Cdx2, *Cdx1*, and *Cdx4* was cloned into the EcoRI site of the pMXs-IRES-Puro retroviral vector (Cell Biolabs Inc., San Diego, CA). How to obtain the full length cDNA for *Cdx2*, *Cdx1*, and *Cdx4* was described in Supplementary materials and methods. Viral constructs encoding shCdx2 targeting 5′-gccaaaggcagctaagataga-3′ and 5′-gcccgaa-cagggacttgttta-3′ sequences of human *Cdx2* ("shCdx2-1" and "shCdx2-2" respectively), shBrm targeting the 5′-gaatgtggtgtt-ggtgctttc-3′ sequence of human *Brm*, shBRG1 targeting the 5′-gtagctccgaggtctgatagt-3′ sequence of human *BRG1*, shIni1 targeting the 5′-gacatgtcagagaaggagaac-3′ sequence of human *Ini1*, and shGFP targeting *GFP* were prepared as described previously [11].

Luciferase reporter assay

SW480 cells (3×10^4) were seeded in 96-well (0.32 cm^2) plates, and transfected with 6.5 ng of Renilla luciferase control vector pGL4.74 (Promega, Madison, WI), and either 200 ng of Firefly luciferase experimental vector pGL4.12 (Promega), pGL4.12-

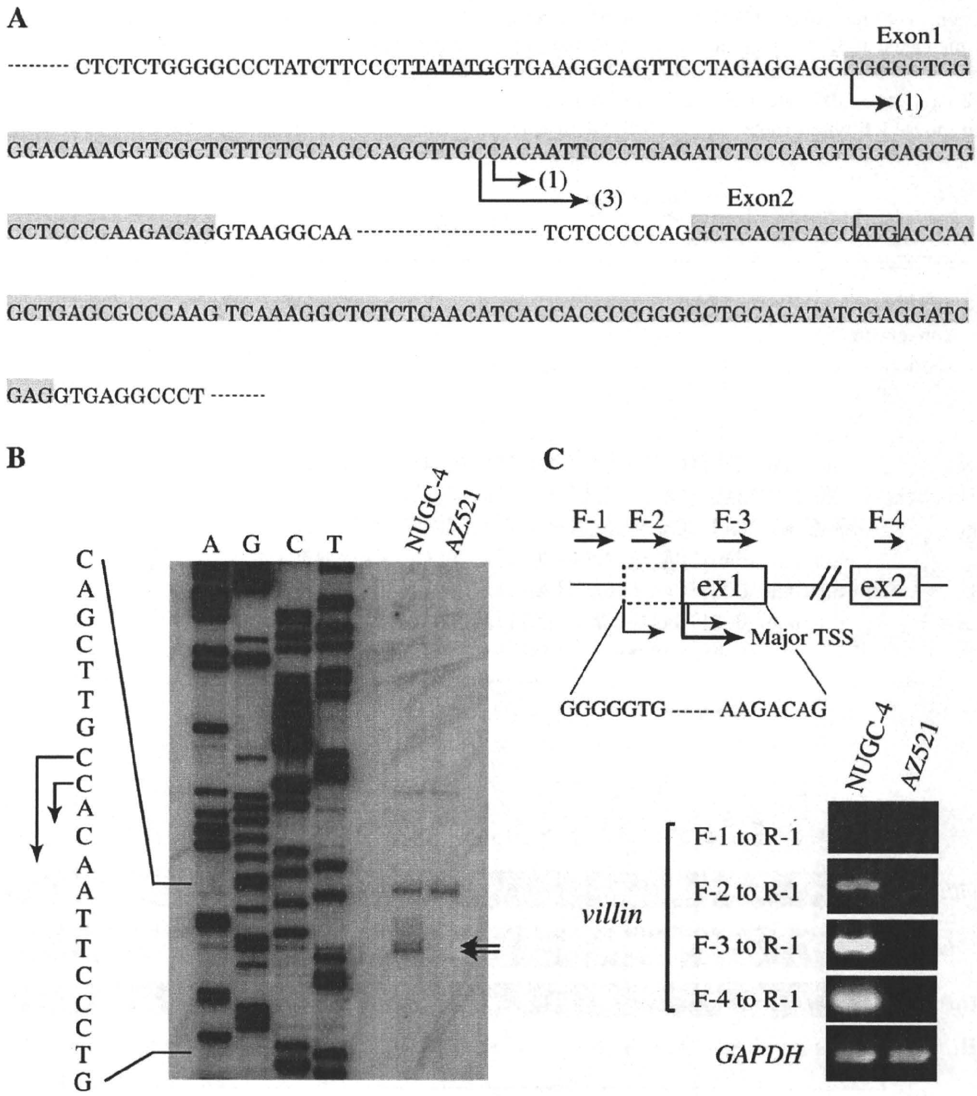

Fig. 1 – (A) Sequences around the transcription initiation sites of the human *villin* gene. Exons 1 and 2 are highlighted by the gray boxes. Three rectangular arrows indicate the 5′-ends of the *villin* transcripts identified using 5′-RACE, and the numbers of clones obtained in this analysis are denoted in parentheses. The ATG translation start codon of the *villin* gene in the exon 2 is also indicated, as is the presumed TATA box in the gene promoter (underlined). (B) Determination of the TSS of the human *villin* gene by primer extension analysis. Villin-expressing NUGC-4 and villin-deficient AZ521 cells were used, and two clear bands (indicated by arrows) were specifically detected in only the NUGC-4 cells. These bands correspond to the TSSs determined by 5′-RACE in (A). (C) RT-PCR analysis of human *villin* using a reverse primer located in the exon 6 (R1) and four forward primers located in the upstream region of exon 2 (F-1/F-2/F-3/F-4). The sequence locations corresponding to each primer are shown in the upper schema, in which the three TSSs determined by primer extension and 5′-RACE are also shown.

villinBX, pGL4.12-villinI, pGL4.12-villinII, or pGL4.12-villinIII. Construction of each vector plasmid was described in the Supplementary materials and methods. All transfections were performed with Lipofectamine2000 Reagent and Lipofectamine PLUS (Invitrogen). Luciferase activities were measured at 48 h post-transfection using the Dual luciferase reporter assay system (Promega).

Chromatin immunoprecipitation (ChIP) assay

ChIP analyses were performed using a ChIP assay kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer's instructions. For crosslinking, SW480 cells or HT-29 cells (5×10^6) were incubated at 37 °C for 15 min in PBS containing 1% formaldehyde. Immunoprecipitations were performed overnight at 4 °C with 2 μg of anti-Cdx2 antibody (CDX2-88; BioGenex), nonimmunized mouse IgG whole molecule (sc-2025; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Brm antibody (ab15597; Abcam), anti-BRG1 antibody (sc-17796; Santa Cruz), or nonimmunized rabbit IgG whole molecule (sc-2027; Santa Cruz). After reverse crosslinking, the obtained DNA was purified using phenol/chloroform extraction followed by ethanol precipitation. PCR amplification procedures detecting the HCR-Cdx, exon 1 and 2 of the *villin*, and the *CD44* promoter region using the purified DNA were described in the Supplementary materials and methods.

GST pull-down assay

Recombinant Cdx2 was expressed in *E. coli* (Rosetta2 strain) as a glutathione S-transferase (GST) fusion protein. ^{35}S-labeled subunits of SWI/SNF complex (Brm, BRG1, Ini1, BAF60a, and β-action) were synthesized in rabbit reticulocyte lysates (Promega) or wheat germ lysates (Promega). For the expression of GST-Cdx2, *Cdx2*

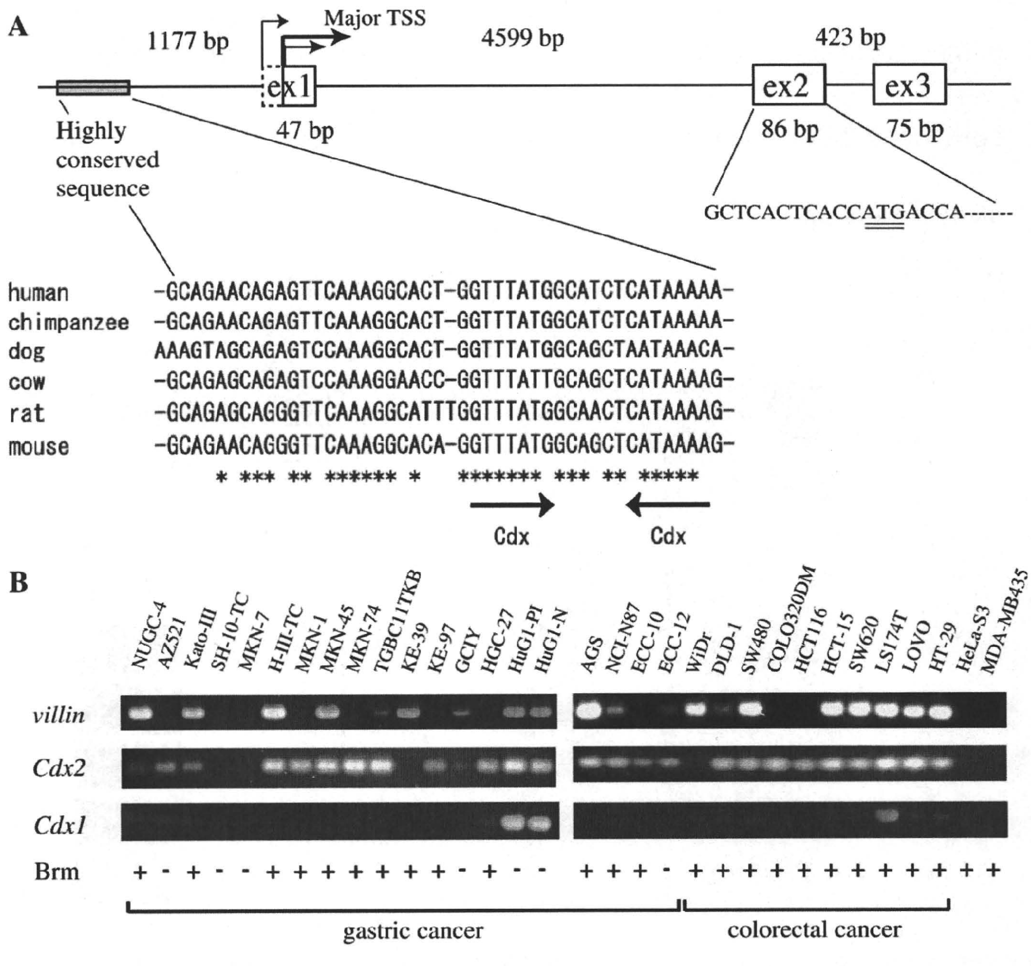

Fig. 2 – (A) Schematic representation of the human *villin* gene structure and sequences of the highly conserved regions harboring two putative Cdx binding sites (HCR-Cdx) in the *villin* promoter regions of six mammal species. In the human *villin* genomic sequence, the HCR-Cdx is located about 1.1 kb upstream of exon 1. The two consensus binding elements for the Cdx family of transcription factors are indicated by arrows. In the schema for the human *villin* gene, the three identified TSSs (including major TSS) are indicated, and the translation start ATG in exon 2 is double underlined. (B) Expression patterns of the *villin*, *Cdx2*, and *Cdx1* mRNAs in a panel of 32 human cancer cell lines. Twenty gastric cancer cell lines, 10 colorectal cell lines, and two non-gastrointestinal cell lines (HeLa-S3 and MDA-MB435) were analyzed by RT-PCR. The presence of Brm protein is denoted by the + and − symbols as in our previous reports [8,11].

cDNA excised from pMXs-Cdx2-IRES-puro was subcloned into the EcoRI site of pGEX-4T2 vector (GE Healthcare, Madison, WI). For the expression of other proteins, *Brm*, *BRG1*, *Ini1*, and *β-action* cDNA inserted into pBluescript-SK(+) (Stratagene) and *BAF60a* cDNA inserted into pOTB7 (Open Systems) were used. GST pull-down assay was performed as described previously [14] except that washing was performed 6 times rather than 4 times. SDS-PAGE was performed as described previously [14].

