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ORIGINAL PAPER

Downregulation of anti-oncomirs miR-143/145 cluster occurs before APC gene aberration in the development of colorectal tumors

Akemi Kamatani · Yoshihito Nakagawa · Yukihiro Akao · Naoko Maruyama · Mitsuo Nagasaka · Tomoyuki Shibata · Tomomitsu Tahara · Ichiro Hirata

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Abstract Accumulating data indicate that some microRNAs (miRNAs or miRs) can function as tumor suppressors or oncogenes and as such are important in cancer development. We previously reported that miR-143 and -145 are frequently downregulated in colon adenomas and cancers, acting as tumor suppressors. In this present study, we investigated the relationship between the downregulation of the miR-143/145 cluster and genetic aberrations of adenomatous polyposis coli (*APC*), which are early genetic events in the development of colorectal tumors. The expression levels of both miRs were determined by performing real-time PCR on tissue samples of familial adenomatous polyposis (FAP), colorectal adenoma, colorectal cancer, and paired non-tumorous tissues. Also, the expression of C- or N-terminus of the APC protein and that of the p53 protein in these tissues were examined immunohistochemically. Our data clearly indicated that the decreased expression of miR-143 and -145 frequently occurred before *APC* gene aberrations. The downregulation of miR-143 and -145 is thus an important genetic event for the initiation step in colorectal tumor development.

Keywords microRNAs-143 and -145 · APC gene · Colorectal tumor development

A. Kamatani · Y. Nakagawa · N. Maruyama · M. Nagasaka · T. Shibata · T. Tahara · I. Hirata
Department of Gastroenterology, School of Medicine, Fujita Health University, Kutsukake-cho, Toyoake, Aichi, Japan

Y. Akao (✉)
The United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1193, Japan
e-mail: yakao@gifu-u.ac.jp

Introduction

MicroRNAs (miRNAs or miRs) are endogenous ~22-nt non-coding RNAs that regulate gene expression by inhibiting the translation of mRNAs in a sequence-specific manner [1–3]. More than 2000 miRNAs in the human genome have already been identified (http://www.mirbase.org/), and up to one-third of all human mRNAs are predicted to be miRNA targets [3]. Each miRNA can target more than 200 different transcripts directly or indirectly [4, 5], and more than 1 miRNA can converge on a single mRNA target [3, 6]. Therefore, the potential regulatory circuitry afforded by miRNAs is enormous. These findings support the notion that alterations of miRNA copy number and their regulatory genes should be highly prevalent in cancer, because genomic aberrations are closely associated with carcinogenesis. Recent increasing evidence shows that the expression of miRNA genes is deregulated in human cancers [7, 8]. Among the tumor-associated miRNAs, miR-143 and -145 are well-established tumor suppressor miRNAs. Since they are transcribed at chromosome position 5q33 as the same primary non-coding RNA (NCR143/145), they are concomitantly down-regulated in most of the cancers [9]. Previously, we reported that both of these miRs are downregulated in colon adenomas as well as in cancers [10].

The adenomatous polyposis coli (*APC*) gene is located in the human chromosome region 5q21-22 [11, 12] and is mutated not only in familial adenomatous polyposis (FAP) but also in most of the sporadic colorectal tumors [11, 13, 14]. Although mutations of *APC* have also been found in gastric and pancreatic cancers, such mutations are not so high in frequency. In tumors other than those found in the alimentary tract, *APC* mutations have rarely been found. Almost all germline and somatic mutations of *APC* found to date are nonsense or frame-shift ones that result in the carboxy

terminal truncation of its gene product (APC). Thus, *APC* is considered to be a tumor suppressor gene [15], and this mutation occurs during the initiation of colon tumor development.

In this study, we investigated the relationship between the expression of miR-143 and -145 and *APC* gene aberrations in the adenoma to carcinoma transition of colorectal cancer.

Materials and methods

Patients and tissue preparation

All human samples were obtained from patients who had undergone a biopsy for diagnosis or surgery for resection at Fujita Health University Hospital (Toyoake, Aichi, Japan), Saiseikai Ibaraki hospital (Ibaraki, Osaka, Japan), Osaka Medical College Hospital (Takatsuki, Osaka, Japan), or Kyoritsu General Hospital (Nagoya, Aichi, Japan) between 2002 and 2011. Two pathologists diagnosed each sample. Informed consent in writing was obtained from each patient. The FAP study group consisted of 2 women and 3 men with a median age of 42.2 (range 21–75). The non-adenoma study group (a patient with Peutz–Jeghers syndrome and Cronkhite–Canada syndrome) consisted of 2 men 67 and 40 years of age, respectively.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from the tissues by the use of TRIzol containing phenol/guanidine isothiocyanate and treatment with DNase I. RNA concentration and purity were assessed by ultraviolet spectrophotometry. RNA integrity was checked electrophoretically. In order to examine the expression levels of miR-143 and -145 in detail, we performed TaqMan® MicroRNA Assays using a real-time PCR apparatus (Life Technologies. Grand Island, NY, USA) [10, 16]. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. The levels of miR-143 and -145 in each tissue were measured and normalized to U6, which was used as an internal control. The expression levels in tumors were designated as down-regulated when the fold change from the expression in the non-tumorous tissue was 0.67, as determined from the results of linear discriminant analysis of miRNA expression patterns from 156 pairs of colon tumors and non-tumorous tissues. The tumor/non-tumor ratio of each miRNA expression in the samples was determined.

