miR-143 and -145. The large transcript (11 kb: open arrow) and 3 or 4 transcripts (7.5, 5.5, and 1.9 kb: closed arrows) were detected (Fig. 3). The 11-kb transcript was hybridized with 6 probes (Fig. 2, a-f; Fig. 3), and the 1.9-kb one was only detected by 143-145 (Fig. 3) and iPCR145 probes (data not shown), and not detected by the 4-35, 41, AKF1-10 or AK126481 probes (data not shown). These results indicate that the host gene was firstly transcribed into the 11-kb transcript and then processed to the mature miR-143 and -145 via 7.5 and 5.5 kb processed variant transcripts. This gene is the non-coding RNA shown by Ebisuya to be subject to splicing [26]. Also, in human normal tissues, miR-145 was consistently expressed at higher levels than miR-143 (Fig. 1A, B). Apart from both miRNAs being produced from the 11-kb transcript, miR-145 would also be generated from the other transcript.
Figure 2: Identification and characterization of the host gene encoding miR-143 and -145. (A) Scheme of the cytogenetic map of chromosomal region 5q33. RT-PCR cloning was performed by using the primer sets that covered the predicted first exon-containing region (clone4-35) of LOC728264 [GeneBank:NR027180], predicted intron-spanning region (clone41), both miR-143 and -145 regions (143-145), predicted pre-miR-145 region (iPCR145), AKF1-10 region (e), and AK126481 region (GeneBank:f) from human placenta and uterus cDNA, and cDNA library. The positions of neighboring genes, PCYOX1L, IL17B, CSNK1A1, and FLJ41603 are also shown as references. The real-time RT-PCR primer sets (LOC3 and 10) specific for this gene are indicated by the arrowheads. The open box (E') represents a novel exon derived from a cDNA clone (a). This exon is not shown in the NCBI database. Region "A" is a cardiac-specific enhancer reported by Cordes [29], and region "B" is p53 and/or Oct4-dependent miR-145 specific promoter reported by Sachdeva [21] and Xu [24].

| Forward primer | Reverse primer |
|----------------|----------------|
| (a) clone4-35  | 5'-CCAACACAGCTCTCAAAACAGGCTGG-3' 5'-TTACAGCGTGTCTTCCTTT-3' |
| (b) clone41    | 5'-GCACTGCACTGAGCTCAGAAGA-3' 5'-GATCACAGCTCAGGACATGAGA-3' |
| (c) 143-145    | 5'-TTGGGTCTGCTGCTCTAAAT-3' 5'-TTCCCAAGAGTACGCGAGT-3' |
| (d) iPCR145    | 5'-GAATCCCCATGATGATGTCATGATG-3' 5'-CTGTGACATTGCTGTGAGGAGT-3' |
| (e) AKF1-10    | 5'-GCGCATAGACCCCACCTCTATC-3' 5'-CTCGTGCATTTGGCTGGGATT-3' |
| (f) AK126481   | 5'-CTGTGACATTGCTGTGAGGAGT-3' 5'-CTCGTGCATTTGGCTGGGATT-3' |
| LOC3          | 5'-AGCACAGACCGAGAAAGA-3' 5'-TTACAGCGTGTCTTCCTTT-3' |
| LOC10         | 5'-AGCGAAGCTCTGGAGAAGCA-3' 5'-GAGGCAGCTGAGGAGGAGT-3' |
| GAPDH(for real-time) | 5'-CCACATCGCGTACACCAT-3' 5'-GCAAAGATACCCATTTCCAGAGTAA-3' |
| GAPDH(for cloning) | 5'-CCACCAGGCAATTCCATGCCA-3' 5'-TCTAGACGCGGAGGCTAGTCCACC-3' |
Figure 3 Northern blot analysis of the host gene encoding miR-143 and -145. Total RNAs (5 μg for human normal tissues, cancer tissue, and cancer cell lines [human stomach tumor RNA was purchased from Takara]) were separated on a 1%(w/v) formaldehyde agarose gel and then blotted onto Hybond N+ nylon membranes (Amersham Biosciences, Piscataway, USA). RNA probes were synthesized from template cDNAs by using a MAXIscript kit (Ambion, Austin, USA) incorporating Digoxigenin-11-UTP (Roche, Penzberg, Germany). Northern blots were hybridized with the AKF1-10 clone (A, upper panel; refer to Fig. 2), 143-145 clone (A, 2nd panel), GAPDH (A, 3rd panel, B, middle panel), and 28S rRNA (A and B, under panel). The primary transcript is marked by the open arrow and other transcripts are marked by closed arrows.
1.9-kb transcript. It is thought that this expression of miR-145 is regulated by a different mechanism dependent on p53 [21] and/or Oct4 [24]. In our preliminary experiments, the expression of host gene and its promoter activity were p53-independent in p53-mutated cancer cell lines (MKN-45 and DLD-1, data not shown). This finding of p53 independency raises the possibility that p53-dependent gene expression and other pathways are abrogated in p53-mutated cancer cell lines. We are currently investigated this point in our laboratory.