Nuclear extraction and immunoprecipitation

Nuclear extracts were prepared from SW480-Cdx2 cells (SW480 cells stably transduced with MuLV-based retrovirus vectors carrying *Cdx2* gene) as described previously. The nuclear extracts were incubated at 4 °C overnight with normal either anti-Brm (Abcam, ab15597) or normal rabbit IgG (Santa Cruz, sc-2027) in TNE buffer [10 mM Tris–HCl (pH 7.8), 1% NP40, 150 mM NaCl, and 1 mM EDTA] with 0.01 M PMSF and protease inhibitors. Protein A/G plus-agarose beads (40 μl) were then added, and samples were incubated at 4 °C for 1 h. The beads were collected and washed three times with TNE buffer, and bound protein complexes were analyzed by SDS/PAGE followed by western blotting using anti-Cdx2 antibody (BioGenex: CDX2-88).

Tumor samples

89 tumor samples (banked at the Fujita Health University School of Medicine, Aichi, Japan) were randomly selected from advanced gastric cancer patients and stained with anti-Cdx2 antibody. We selected 39 samples from this group in which immunoreactivity of Cdx2 was clearly evident. In this group of 39 tissue specimens (Table S1), we then analyzed the expression pattern of villin and Cdx2. The corresponding patients were aged from 37 to 92 years (a mean of 67.5 ± 11.0 years), and comprised 26 males and 13 females. The clinical stage distribution, according to the UICC (the International Union Against Cancer) classification, was as follows: I in 4 patients (10.3%), II in 7 patients (17.9%), III in 20 patients (51.3%), and IV in 8 patients (20.5%). This study was approved by the institutional ethical review board for human investigation at Fujita Health University.

Results

Detection of the major transcription start site for the human villin gene in gastrointestinal cells

In our recent study [8], we employed 5′-RACE analysis and detected a transcription start site (TSS) for the human *villin* gene, which differs from the previously reported site at 20 bp upstream from the ATG translation start codon [15]. By 5′-RACE, we here isolated two additional *villin* TSSs, which are closely located to our previous one (Fig. 1A). Using villin-positive NUGC-4 and villin-negative AZ-521 cells, we next performed the primer extension experiments, and two TSSs obtained from our 5′-RACE analysis were clearly detected (Fig. 1B). The longest transcript (shown in Fig. 1A) and the earlier reported TSS [15] were not detected in this analysis.

We additionally performed RT-PCR analysis of *villin* using the same cell lines. One fixed reverse primer (present in exon 6) and four forward primers were used (Fig. 1C), and these data were consistent with 5′-RACE results and with the predicted exon/intron structures. Genomic sequences from six mammal species were further compared, and the non-coding exon 1 we identified was found to be highly conserved (Fig. S1). Taken together, we concluded that the major TSS for the human *villin* gene in gut cells is the 5′-end cytosine residue of the second longest transcript (Figs. 1A, C).

A highly conserved region containing two putative Cdx binding sites (HCR-Cdx) is present in the promoter of the villin gene

To examine the transcriptional regulation of the *villin* gene, the genomic sequences upstream of its non-coding exons in six mammal species were screened using NCBI Blast2 Sequence [16] and Clustal_W [17]. About 1.1 kb upstream of our identified human *villin* TSS, there is a highly conserved region (Fig. 2A), within which was found a consensus binding element for the chicken *CdxA* homeobox gene, a homologue of the mammalian *Cdx1*, *Cdx2*, and *Cdx4* gene [18,19]. Among these Cdx proteins, Cdx1 and Cdx2 are essential for intestinal development and homeostasis [20], and Cdx2 is thought to play a very important role in the mammalian gut as a regulator of many genes such as *sucrase isomaltase* [21], *MUC2* [22], *KLF4* [23], *guanylyl cyclase C* [24], and *LI-cadherin* [18].

We next screened 32 human cell lines (20 gastric, 10 colorectal, and two non-gastrointestinal cells) for the expression of *villin*,

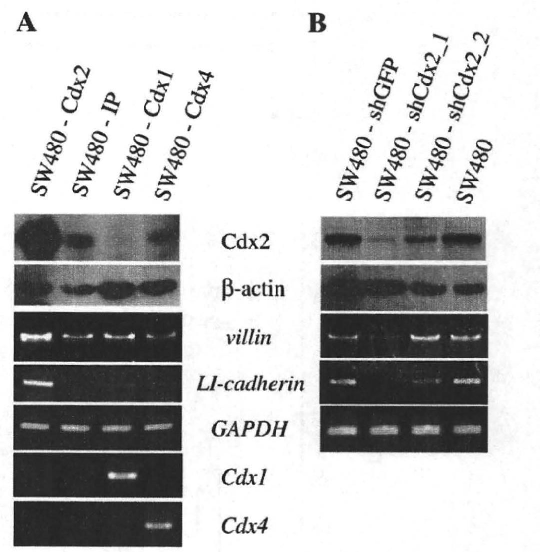

Fig. 3 – (A) Western blotting analysis of Cdx2 and β-actin (internal control) expression in SW480 cells virally transduced with pMXs-Cdx2-IRES-Puro (SW480-Cdx2), pMXs-IRES-Puro (SW480-IP), pMXs-Cdx1-IRES-Puro (SW480-Cdx1), or pMXs-Cdx4-IRES-Puro (SW480-Cdx4). The expression of *Cdx1*, *Cdx4*, *villin*, *LI-cadherin*, and *GAPDH* (internal control) was analyzed by RT-PCR. (B) Western blotting analysis of Cdx2 and β-actin expression in SW480 parental cells and established infectant SW480 cells expressing shRNAs targeting the *Cdx2* (shCdx2_1 and shCdx2_2) or *GFP* (shGFP) genes. The expression of *villin*, *LI-cadherin*, and *GAPDH* was analyzed by RT-PCR.

Cdx2, and *Cdx1*. As shown in Fig. 2B, many gastric and colorectal cells express *villin*, and in each case also express *Cdx2*, with two exceptions, KE-39 and WiDr. On the contrary, *Cdx1* expression is not as common in gastric and colorectal cancer cell lines. These expression patterns suggest that Cdx2 regulates the transcription of the *villin* gene. The non-gastrointestinal cell lines HeLa-S3 and MDA-MB435 were negative for both *villin* and *Cdx2* as expected.

Exogenous modulation of Cdx2 expression level affects the endogenous villin gene expression

We constructed a series of MuLV-based retrovirus vectors carrying either the *Cdx2*, *Cdx1*, or *Cdx4* gene, and transduced them individually into the SW480 colorectal cancer cell line. In the resulting stable SW480 transductants, *villin*, *LI-cadherin*, and *GAPDH* were analyzed by RT-PCR (Fig. 3A). *LI-cadherin*, whose expression is regulated by Cdx2 [18], was found to be strongly induced in SW480-Cdx2 cells, and marginally induced in SW480-Cdx1 cells. The *villin* gene response was very similar; a strong upregulation in SW480-Cdx2 cells but a weak induction in SW480-Cdx1 cells (Fig. 3A). No expression changes were detected in SW480-Cdx4 cells for either *LI-cadherin* or *villin*.

It is noteworthy that the endogenous Cdx2 protein expression levels were efficiently suppressed by exogenous Cdx1 expression, suggesting that Cdx1 protein negatively regulates *Cdx2* gene expression. This effect of Cdx1 upon *Cdx2* has been revealed also in previous reports of *Cdx1* knockout mice and *Cdx1* transgenic mice [25,26]. In SW480-Cdx1 cells, therefore, exogenous Cdx1 appears to act as a somewhat effective substitute for endogenous Cdx2 and thereby facilitate *villin* and *LI-cadherin* expression, although to a lesser extent.

We constructed additional retrovirus vectors carrying short-hairpin RNAs (shRNAs) that were designed to target two different regions of the *Cdx2* gene. These vectors were transduced into SW480 cells to obtain stable transfectants. Western blotting analysis revealed a drastic decrease of Cdx2 expression in SW480-shCdx2_1 cells and a moderate decrease in SW480-shCdx2_2 cells (Fig. 3B). To analyze the effects of Cdx2 upon endogenous *villin* and *LI-cadherin* expression, RT-PCR analyses were performed (Fig. 3B). *LI-cadherin* expression was found to be decreased in both the SW480-shCdx2_1 and SW480-shCdx2_2 transduced cells, but was more prominent in the former probably reflecting its greater knockdown efficiency of Cdx2. *Villin* expression was reduced in SW480-shCdx2_1 cells only, suggesting that Cdx2 knockdown in SW480-shCdx2_2 was