Immunohistochemical examination

A series of four 3-mm sections cut from paraffin-embedded tissues of each tissue specimen was prepared. One section was stained with hematoxylin eosin, and the other 3 were used for immunohistochemical staining performed with a Vector Stain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). The method of immunohistochemical staining was performed as recommended by the manufacturer and described in a previous report [17]. The antibodies used included antibodies against the human C-terminus of APC (C20, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), N-terminus of APC (Epitomics, Inc. Burlingame, CA, USA), and p53 protein (DO7, Leica Biosystems, Buffalo Grove, IL, USA). The localization of immunoreactive complexes was visualized by incubation of the section for 5–10 min in 1 M Tris–HCl, pH 7.6, contain 0.02 % (w/v) 3,3′-diaminobenzine tetrahydrochloride, and 0.03 % (v/v) hydrogen peroxidase. Counterstaining was performed with hematoxylin. Evaluation of staining for APC was based on the tone of the brown stain in the cytoplasm. We divided APC staining into 3 levels according to its tone (Fig. 1). The evaluation of mutant p53 expression was performed by observing the nuclear staining.

Statistics and data analysis

Statistical differences between miRNA levels and immunohistochemical data of tumor samples were evaluated by using Pearson's chi-square test or Fisher's extract test for differences between the 2 groups. A p value of 0.05 was considered to be significant. All calculations were performed by using the software JMP (version 5.1, SAS Inc, Cary, NC, USA).

Results

The frequency of downregulated expression of miR-143 and -145 in colon tumors

We firstly analyzed the expression levels of miR-143 and -145 by performing real-time PCR on 23 tumor samples from the 5 patients with FAP, 67 samples of sporadic adenomas, and 89 samples of colorectal cancers, and 5 polyp samples from 2 patients, one with Peutz–Jeghers syndrome (PJS) and the other with Cronkhite–Canada syndrome (CCS), compared with those in the paired normal samples (Table 1). The expression levels of miR-143 and -145 were different between the tumor and non-tumor adjacent mucosa. The downregulated expression of both miRs was found in the majority of adenomas tested except those from PJS and CCS patients, as well as in most of the carcinomas (Tables 1, 2), which suggests that the decreased expression of miR-143 and -145 was closely associated with the initiation of tumor development. Importantly, both