**Regulation of NCR143/145 expression in cancers**

Most miRNAs located within protein-coding or non-coding genes are transcriptionally linked to the expression of their host genes [27]. In order to investigate the coordinated expression of the host gene identified in this study with mature miR-143 and -145, we performed real-time RT-PCR analysis by using the host gene-specific primer set shown in Fig. 2. In human normal tissues, the host gene was highly expressed, as were both miRNAs; but in the corresponding cancer cell lines, the signal was hardly detected (Fig. 4A), though the host gene and both miRNAs were highly expressed in normal human cell lines (Additional files 1 - Figure S1 & S2). Also in human cancer tissues, the host gene was down-regulated compared with its expression in normal human tissues (Figs. 3, 4B and additional file 1 - Figure S3). As a result, the down-regulation of host gene NCR143/145 expression caused low expression of both mature miRNAs in human cancer cell lines. Thus, the aberrant transcription of NCR143/145 could contribute to the low expression of miR-143 and -145.

The expression level of miRNAs that act as tumor suppressors is frequently reduced in cancers because of chromosome deletions, epigenetical changes, aberrant transcription, and disturbances in miRNA processing. Michael et al. [13] reported that in colorectal cancers the decreased levels of mature miR-143 and -145 were due to reduced Dicer-processing activities. In our study, the activity and expression of Dicer and RISC proteins seemed to be intact in colorectal cancer cells, because the expression levels of miR-143 and -145 were up-regulated by stimulation with a growth inhibitor [7]. Therefore, we propose that the inadequate expression of miR-143 and -145 was due to the perturbation of transcription and/or that of the another processing enzyme, Drosha, which causes the transit from primary miRNAs to precursor ones. Recently, it was reported that p53 interacts with the Drosha processing complex through association with DDX5 and facilitates the processing of primary miRNAs to precursor ones [28]. That report also indicated that mutant p53 interfered with these processing activities. These findings suggest that the inappropriate p53-dependent modulation of miRNA biogenesis also affects the expression of mature miRNAs in cancer cells. But, in our study, Drosha expression and its processing activity seemed to be normal in p53-mutated MKN-45 and K562 cells (data not shown).

To exclude the possibility that the DNA of the loci and histone were epigenetically methylated, in earlier studies [6,7,9] we examined by qRT-PCR the expression of both miRNAs in DLD-1, SW480 and EBV-transformed L25 cells after treatment of the cells with 5-aza-2’-deoxycytidine and tricostatin A. As a result, the levels of both miRNAs were almost unchanged by either treatment [6,7,9].
To confirm the presence of the genomic locus including both miRNAs at chromosome 5q33, we carried out genomic PCR on several cancer cell lines (Additional file 1 - figure S4) [6,7,9]. In HeLa, U937, and PC3 cells, one allele of the locus might have been deleted. This locus is frequently involved in chromosome copy number loss in various types of cancers including non-small cell lung cancer and gastric cancer according to the CGH database http://www.cghtmd.jp/CGHDatabase/index_j.jsp. Therefore, further detailed cytogenetic study will be needed to understand the mechanism of miR-143 and -145 down-regulation in many cancer cell lines.

Additional material

Additional file 1 Supplementary figures. Figure S1: Real-time RT-PCR analysis of mature miR-143 and -145 expression in human cell lines. Relative miR-143 (A) and -145 (B) expression levels are indicated on the left axis by using the comparative ΔCt method (value of 2^(-ΔΔCt)(miR-143/18s)). Figure S2: Real-time RT-PCR analysis of NCR143/145 expression in human cancer cell lines and normal cell lines. The relative expression level of NCR143/145 in human cancer cell lines was compared with that in human normal cell lines (WI-38 and IMR-90) by using the LOC10 primer set (see Fig. 2). Figure S3: Real-time RT-PCR analysis of NCR143/145 expression in human stomach. The relative expression level of NCR143/145 in human stomach tumor was compared with that in normal human stomach by using the LOC10 primer set (see Fig. 2). Figure S4: Confirmation of the presence of genomic loci of miR-143 and -145 at chromosome 5q33 by genomic PCR. We extracted genomic DNAs from 2 cell lines and normal human oral squamous cells by using DNAzol (Invitrogen, Carlsbad, USA) and used them for PCR. The 143-145 primer set was used for genomic loci of miR-143 and -145 (see Fig. 28). The genomic locus of GAPDH was used as an internal control.

Abbreviations

ERRX: extracellular signal-regulated kinase 5; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; IRS-1: insulin receptor substrate 1; OCT4: Octamer-4; RISC: RNA-induced silencing complex; DDX5: DEAD box polypeptide 5; EBV: Epstein-Barr virus; CGH: Comparative genomic hybridization; UTP: uridine triphosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; rRNA: ribosomal RNA.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

AI and YA conceived and planned the experiments. TN and YA provided the human cancer cell lines. YN and IH collected the clinical specimens. AI performed all experiments. All authors read and approved the manuscript.

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