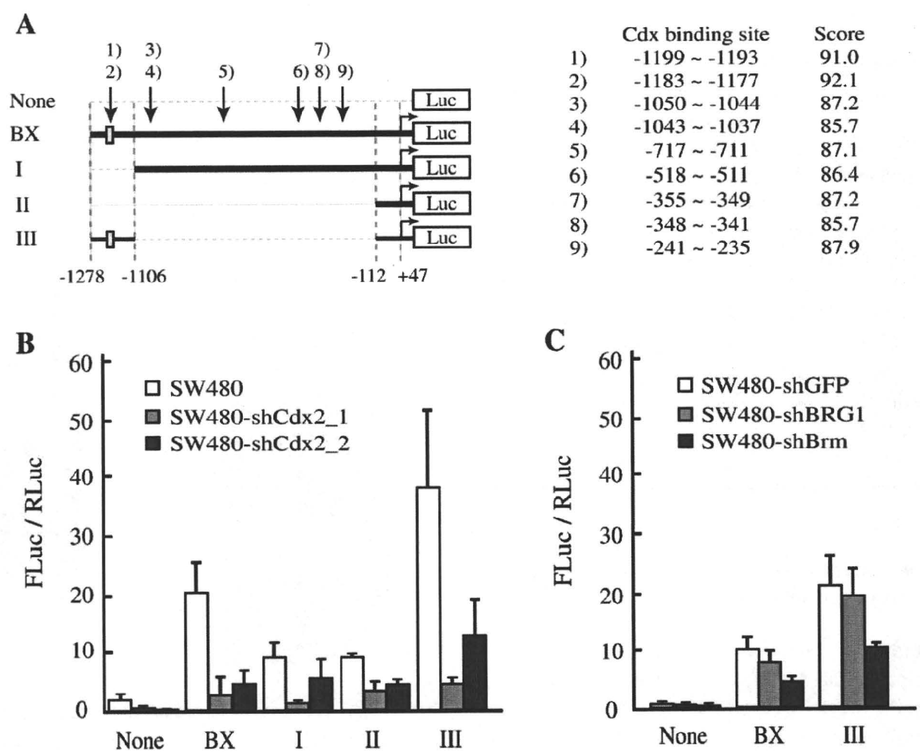

Fig. 4 – (A) Schematic representation of the human *villin* gene reporter constructs used in this study. HCR-Cdx is denoted by the grayish box, with + 1 assigned to the major 5′-end base of the *villin* cDNA sequence determined by both 5′-RACE and primer extension analysis. The nine predicted Cdx binding sites shown with scores were revealed using TFSEARCH programs (ver. 1.3). (B) Reporter analyses of a series of *villin* promoter constructs in parental SW480 cells and established SW480 cells expressing shRNAs targeting *Cdx2* (shCdx2_1 and shCdx2_2). Luciferase activities were measured at 48 h after transfections, and the data shown are the mean values of triplicate experiments, with the error bars correspond to the standard errors. (C) Reporter analyses of *villin* gene constructs in SW480 cells expressing shRNAs targeting *GFP*, *BRG1*, and *Brm*. 48 h after transfection of each reporter plasmid, luciferase activities were measured. Data shown are the mean values of triplicate experiments, with the error bars corresponding to the standard errors.

insufficient to suppress the *villin* transcription. From these results, we conclude that the transcription factor Cdx2 plays essential roles in *villin* expression in intestinal cells.

Since we have previously reported that Brm is required for *villin* expression in gastric cancer cells [8], we tested whether the loss of *Cdx2* expression would lead to a down-regulation of *Brm* expression and *vice versa*. Using SW480 and AGS cells stably expressing shCdx2_1, shCdx2_2, shBrm, shBRG1, shIni1, or shGFP, we performed western blotting for Cdx2 and Brm. However, no reciprocal effects could be detected in either cell line; the expression of these two genes seems to be mutually independent (Fig. S2).

The HCR-Cdx in the villin promoter is critical for both the Cdx2-dependent and Brm-dependent expression of villin

To test whether the highly conserved region of the *villin* promoter containing two putative Cdx2 binding sequences (HCR-Cdx) is important for intestinal *villin* expression, we designed a series of reporter constructs for this region and performed luciferase assays in SW480 cells (Fig. 4A). As shown in Fig. 4B, SW480 cells transfected with constructs harboring HCR-Cdx (BX and III) showed significantly higher levels of luciferase activity compared with constructs in which this region is deleted (I and II). These data imply that *villin* transcription is efficiently activated through the HCR-Cdx of its promoter.

When similar experiments were performed using the stable transductants, SW480-shCdx2_1 and SW480-shCdx2_2 (Fig. 3B), a strong decrease in reporter activity was observed for the BX and III reporter constructs (Fig. 4B). These data indicate that transactivation of the *villin* gene via HCR-Cdx strongly depends on Cdx2 expression. Compared with the BX and III reporters, reporter I, which lacks HCR-Cdx, also showed slight dependency to shCdx2_1 and shCdx2_2. This might be explained by several Cdx consensus sequences present between −1106 and −112 of the *villin* promoter (Fig. 4A), which have lower binding scores and are not conserved in mammals.

Using the BX and III reporters, we further examined the effects of Brm and BRG1 knockdown in SW480 cells. For both reporters, shBrm, but not shBRG1, efficiently suppressed luciferase activity (Fig. 4C), implying that intestinal *villin* gene expression is dependent on Brm as well as Cdx2, consistent with our previous report [8].

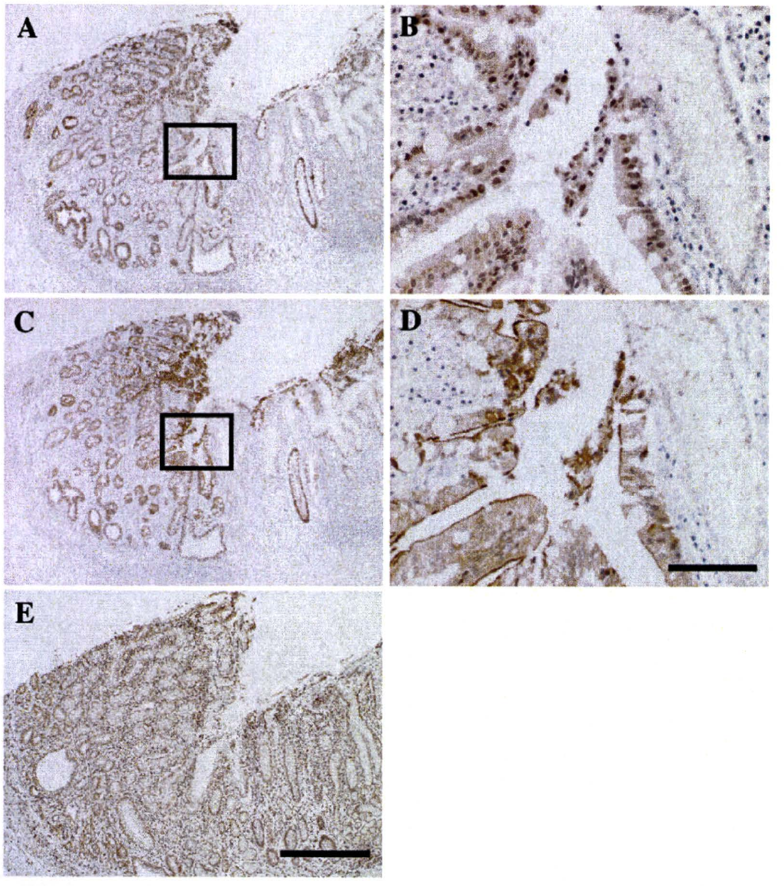

Fig. 5 – Immunostaining of Cdx2 (A, B), villin (C, D), and Brm (E) in sequential sections of non-malignant gastric mucosa, in which normal gastric mucosa (villin negative) and intestinal metaplasia (villin positive) coexist. (B) and (D) are magnified images corresponding to the framed areas in panels A and C, respectively. Cdx2 is an intranuclear protein, whereas villin is expressed in the cytoplasm and/or on the brush border structure of the cellular membrane. Scale bars, 500 μm (A, C, E); 100 μm (B, D).

The expression of Cdx2 and villin is tightly coupled in gastrointestinal tissues

It has been previously reported that the expression of Cdx2 in normal adult tissues is mostly restricted to the epithelial cells of the small and large intestine [27]. It was also reported in an earlier study that Cdx2 and Cdx1 are almost always induced in gastric intestinal metaplasia [28]. Additionally, from previous analysis of multiple human tumor samples, it has been observed that adenocarcinomas of intestinal origin are mostly Cdx2-positive [7]. These accumulative findings in vivo suggest that the distribution of Cdx2 is similar to that of villin, which supports the results of our biochemical analysis that the *villin* gene is regulated by Cdx2. To more precisely evaluate whether a correlation exists between the expression of Cdx2 and villin, we performed an immunohistochemical survey at the resolution of a single cell.

Normal gastric mucosa expresses neither Cdx2 nor villin, but both proteins are detectable in a variety of stomach disorders [28]. We thus speculated that gastric tissues would be the most suitable for evaluating the correlation between Cdx2 and villin, and analyzed 39 gastric cancer tissue samples, in which immunoreactivity against Cdx2 was clearly evident (Table S1). As both normal gastric mucosa and non-malignant intestinal metaplasia were present on all of these samples, we first evaluated the areas harboring intestinal metaplastic changes. In all of the 39 cases examined, the expression patterns of villin and Cdx2 were found to be completely identical at a single cell resolution (Figs. 5A–D). As we previously reported [8], Brm was detected in normal gastric mucosa and non-malignant intestinal metaplasia as well as mesenchymal cells (Fig. 5E). The expression patterns of these proteins in gastric cancer tissues were then evaluated, and we again observed a perfect coupling of both proteins at a single cell resolution (Figs. 6A–D). These clear co-expression patterns *in vivo* strongly support our contention that Cdx2 is a principal regulator of *villin* expression. As shown in Figs. 6E and F, all the malignant cells were found to express Brm, which is consistent with the idea that both Cdx2 and Brm are required for *villin* expression.

It is noteworthy that *villin* is also expressed in some cells that are not of gastrointestinal origin, including those of the kidney proximal tubules, intrahepatic bile ducts, and pancreatic ducts [1]. To test whether Cdx2 is always required for the *villin* expression, sequential sections of proximal tubules from a normal kidney were immunostained for both Cdx2 and villin (Fig. S3). The results clearly indicated that *villin* expression does not always require Cdx2 expression in these non-gastrointestinal cells.