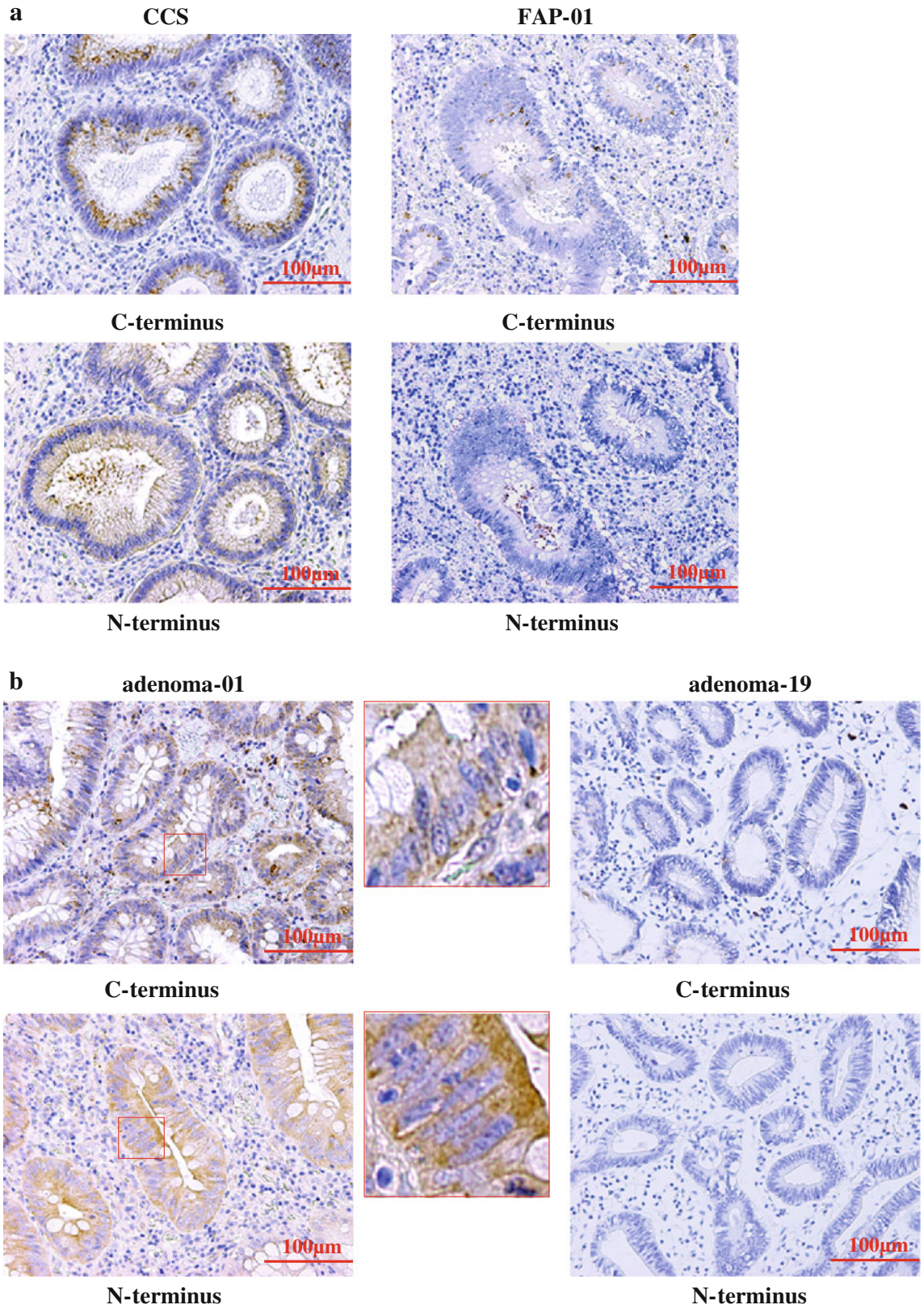

Fig. 1 Immunohistochemical staining for N-terminus or C-terminus of APC protein in human colorectal tumors. APC detected with antibodies specific for the N-terminus or C-terminus of APC is detectable in the cytoplasm of adenoma cells from CCS (**a**) and adenoma-01 (**b**); whereas FAP-01 (**a**) and adenoma-19 (**b**) show no staining

Table 1 Characteristics of the study population and the frequency of down-regulated miR-143 and -145 in colon tumors

	Colorectal cancer ($n = 89$)	Colorectal adenoma ($n = 67$)	Familial adenomatous polyposis ($n = 23$)	CCS or PJS ($n = 5$)
miR-143↓	64/89 (71.9 %)	46/67 (68.7 %)	20/23 (87.0 %)	2/5 (40.00)
miR-145↓	69/89 (77.5 %)	46/67 (68.7 %)	20/23 (87.0 %)	1/5 (20.00)

CCS Cronkhite–Canada syndrome, *PJS* Peutz–Jeghers syndrome

Table 2 Expression profiles of miR-143 and -145, APC, and p53

Pathological findings	Immunohistochemistry			miRNA	
	APC C-terminus 1:100	APC N-terminus 1:100	p53 D07 1:100	miR-143	miR-145
FAP-01	−	−	N	0.03	0.07
FAP-02	−	−	N	0.24	0.2
FAP-03	−	−	N	0.47	0.43
FAP-04	−	−	N	0.82	0.67
Adenoma-01	++	++	N	0.06	0.09
Adenoma-02	++	+	N	0.17	0.13
Adenoma-03	++	+	N	0.02	0.03
Adenoma-04	−	+	N	0.06	0.01
Adenoma-05	+	++	N	0.04	0.05
Adenoma-06	+	++	N	0.11	0.12
Adenoma-07	++	+	N	0.08	0.21
Adenoma-08	+	+	N	0.19	0.28
Adenoma-09	+	+	N	0.33	0.36
Adenoma-10	++	+	N	0.23	0.36
Adenoma-11	−	+	N	0.28	0.18
Adenoma-12	+	+	N	0.1	0.13
Adenoma-13	++	+	N	0.02	0.02
Adenoma-14	++	+	N	3.8	2.08
Adenoma-15	++	+	N	2.49	1.81
Adenoma-16	++	+	N	1.83	1.1
Adenoma-17	+	−	N	0.92	1.61
Adenoma-18	++	+	N	0.69	0.7
Adenoma-19	−	−	N	0.77	0.625
Carcinoma-01	−	−	N	0.2	0.3
Carcinoma-02	−	−	P	0.06	0.1
Carcinoma-03	−	−	N	0.29	0.28
Carcinoma-04	−	−	N	0.07	0.13
CCS	++	+	N	0.86	7.67
PJS	+	+	N	0.75	0.9

	miR-143↓ and miR-145↓	Mutated *APC*
Sporadic adenoma	13/19	4/19
p value	$p = 0.0033$	

APC protein: Positive staining is indicated as ++ (strong staining) or + (weak staining). Negative staining is − (no staining). p53 protein: Positive staining is indicated as "P" and negative staining as "N." (negative staining)

miRNAs were down-regulated concomitantly in most cases (Table 2), which is not surprising because they are transactivated as the same primary miRNA, NCR143/145 [9]. On the other hand, in PJS and CCS samples, the expression levels of miR-143 and -145 were almost the same as those in the paired normal samples in most cases (Table 1).