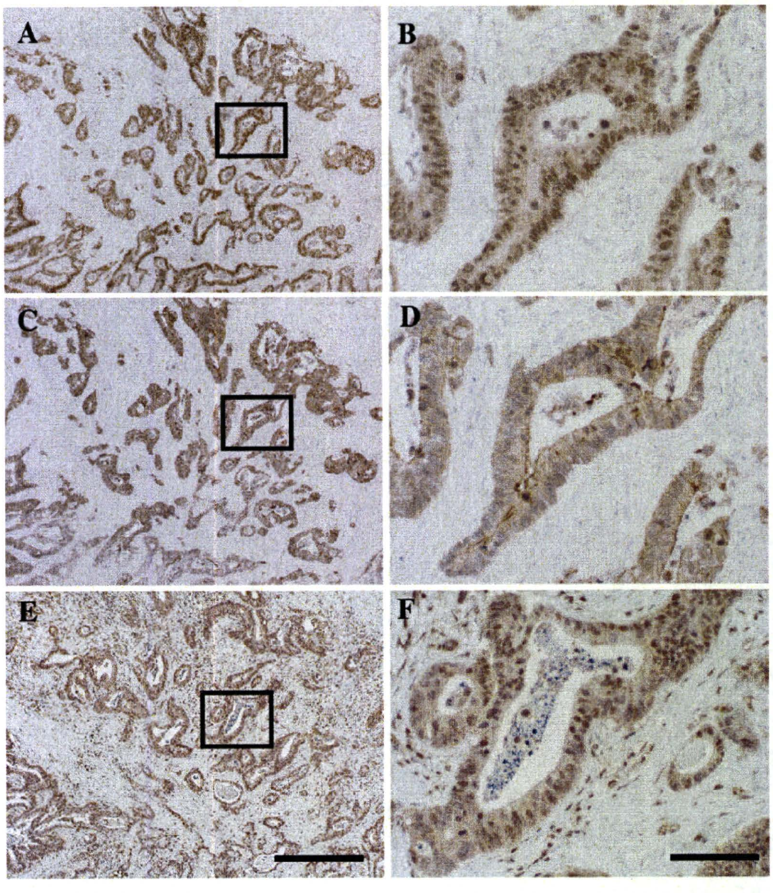

Fig. 6 – Immunostaining of Cdx2 (A, B), villin (C, D), and Brm (E, F) in sequential sections of primary gastric cancers (moderately differentiated tubular adenocarcinomas). (B), (D), and (F) are magnified images corresponding to the framed areas in panels A, C, and E respectively. Scale bars, 500 μm (A, C, E); 100 μm (B, D, F).

Cdx2 directly binds to several subunits of the SWI/SNF chromatin remodeling complex

Considering the cooperative activity between Cdx2 and Brm (Brm-type SWI/SNF complex) in the regulation of *villin* expression, we examined whether Cdx2 protein directly interacts with Brm or other components of the SWI/SNF complex. Brm, BRG1, BAF60a, Inil and β-actin proteins were synthesized *in vitro*, and each was mixed with a GST-Cdx2 fusion protein. As shown in Fig. 7A, the GST pull-down assay clearly demonstrated that Brm strongly binds to Cdx2. BRG1, an alternative catalytic subunit of SWI/SNF complex, also showed a similar binding affinity to Cdx2, although this protein does not appear to be required for intestinal *villin* expression, as judged by our current findings (Fig. 4C) and also our previous report [8]. With regard to the three other subunits of the SWI/SNF complex, Ini1 and BAF60a can directly bind to Cdx2 protein, whereas β-actin cannot (Fig. 7A). These results suggest that SWI/SNF complex can interact with Cdx2 protein at multiple points on its surface.

Using the nuclear extracts from SW480-Cdx2 cells, we further performed the coimmunoprecipitation assay. As shown in Fig. 7B, Cdx2 protein was efficiently detected by western blotting in the immunoprecipitates of anti-Brm antibody. From this result, we concluded that Brm binds to Cdx2 *in vivo*.

Cdx2 and Brm interact with the villin promoter region

As both Brm and Cdx2 are required for intestinal *villin* expression, we tested whether these two proteins associate with the *villin* promoter. ChIP assays using SW480 cells were performed, and the HCR-Cdx, and in a lesser extent the exon 1 region of the *villin* gene, were efficiently coimmunoprecipitated with either Cdx2 or Brm (Fig. 7C). This indicates that both Cdx2 and Brm are present around the HCR-Cdx motif in the *villin* promoter.

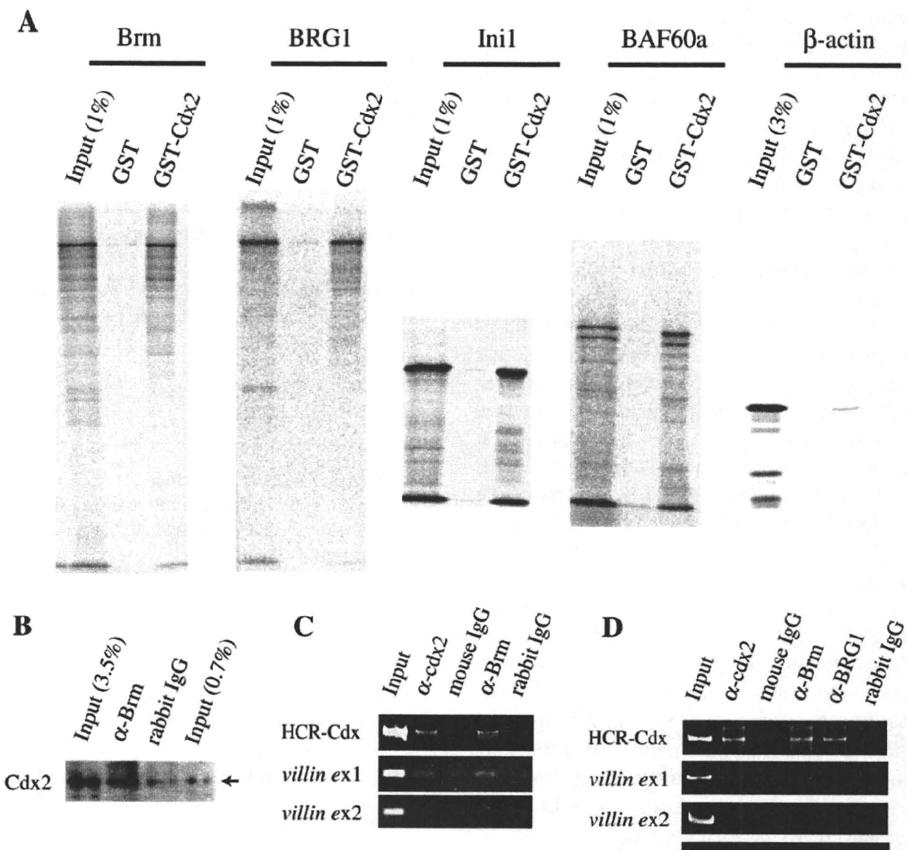

Fig. 7 – **(A) The binding of Cdx2 to subunits of the SWI/SNF complex analyzed by GST pull-down. Purified GST-Cdx2 and GST (control) proteins attached to GSH-Sepharose beads were prepared, and their binding to Brm, BRG1, Ini1, BAF60a, and β-actin (synthesized *in vitro* in the presence of [^{35}S]methionine) was analyzed. (B) An immunoprecipitation assay using nuclear extracts derived from SW480-Cdx2 cells (SW480 cells stably transduced with MuLV-based retrovirus vectors carrying *Cdx2* gene). Immunoprecipitates with anti-Brm antibody or non-specific rabbit IgG (control) were analyzed by western blotting using anti-Cdx2 antibody. (C, D) ChIP analyses of the human *villin* gene in SW480 (C) or HT-29 cells (D). For immunoprecipitations, anti-Cdx2 antibody, nonimmunized mouse IgG whole molecule, anti-Brm antibody, anti-BRG1 antibody, and nonimmunized rabbit IgG whole molecule were used. PCR was performed with primers that recognize the HCR-Cdx, exons 1 and 2 of the human *villin* gene, and the promoter region of the human *CD44* gene.**

We performed another series of ChIP analysis using colorectal HT-29 cells, which are reported to express *CD44* [29], a target of both the Brm- and BRG1-type SWI/SNF complex [9,10]. As shown in Fig. 7D, *CD44* promoter was detected in the immunoprecipitates of Brm and BRG1 antibodies as expected, but not in those of Cdx2 antibodies. In contrast, the HCR-Cdx region was efficiently coimmunoprecipitated with Cdx2 and Brm as well as BRG1. From these results, we concluded that Cdx2 and Brm interact with the *villin* promoter region in gastrointestinal cells.

Discussion

Cdx2 and the Brm-type SWI/SNF complex act cooperatively to regulate villin expression in gastrointestinal cells

Our ChIP analyses revealed that in human gastrointestinal cells, Cdx2 and Brm are present around the HCR-Cdx in the *villin* promoter (Figs. 7B, C). Luciferase reporter assays further suggested that the Cdx binding sites present in the HCR-Cdx region are responsible for the Cdx2- and Brm-dependent transactivation of human *villin* (Fig. 4). These results indicate that Cdx2 would recruit the Brm-type SWI/SNF complex to facilitate the stable expression of the *villin* gene. Through the direct binding of several subunits (Fig. 7A), Cdx2 is likely to efficiently recruit the SWI/SNF complex in the process of transactivation of target genes, like such other transcription factors as AP-1 [30], CREB [31], C/EBP-β [32], MyoD [33], c-Myc [34,35], glucocorticoid receptor [36,37], estrogen receptor [38], androgen receptor [39], GATA-1 [40], SP-1 [41], p54nrb [14], and so on.

The BRG1-type SWI/SNF complex does not seem to be required for the *villin* gene expression (Fig. 4C). It is of note, however, that Brm knockout (Brm (−/−)) mouse is alive without an apparent phenotype [42]. We believe that in cells which completely lack Brm, BRG1 would compensate for it in a very low efficiency. It should be pointed out that BRG1 can also bind Cdx2 *in vitro* and is recruited to the *villin* promoter (Figs. 7A and B). The molecular mechanisms for preferential transactivation activity of the Brm-type SWI/SNF complex remain to be elucidated. Our present data show that Cdx2 regulates the *villin* gene in gastrointestinal cells.

It is noteworthy in this regard, however, that in non-intestinal tissues such as renal proximal tubules, *villin* expression is not accompanied by Cdx2 expression (Fig. S3). This suggests that other transcription factors unique to non-intestinal tissues can substitute for Cdx2 and fully activate the *villin* gene expression. Among the 30 gastrointestinal tumor cell lines examined in our current study, we observed two villin-positive but Cdx2-negative cell lines, WiDr and KE-39 (Fig. 2B). In these malignant cells, such substitutive transcription factors other than Cdx2 might be ectopically expressed.

Cdx2 should be one of the key molecules underlying the association between the Brm-deficiency and the histologic features of gastric cancer

In our previous study, we showed that loss of Brm expression is frequent in gastric cancer, and that the Brm-deficiency correlates well with undifferentiated status of gastric malignancy [8]. We also found the involvement of Brm in the expression of *villin*, which is an important gut differentiation marker. It is apparent, however, that villin alone cannot account for the relationship between Brm expression and the gastric cancer classification. Therefore, it is of considerable interest that Cdx2 can recruit the Brm-type SWI/SNF complex to the *villin* promoter, through its direct binding to several subunits of the SWI/SNF complex. It is intuitive that Cdx2 cooperates with the Brm-type SWI/SNF complex to regulate not only the *villin* gene but also many genes important for intestinal differentiation, maintenance, function, and structure [18,20–24]. We are convinced that Cdx2 is a key linker between the loss of Brm and undifferentiated status of gastric cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2009.01.006.

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Locked Nucleic Acid *In situ* Hybridization Analysis of miR-21 Expression during Colorectal Cancer Development

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Abstract **Purpose:** To better understand microRNA miR-21 function in carcinogenesis, we analyzed miR-21 expression patterns in different stages of colorectal cancer development using *in situ* hybridization (ISH).

Experimental Design: Locked nucleic acid (LNA)/DNA probes and a biotin-free tyramide signal amplification system were used in ISH analyses of miRNA expression. Conditions for specific detection of miR-21 were determined using human cell lines and miR-21–expressing lentiviral vectors. Expression was determined in 39 surgically excised colorectal tumors and 34 endoscopically resected colorectal polyps.

Results: In the surgical samples, miR-21 expression was much higher in colorectal cancers than in normal mucosa. Strong miR-21 expression was also observed in cancer-associated stromal fibroblasts, suggesting miR-21 induction by cancer-secreted cytokines. Protein expression of PDCD4, a miR-21 target, was inversely correlated with miR-21 expression, confirming that miR-21 is indeed a negative regulator of PDCD4 *in vivo*. In the endoscopic samples, miR-21 expression was very high in malignant adenocarcinomas but was not elevated in nontumorigenic polyps. Precancerous adenomas also frequently showed miR-21 up-regulation.

Conclusion: Using the LNA-ISH system for miRNA detection, miR-21 was detectable in precancerous adenomas. The frequency and extent of miR-21 expression increased during the transition from precancerous colorectal adenoma to advanced carcinoma. Expression patterns of miR-21 RNA and its target, tumor suppressor protein PDCD4, were mutually exclusive. This pattern may have clinical application as a biomarker for colorectal cancer development and might be emphasized by self-reinforcing regulatory systems integrated with the *miR-21* gene, which has been previously shown in cell culture.

Novel mechanisms of human gene regulation mediated by microRNAs (miRNA) have recently been established (1). MiRNAs control gene expression at the posttranscriptional level by targeting mRNAs for translational repression or mRNA degradation (1). Evidence is accumulating that many miRNAs are differentially regulated in normal development and cancers, and that deregulation of specific miRNAs may contribute to human cancer development and progression (1). To better understand the molecular mechanisms that regulate miRNA gene expression, our group has recently developed an algorithm to predict the promoters of human miRNA genes that are likely to be involved in evolutionarily conserved regulatory systems (2). Using this algorithm, we predicted 59 putative miRNA gene promoter regions, one of which was the *miR-21* miRNA promoter. Because high miR-21 expression levels have been reported in various malignancies, including breast cancer (3–5), pancreatic cancer (3, 6, 7), cholangiocarcinoma (8), hepatocellular carcinoma (9), brain tumor (10), leukemia (11, 12), cervical cancer (13), ovarian cancer (14), colorectal cancer (3, 15), prostate cancer (3), lung cancer (3), and gastric cancer (3), we concentrated on miR-21 analysis. Our biochemical analyses

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Translational Relevance

To better understand microRNA (miRNA) function in carcinogenesis, sensitive and reproducible methods for *in situ* hybridization (ISH) are needed. For experimental analysis, we have chosen analysis of miR-21 expression during the process of colorectal cancer development, and strictly determined conditions for specific detection of miR-21 using several human cell lines and miR-21–expressing lentiviral vectors. ISH analysis of miRNA expression levels was finally established with locked nucleic acid (LNA)/DNA probes and biotin-free tyramide signal amplification system, which is finally applicable to formalin-fixed paraffin-embedded clinical samples. The established LNA-ISH system for miRNA detection showed that elevation of miR-21 becomes detectable from precancerous adenomas, and the extent and frequency of miR-21 expression increase during the colorectal canceration from precancerous adenoma to advanced carcinoma. Mutually exclusive expression patterns between miR-21 RNA and its target, tumor suppression protein PDCD4, in adenocarcinomas and precancerous adenomas will have a potential clinical application as a biomarker for colorectal cancer development.

confirmed that the *miR-21* promoter predicted by the algorithm was correct (2). Transcription of primary miR-21 transcripts (pri-mir-21) was initiated 30 bp downstream of the promoter TATA box. Other evolutionally conserved regulatory elements present in the miR-21 promoter included the binding sites of activator protein (AP-1), Ets/PU.1, SP1, CCAAT/enhancer binding protein-α, nuclear factor I (NFI), serum response factor, p53, and signal transducer and activator of transcription 3. We further showed that phorbol 12-myristate 13-acetate activated miR-21 transcription through the several AP-1 and Ets/PU.1 binding sites in the miR-21 promoter, and also showed that miR-21 was induced during macrophage differentiation of HL-60 cells (16). Therefore, we hypothesized that increased miR-21 expression, which has been validated in many cancers, may reflect elevated tumor cell AP-1 activity. We also identified the negative transcriptional regulator nuclear factor I-B (NFIB) as a target for miR-21 regulation (16). Because the *miR-21* promoter contains an NFIB binding site and NFIB efficiently suppresses AP-1–dependent miR-21 transactivation, we concluded that a double-negative feedback loop consisting of miR-21, NFIB, and the *miR-21* promoter could self-reinforce miR-21 expression (16).

Several other miR-21 targets have also been suggested such as *RECK* (17), *Sprouty2* (18), *PTEN* (8, 9), *TPM1* (19), and *PDCD4* (20–23). Tumor suppressor PDCD4 is reported to suppress protein synthesis (24, 25) through cytoplasmic binding of eIF4A and is also known to inhibit several transcription factors, including AP-1 (26, 27), SP1 (28), and β-catenin (26). Because DNA binding sites for some of these factors are present in miR-21 promoters, PDCD4 might also contribute to the down-regulation of miR-21 expression through double-negative feedback regulation. Indeed, it was recently reported that in Ras-induced cellular transformation of a rat thyroid cell line, PDCD4 reduces miR-21 activity, at least partly, by suppressing AP-1 activity (29). However, at this moment, there is even no clear proof that PDCD4 is a target of miR-21 *in vivo* because of the lack of efficient tools to detect miR-21 expression in a single-cell resolution.

As a first step toward understanding mechanisms regulating *miR-21* expression *in vivo*, we examined miR-21 expression patterns during the cancer development. Colorectal tumors were chosen for the experimental analyses because the clinical and histologic features of colorectal cancers are relatively simple compared with other malignancies, and most of colorectal cancers are thought to develop from precancerous adenomas (30). For this purpose, we established a sensitive and stable *in situ* hybridization (ISH) method using formalin-fixed, paraffin-embedded (FFPE) tissues. Experimental results indicate that the frequency and extent of miR-21 expression increase during colorectal cancer progression from precancerous adenoma to advanced carcinoma.

Materials and Methods

Human cell lines. HEK293 (originated from embryonic kidney), HeLa (cervical carcinoma), MDA-MB435 (breast ductal carcinoma), PA-1 (embryonic carcinoma), NCC-IT (embryonic carcinoma), A427 (non small cell lung carcinoma), G401 (rhabdoid tumor), SW620 (colorectal adenocarcinoma), and AZ521 (gastric cancer) cells were maintained at 37°C in high-glucose DMEM supplemented with 10% FCS (Gibco/Invitrogen).

Locked nucleic acid–modified oligonucleotide probes. Locked nucleic acid (LNA)–modified oligonucleotide probes labeled with FITC at their 3′-ends were obtained from Molecular Biology of ThermoElectron GmbH. The sequences of miR-21 probe and the scramble control probe for a negative control were 5′-TLcAALcATLcAGLtCTLgATLaAGLcTA-3′ and 5′-CLaTTLaATLgTCLgGALcAALcTCLaAT-3′, respectively. La, Lt, Lc, and Lg were LNA monomers corresponding to the bases A, T, C, and G, respectively.

ISH with LNA-modified oligonucleotide probes. Five-micrometer-thin sections of FFPE tissues adhered to glass slides were deparaffinized in three consecutive xylene baths for 1 min each, followed by 1 min each in serial dilutions of ethanol (100%, 100%, 95%, 95%) and three changes of diethyl pyrocarbonate–treated water. Slides were then immersed in 0.3% H_2O_2 for 30 min at room temperature, washed thrice with diethyl pyrocarbonate–treated water, digested with 400 μg/mL proteinase K (Roche) at 37°C for 15 min, washed thrice with diethyl pyrocarbonate–treated water, submerged in 95% ethanol for 1 min, and air-dried completely. Slides were then hybridized in incubation chambers overnight at 37°C in an oven, using 0.2 μmol/L LNA–modified probes diluted with mRNA ISH solution (DAKO). After hybridization, slides were rinsed thrice in 0.5× SSC, washed for 30 min at 50°C in 0.5× SSC/0.1% Brij35 (Sigma), and rinsed twice in TBS. An anti-FITC horseradish peroxidase–conjugated antibody (DAKO, P5100) at 1:100 dilution in TBS/1% bovine serum albumin was applied to the slides for 60 min at room temperature, followed by three washes in TBS/0.1% Tween 20 (TBS-T). For amplification of antibody signals, FITC-conjugated phenol (fluorescyl-tyramide, DAKO, K1497) was applied to the slides for 30 min at room temperature, followed by three washes in TBS-T. Finally, an anti-FITC antibody conjugated to horseradish peroxidase (DAKO, K1497) was added to the slides for 30 min at room temperature, followed by three washes in TBS-T. The reaction products were visualized using a 50 mg/dL 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide.

Immunohistochemical staining. Deparaffinization, endogenous peroxidase inactivation, antigen retrieval of FFPE clinical tissues, and immunostaining with anti-PDCD4 (ab51495, Abcam) antibody were done as described previously (31). The immunostained sections were evaluated independently by two pathologists in conjunction with the H&E-stained sections from the same lesions.

Lentivirus vectors. For the polymerase II–driven (SV40 promoter) vector, a part of pri-miR-21 sequence (from −6 bp to +65 bp of the 5′-end of mature miR-21 sequence) was inserted between the *Bam*HI and *Eco*RI sites of pLSP (22) to generate pLSP-pre-mir21_short. For the polymerase III–driven (U6 promoter) vector, the mouse U6 promoter and a part of the pri-miR-21 sequence (from −57 bp to +115 bp of the 5′-end of mature miR-21 sequence) were inserted into the *Bam*HI and *Eco*RI sites of pLSP to generate pLSP-mU6-pre-mir21_long. Sequences of synthetic oligonucleotide pairs for shCre (targeting Cre-recombinase of P1phage) and shU3-12 (targeting part of MuLV-LTR) were described previously. They were inserted into pLPS as described above to generate pLPS-shCre and pLSP-shU3-12 (32), respectively, and used for the negative controls. Vesicular stomatitis virus-G pseudotyped lentiviral vectors were produced using the ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's instructions.