Correlation between the downregulation of miR-143/145 cluster and APC gene aberrations

Based on these results, we focused on the relationship between the aberrations of the *APC* gene and the downregulation of miR-143 and -145. We examined the expression levels of the C- and N- terminus of APC and mutant p53 protein as estimated by immunohistochemical staining of the tumor samples (Table 2). We defined a case as having a mutated *APC* when the staining for either N- or C-terminus APC was judged to be negative [15]. We confirmed the expression of APC protein in the samples from PJS and CCS as positive controls. As shown in Fig. 1, the staining with antibody specific for either N- or C-terminus APC was evident in the cytoplasm of adenoma cells of CCS. In contrast, the staining in adenomas from FAP (Fig. 1) and a few carcinomas was considerably weaker with either antibody. The *APC* mutations (i.e., negative staining) occurred in all of the samples from the patients with FAP or cancer. On the other hand, 15 out of 19 sporadic adenomas showed good expression levels of both N- and C-terminus APC (Fig. 1; Table 2). When we compared the ratios of the down-regulation of miR-143 or -145 to either the N- or C-terminus APC mutation in the sporadic adenomas, 12 out of 15 cases expressing non-mutated APC showed significantly reduced expression levels of miR-143 and -145 ($p = 0.0033$; Table 2). Of course, most FAP cases showed the reduced expression of miR-143 and -145. As to mutant p53, only 1 sample among the 4 carcinomas, all of which had APC mutations, was positive for p53 staining. These findings indicate that the downregulation of both miR-143 and -145 could have occurred before the expression of *APC* gene aberrations during tumor development.

Discussion

Familial adenomatous polyposis, in which adenomas transform obviously into adenocarcinomas, has been considered to be a typical model for the adenoma–carcinoma sequence theory. In this theory, no matter whether the tumors are hereditary colon cancers or non-hereditary, they are thought to become malignant in the following manner: normal epithelium → adenoma → carcinoma → metastasis. In addition, abnormalities of many oncogenes and tumor suppressor genes are involved in this sequence of events [18, 19]. The APC gene is mutated at the earliest step, and p53 gene aberration is later in this sequence of events [19, 20]. In the present study, this sequential event was also true, because APC aberrations were frequently observed in FAP and sporadic adenomas, but not in carcinomas. And mutant p53 was positive in one of the 4 carcinomas.

On the other hand, the inappropriate expression of miRNAs is closely associated with cancer development. The combined decreased expressions of miR-143 and -145 are frequently observed in most of the cancers and even in colon adenomas, and these miRNAs function as tumor suppressors [9, 10, 16]. We previously reported that the downregulation of both mature miRNAs is due to aberrant transcription of the primary miRNA (NCR143/145) [9]. MiR-145 targets *c-myc* mRNA and inhibits the expression of its protein at the translational level [21]. Also, miR-143 targets *Erk5*, which downstream target molecule in a signaling pathway leads to transactivation of *c-myc* expression [22]. Therefore, the downregulation of both miRNAs results in the upregulation of c-myc, which would be an essential event in colon tumor development. Although the sequential genetic events of downregulation of miR-143 and -145 clusters and APC mutation are mutually independent, both events could contribute to constitutional activation of Wnt/b-catenin signaling, which is a central major growth signaling in colon cancer cells. In conclusion, we determined that the downregulation of miR-143 and -145 preceded *APC* aberrations in the early stage of colorectal tumor development. To elucidate the significance of downregulation of miR-143 and -145 clusters before *APC* mutation will be needed for further understanding the colon tumor development.

Conclusion

The expression levels of miR-143 and -145 were different between the tumor and non-tumor adjacent mucosa. The downregulated expression of both miRs was found in the majority of adenomas as well as in most of the carcinomas. We compared the ratios of the downregulation of miR-143 or -145 to either the N- or C-terminus APC mutation in the sporadic adenomas, and the majority of cases expressing non-mutated APC showed significantly reduced expression levels of miR-143 and -145. These findings indicate that the downregulation of both miR-143 and -145 could have occurred before the expression of *APC* gene aberrations during tumor development.

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