Virus transduction and protein analysis. HEK293 cells were transduced with vesicular stomatitis virus-G pseudotyped vectors at multiplicity of infection of 1 to 2 and selected with puromycin for 1 wk. About 1 wk after the end of selection, cells were disrupted for total protein preparation. Western blotting analysis with anti-PDCD4 and anti-actin antibodies was done as described previously (22).

Colorectal tissue samples. Thirty-four endoscopically resected colorectal polyps and 39 surgically excised colorectal tumors were selected from a list of patients with colorectal lesions who underwent endoscopic or surgical operation in 2006. All tissue samples were banked at the Fujita Health University School of Medicine, Aichi, Japan. For the endoscopically resected samples, the patients were between 35 and 83 y old (mean age, 58.8 ± 12.0 y) and included 23 males and 11 females. For the surgically excised samples, the patients were between 41 and 83 y old (mean age, 65.7 ± 9.4 y) and included 23 males and 16 females. Among the 35 cases of surgically removed colorectal cancer, the clinical tumor stage distribution was stage I in 1 patient, stage II in 16 patients, stage III in 15 patients, and stage IV in 2 patients, according to the International Union Against Cancer classification. This study was approved by the institutional ethical review board for human investigation at Fujita Health University.

Results

Detection of miR-21 expression levels by ISH with LNA-modified oligonucleotide probes. To screen for miR-21–positive and miR-21–negative control cultures for ISH, levels of primary transcript of miR-21 (pri-miR-21) and mature miR-21 were determined (Supplementary Fig. S1A and B). Expression level patterns of pri-miR-21 and mature miR-21 were very similar, suggesting that there were no significant rate-limiting steps in the processing of miR-21 production among these cell lines. Among the nine cell lines tested, we used malignant HeLa and MDA-MB435 cells as positive controls and nonmalignant HEK293 cells as a negative control.

A LNA-modified probe was designed in which every third DNA nucleotide was substituted with a corresponding LNA monomer, which was subsequently used for ISH with LNA-modified oligonucleotide probes (LNA-ISH). With fluorescent microscopic observation, the FITC-labeled probe clearly stained the cytoplasm of HeLa and MDA-MB435 cells, whereas HEK293 cell cytoplasmic staining was much weaker (Fig. 1A). Because mature miRNAs are present in the cytoplasm (33), the probe most likely detected the mature miR-21 signals. To apply this LNA-ISH to clinical FFPE samples, we also attempted to detect miR-21 by combining LNA-ISH with the biotin-free tyramide signal amplification system. Using this method, 3,3′-diaminobenzidine staining of miR-21 was visible in the cytoplasm (*brown*; Fig. 1A).

To confirm the specificity of miR-21 detection and more precisely quantify miR-21 expression levels, we produced two lentiviral vectors carrying a portion of pri-miR-21 (including the entire pre-miR-21) driven by the SV40 promoter (polymerase II–driven pLSP-pre-mir21_short) or the mouse U6 promoter (polymerase III–driven pLSP-mU6-pre-mir21_long; Supplementary Fig. S2). HEK293 cells were transduced with pLSP-pre-mir21_short, pLSP-mU6-pre-mir21_long, and pLSP-shGFP (control), respectively, and stable transductants were selected for further analyses. Expression of miR-21 in these established cell lines was analyzed (Supplementary Fig. S3), and LNA-ISH for miR-21 was done. As shown in Fig. 1B, HEK293 cells transduced with miR-21–expressing vectors exhibited a clear cytoplasmic staining when compared with control cells, and pLSP-mU6-pre-mir21_long–transduced cells showed denser staining than pLSP-pre-mir21_short–transduced cells. From these results, we concluded that our LNA-ISH could detect miR-21 in a semiquantitative manner.

High miR-21 expression levels were observed not only in cancer cells but also in cancer-associated fibroblasts from colorectal FFPE tissues. We first performed the LNA-ISH for miR-21 using the surgically excised advanced colorectal cancer tissues. In the 34 slides examined, precancerous adenomatous lesions were found in five cases: three were separate polyps and two were adenomatous masses adjacent to malignant adenocarcinoma. Including these five lesions, we performed the LNA-ISH for miR-21 on 39 lesions (Supplementary Table S1). The expression of miR-21 in adenocarcinoma was much higher than the expression in normal mucosa (Fig. 2; Table 1). In the surgical samples, however, increased miR-21 expression was barely detectable in precancerous adenomas. Unexpectedly, up-regulation of miR-21 was observed not only in malignant cells but also in the stromal fibroblasts adjacent to the tumor (Fig. 2B; Supplementary Fig. S4A; Supplementary Table S1). Overexpression of miR-21 was never observed in fibroblasts far from the tumor mass (Fig. 2B). When an equivalent LNA probe but in which the miR-21 oligonucleotide sequence has been scrambled was used instead, this control probe showed no significant staining in tumor regions, normal tissue, and the stromal fibroblasts adjacent to the tumor (Supplementary Fig. S5B and D), showing clear contrast to the staining by the miR-21 probe in the sequential FFPE colorectal tissue sections (Supplementary Fig. S5A and C). All these results suggest that nonmalignant stromal fibroblasts adjacent to tumors might induce miR-21 expression due to factors secreted from the nearby tumors.

Up-regulation of miR-21 was frequently observed in precancerous adenomas but never in nontumorigenic polyps. We next analyzed miR-21 expression levels in endoscopically resected colorectal polyps that were <15 mm in diameter. Among the 34 samples examined, 28 cases were tumorigenic and 6 cases were nontumorigenic (Table 2; Supplementary Table S2). The ISH signal intensities of these endoscopically resected adenomas were far stronger than those of the five surgically excised adenomas (Supplementary Table S1; Table 1). Importantly, the RNA preservation of endoscopically resected tissues was much better than that of surgical excised tissues. This difference could be due to a longer time before immersion in formalin for the surgical samples (1-3 hours) compared with that for

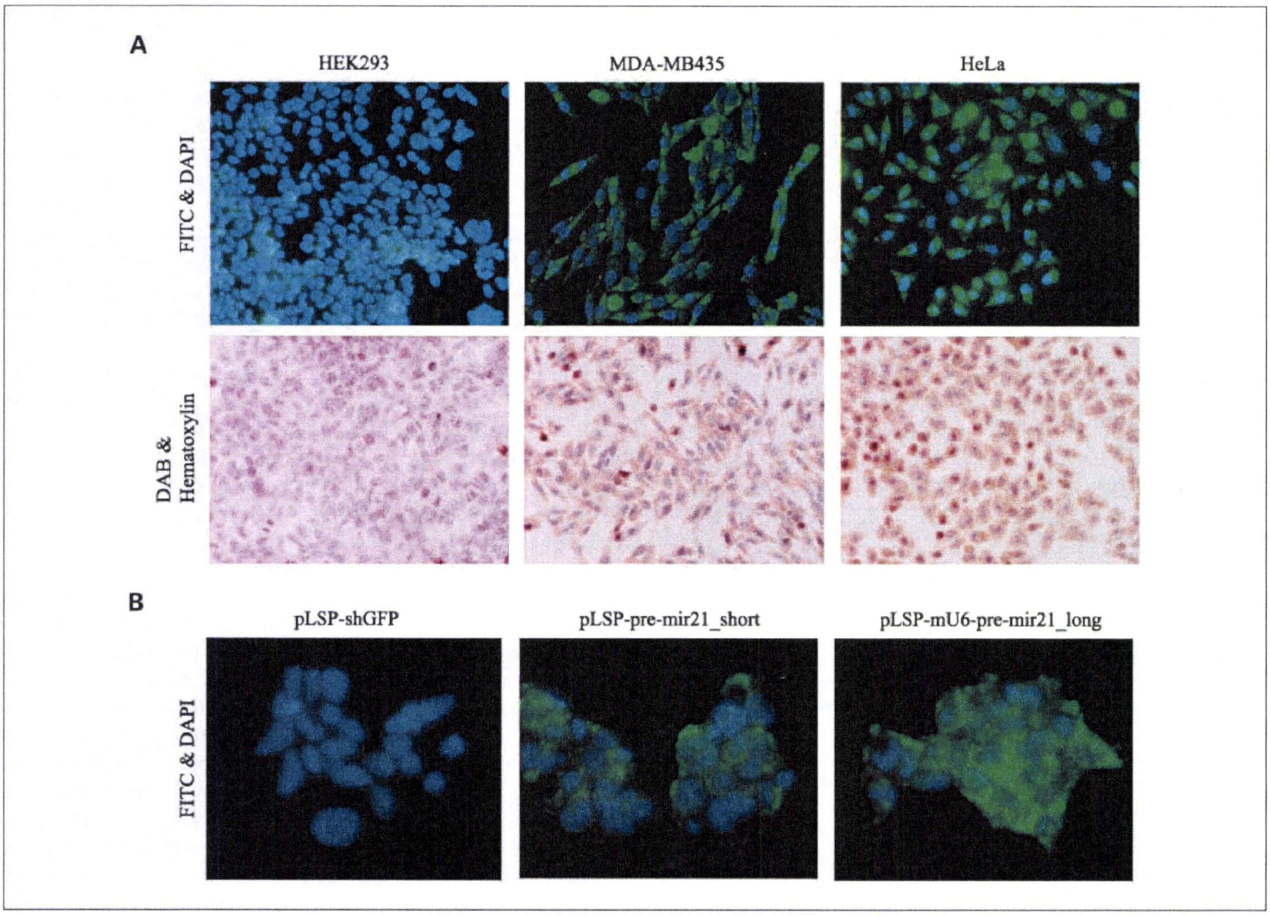

Fig. 1. ISH with FITC-labeled LNA-modified probe (LNA-ISH) to detect miR-21 expression levels in formalin-fixed human cell lines. *A,* top, combined images of cytoplasmic miR-21 highlighted with FITC signal (*green*) and nuclear DNA counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). Bottom, FITC in the probes were detected by the biotin-free tyramide signal amplification system. MiR-21 is finally stained with 3,3′-diaminobenzidine (*DAB*; *brown*), whereas the nuclei were counterstained with hematoxylin (*blue*). *B,* LNA-ISH detecting miR-21 of HEK293 cells, which were transduced with miR-21 expression vectors (pLSP-pre-mir21_short and pLSP-mU6-pre-mir21_long). Combined images of cytoplasmic miR-21 detected with FITC signal (*green*) and nuclear DNA counterstained with 4′,6-diamidino-2-phenylindole (*blue*). HEK293 cells transduced with pLSP-shGFP were used as a negative control.

endoscopic ones (1-10 minutes) and also to the the much longer formalin fixation time for surgical tissues (24-96 hours) compared with the fixation time for endoscopic ones (<6 hours).

Expression of miR-21 in nontumorigenic lesions was not elevated above levels detected in normal colorectal mucosa (Fig. 3A). On the contrary, increased miR-21 expression was frequently observed in both precancerous adenomas and adenocarcinomas (Fig. 3B and C). As shown in Table 2 and Fig. 3, miR-21 expression was obviously higher in malignant adenocarcinoma than in precancerous adenoma. From these results, we concluded that elevation of miR-21 expression accompanies colorectal tumor development from precancerous adenoma to advanced carcinoma. We also expect that early detection of miR-21 up-regulation may have potential clinical application as a new biomarker for colorectal tumorigenesis.

MiR-21 and PDCD4 expression show mutually exclusive patterns in the areas around colorectal cancer tissues. It has been reported that PDCD4 is a target of miR-21 mostly by transiently introducing miR-21 RNA or antisense oligonucleotides for miR-21 exogenously (20–23). Recently, we have also shown that PDCD4 protein is up-regulated when cells were transduced with lentivirus vectors expressing newly developed decoy RNAs that specifically inhibit miR-21 function (22). We here tested whether HEK293 cells, which show marginal miR-21 expression (Fig. 1), reduce endogenous PDCD4 expression by stable expression of exogenous miR-21. By comparing PDCD4 protein levels in cells transduced with miR-21 expression lentivirus vector (pLSP-mU6-pre-mir21_long) and those with control vectors (or untransduced cells) by Western blotting, we have observed clear reduction in the steady-state levels of PDCD4 by exogenous miR-21 expression (Fig. 2C).

We next performed ISH for miR-21 and immunostaining of PDCD4 using sequential sections obtained from surgically resected colorectal cancer tissues. As shown in Fig. 4A, expression of PDCD4 was high in normal tissues (*right*) but was nondetectable in colorectal cancer cells. Areas with abundant miR-21 expression wrapped over the areas with low PDCD4 expression. Even when endoscopically resected samples were used, mutually exclusive expression patterns were frequently observed between miR-21 and PDCD4 in both malignant adenocarcinoma (Figs. 4B; Supplementary Fig. S6A) and precancerous adenoma

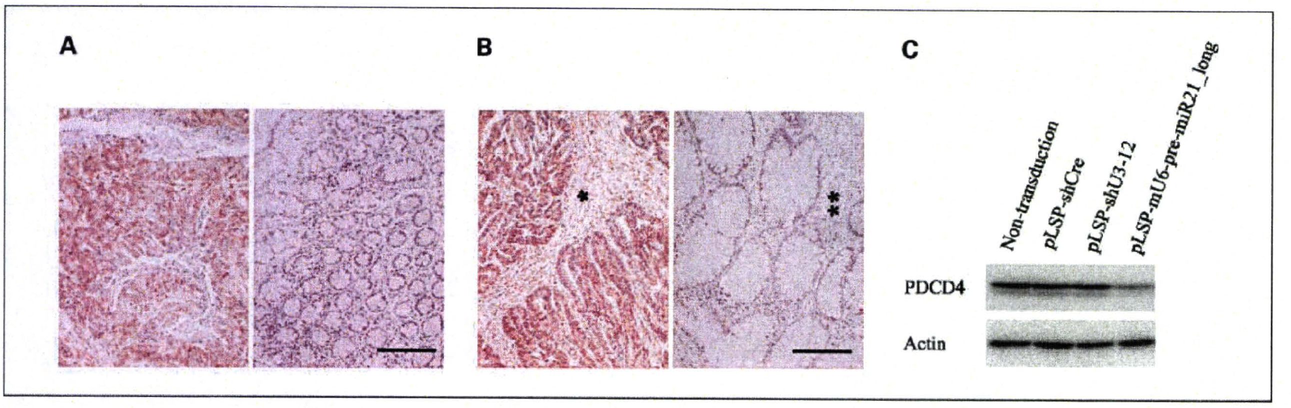

Fig. 2. *A,* LNA-ISH detection of miR-21 in the colorectal adenocarcinoma (*left*) and normal colorectal mucosa (*right*) on the same slide. Bar, 200 μm. *B,* LNA-ISH detection of miR-21 in colorectal tissue from another patient; colorectal adenocarcinoma (*left*) and normal colorectal mucosa (*right*) on the same slide. *, fibroblasts around the cancer; **, fibroblasts around the normal gland. FFPE colorectal tissues were obtained from surgical operation. Bar, 200 μm. *C,* expression of PDCD4 and actin proteins in HEK293 cells transduced with miR-21 expression lentiviral vectors (pLSP-mU6-pre-mir21_long) or two control viral vectors (pLSP-shCre and pLSP-shU3-12). Total protein samples were isolated 2 wk after the transduction and analyzed by Western blotting. Untreated HEK293 cells (nontransduction) were also used as a control.

(Figs. 4C; Supplementary Fig. S6B). These results support the hypothesis that PDCD4 is an *in vivo* target of miR-21 and further suggest that an early increase in miR-21 expression during colorectal tumorigenesis results in a decrease in PDCD4 expression.

Discussion

MiR-21 expression and cancer development. MiR-21 expression has been reported to be one of the best hit miRNA in many profiling experiments designed to detect up-regulated miRNA in human cancer including colorectal carcinoma (3). Our ISH analysis on the colorectal carcinomas clearly detected high level expression of miR-21 in most of them (Fig. 2; Table 1), and importantly, we further showed that this miR-21 increase can be frequently detected from the adenoma stage in the section of endoscopic mucosal resection (Fig. 3; Table 2). Even in this early stage, we observed that PDCD4, a target of miR-21, was concomitantly reduced in miR-21-up-regulated regions (Fig. 4C). These results indicate the importance of up-regulation of miR-21 and down-regulation of PDCD4 as diagnostic biomarkers of colorectal carcinogenesis.

Recently, extensive analysis on miRNA profiles that are associated with prognosis and therapeutic outcome in colon adenocarcinoma was reported (15). Importantly, more advanced tumors expressed higher levels of miR-21 using either microarray data from the test cohort or the quantitative reverse transcription-PCR data from the validation cohort. By analysis on pooled cohorts, they further showed that high miR-21 expression is associated with a poor prognosis in either stage

Table 1. Summary of miR-21 expression levels in surgically excised colorectal adenocarcinoma (malignant tumor) and colorectal adenoma (benign tumor with cancerous potential)

Histologic features of colorectal tumors	Evaluation of miR-21 expression					
	5	4	3	2	1	Total
Adenocarcinoma (malignant tumor)	6	10	8	6	4	34
Adenoma (benign tumor with cancerous potential)	0	0	0	1	4	5

NOTE: Evaluations of the miR-21 expression based on the LNA-ISH staining compared with normal colorectal mucosa on the same slides. Values assigned to the staining (from 1 to 5) were decided as follows: 5, most tumor cells (>80%) show much stronger staining than normal epithelial cells; 4, most tumor cells (>80%) show stronger staining than normal epithelial cells; 3, a part of tumor cells (~20-80%) show stronger staining than normal epithelial cells; 2, some tumor cells (more than 20%) show slightly stronger staining than normal epithelial cells; 1, almost all tumor cells show same staining intensity as normal epithelial cells.

Table 2. Summary of the association between high level expression of miR-21 expression and the histologic features of endoscopically resected colorectal polyps

Histologic features of colorectal polyps		Up-regulation of miR-21			Total
		++	+	−	
Tumorigenic polyp	Noninvasive carcinoma	7	0	1	8
	Adenoma (high or low grade)	8	2	10	20
Nontumorigenic polyp	Hyperplastic	0	0	3	3
	Juvenile	0	0	1	1
	Peutz-Jegher's (hamartomatous)	0	0	2	2

NOTE: Pathohistologic diagnoses were judged by the Vienna classification of gastrointestinal epithelial neoplasia. Evaluations of the intensity of miR-21 staining by LNA-ISH were decided as follows: ++, cells in the polyp show much stronger staining compared with normal epithelial cells on the same slide; +, cells in the polyp show stronger staining compared with normal epithelial cells on the same slide; −, cells in the polyp show the same staining intensity as normal epithelial cells on the same slide.

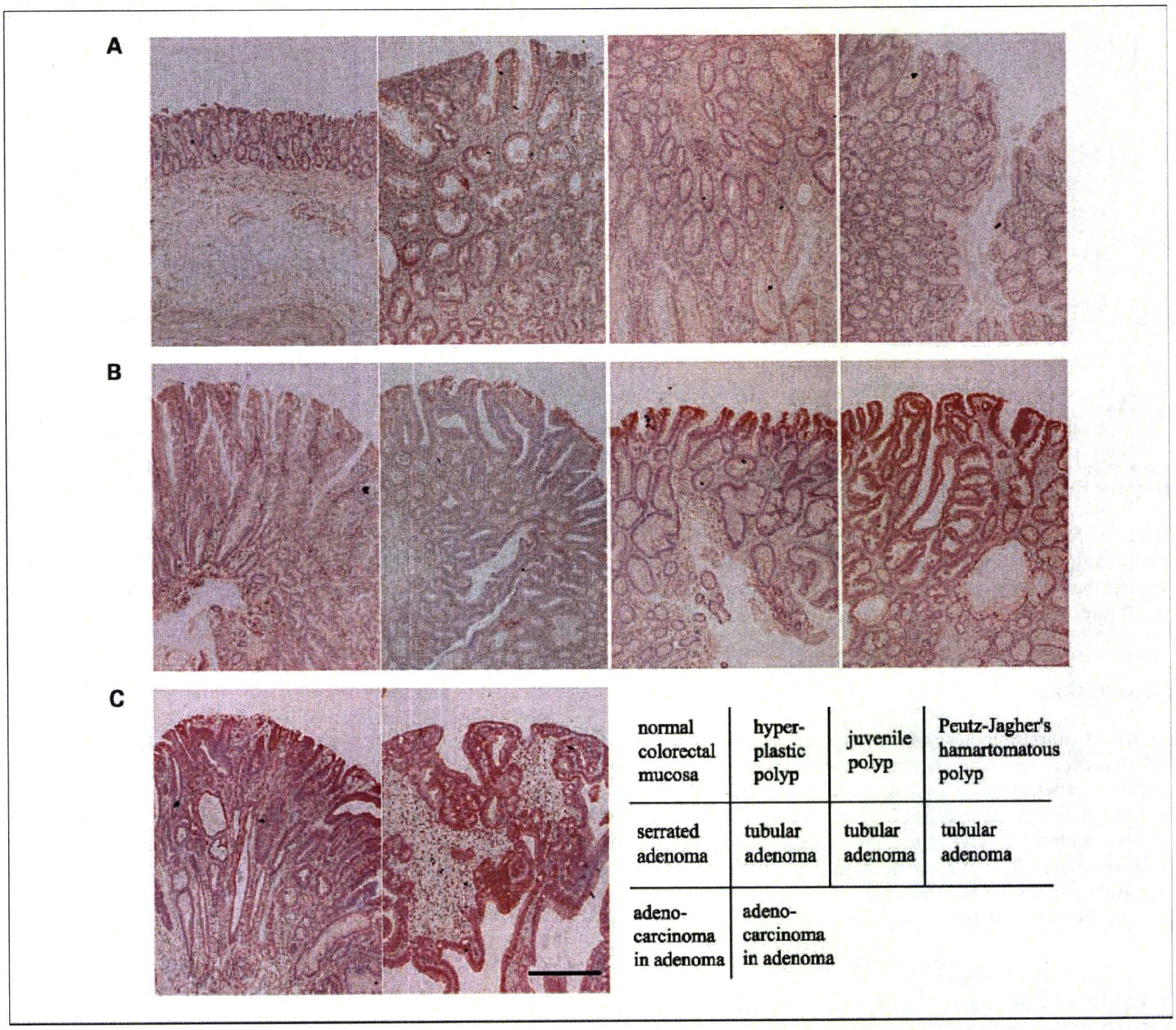

Fig. 3. LNA-ISH detecting miR-21 with FFPE colorectal tissues obtained from endoscopic mucosal resection. Bar, 500 µm. *A*, nontumorous colorectal tissues. Photos from left to right are normal colorectal mucosa, hyperplastic polyp, juvenile polyp, and Peutz-Jagher's hamartomatous polyp. *B*, tumorous but nonmalignant colorectal lesions. Photos from left to right are serrated adenoma (negative for miR-21), tubular adenoma (negative for miR-21), tubular adenoma (miR-21 positive in the edge), and tubular adenoma (positive for miR-21). *C*, tumorous and malignant colorectal lesions. In each photo, adenocarcinoma in adenoma, strongly positive for miR-21, is shown.

II or stage III colon cancer patients, indicating its potential as a prognostic biomarker (15).

LNA-ISH combined with biotin-free tyramide signal amplification system is a useful technique for determination of miRNA expression levels in FFPE tissues. The LNA-modified oligonucleotide would be one of the most sensitive probes currently available for miRNA detection (34, 35). Nevertheless, it is quite difficult to detect miRNAs by ISH, especially in FFPE clinical tissues. It has been reported that the LNA-ISH technique could detect some miRNA species in FFPE samples (36). However, using LNA-modified probes alone or LNA-ISH combined with a universal immunoenzyme polymer method (Histofine Simple Stain MAX PO_MULTI purchased from Nichirei), we were not able to detect miR-21 expression in FFPE samples (data not shown). In our previous study (31), the biotin-free tyramide signal amplification system was used to detect the nuclear protein Brm, which is difficult to detect by immunohistologic methods (37). In the present study, we applied this method to LNA-ISH and were able to sensitively detect miR-21 expression. Because detection of nonspecific signals is not infrequent when using tyramide amplification, we confirmed that staining by the scramble control probe (Supplementary Fig. S5B) as well as staining unrelated to the LNA/DNA probe (Supplementary Fig. S4B) was only rarely detected.

High-level expression of miR-21 in cancer-associated fibroblasts may be induced by secreting factors originating from cancer cells. It is very interesting that the stromal fibroblasts around tumors frequently express miR-21 at high levels. In most cases from the present study, these cancer-associated fibroblasts showed strong miR-21 expression compared with distant normal fibroblasts and normal epithelial cells (Supplementary Table S1). In some cases, the fibroblast staining intensities were even more intense than those of adjacent malignant cells (Supplementary Fig. S4A). Therefore, we hypothesized that this is a non–cell-autonomous phenomenon and that cytokines secreted from the adjacent malignant tumors might contribute to miR-21 induction. In this regard, it has been reported that interleukin-6 levels are elevated in the cancer-associated fibroblasts around a colon cancer, in serum, and in tumor tissues from colorectal cancer patients (38, 39). It is also noteworthy that interleukin-6 induces the transcription of miR-21 in multiple myeloma cells through mediation of signal transducer and activator of transcription-3 activity (12). Importantly, signal transducer and activator of transcription-3 binding sites are present just upstream of the transcriptional start site in the *miR-21* promoter. Therefore, interleukin-6 is a candidate for miR-21 induction in these cancer-associated fibroblasts.

MiR-21 RNA and PDCD4 protein expression patterns were mutually exclusive in colorectal epithelial cells. PDCD4 was highly expressed in normal colorectal epithelium, but PDCD4 expression was often reduced in precancerous colorectal regions (Fig. 4). This observation is consistent with a recent report that normal mucosa showed strong nuclear PDCD4, which was significantly reduced in adenomas (40). Our ISH analyses further indicated that cells with reduced PDCD4 expression frequently had elevated miR-21 expression, which was nondetectable in normal colorectal epithelium (Fig. 4). In progressive colorectal cancers, almost all cells expressed miR-21, whereas PDCD4 was almost undetectable. In summary of our ISH analysis, expression of miR-21 RNA and PDCD4 protein showed mutually exclusive patterns in colorectal epithelial cells. These observations support that PDCD4 is a good target of miR-21 *in vivo*. Because PDCD4 is a potent tumor suppresser, miR-21 may perform oncogenic functions, at least in part, through down-regulation of PDCD4.

We have previously shown that in human cell culture systems, a double-negative feedback loop operates between miR-21 and its target protein, NFIB, through the miR-21 promoter, which has the binding site of this negative transcriptional regulator (16). This means that the *miR-21* gene integrates a system that self-reinforces its own expression. Whereas in adult rats *NFIB* mRNA is highly expressed (41), we are currently not able to perform specific immunohistochemical staining due to the absence of a specific anti-NFIB antibody that is applicable to FFPE clinical samples and is non–cross-reactive with other NFI family members. Therefore, direct evidence of NFIB involvement *in vivo* remains to be established. Using rat cell culture system, Dr. Verde's group very recently indicated that PDCD4 suppressed miR-21 promoter activity, at least in part, by inhibiting AP-1 activity

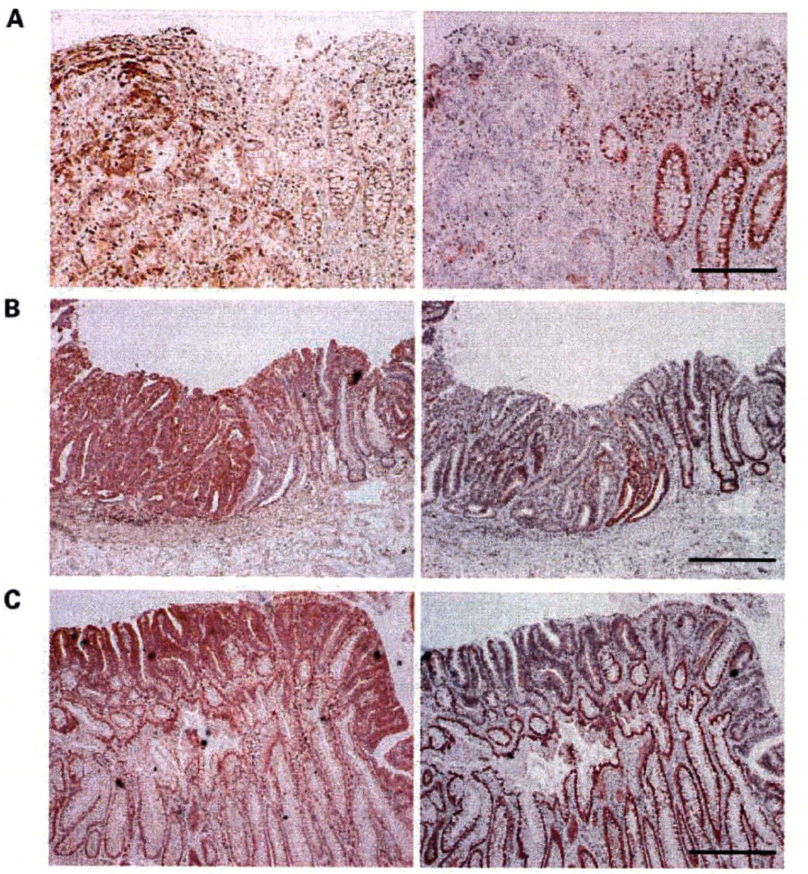

Fig. 4. Images from sequential FFPE colorectal tissue sections in which LNA-ISH for miR-21 RNA (*left*) and immunostaining for PDCD4 protein (*right*) were done. *A,* a surgically resected colorectal adenocarcinoma. The border of the colorectal cancer (*left half*) and normal colorectal mucosa (*right half*) is apparent in the two photos. Bar, 200 μm. *B,* a colorectal polyp containing adenocarcinoma and adenoma obtained from endoscopic mucosal resection. In the two photos, evidence of malignant change is apparent in the left half. Bar, 500 μm. *C,* a nonmalignant adenomatous colorectal polyp obtained by endoscopic mucosal resection. Adenomatous change is apparent at the edge of the colorectal mucosa. Bar, 500 μm.

(29). Therefore, these double-negative feedback loops operating through the miR-21 promoter may contribute to the self-reinforced expression of miR-21. This feedback could further lead to the mutually exclusive expression patterns observed between miR-21 RNA and PDCD4 protein in colorectal cancer. It also remains to be determined which transcriptional factors that are normally inhibited by PDCD4 are crucial for *miR-21* gene induction in colorectal cancer. The molecular mechanisms involved in functional suppression of these transcription factors by PDCD4 could significantly advance our understanding of cancer progression. Therefore, elucidation of the molecular mechanisms involved in the entire regulatory network formed by miR-21 is important for understanding the initial stages of colorectal carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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GASTROENTEROLOGY

Multi-center survey regarding the management of anticoagulation and antiplatelet therapy for endoscopic procedures in Japan

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Key words
aspirin, endoscopic biopsy, endoscopic mucosal resection, endoscopic submucosal dissection, warfarin.

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The questionnaires were collected from participants at the meeting of Tokyo Gastrology Clinical Diagnosis Conference (TGCDC), supported by Eisai Co. and the contents were partially presented at the 7th TGCDC meeting, Tokyo, Japan, on 11 January, 2008.

Abstract

Background: A guideline on the management of anticoagulation and antiplatelet therapy for endoscopic procedures has been established from Japan Gastroenterological Endoscopy Society in 2005. However, it is unknown whether consensus on the management of these conditions is obtained among endoscopists in daily practice owing to the guideline.
Methods: To study the current practice on the management, survey questionnaires were sent to 13 representative endoscopists of 13 middle or high-volume hospitals in the Tokyo area.
Results: Responses were obtained from all 13 endoscopists. The results showed that only five (38%) and six (46%) hospitals had their own standard protocols regarding the management for endoscopic biopsy and endoscopic mucosal resection (EMR), respectively. There was a wide variation among endoscopists in terms of discontinuation of each agent. When the patients had a major risk of thromboembolism due to discontinuation of anticoagulants and antiplatelet agents, seven (54%) and five (38%) endoscopists, respectively, never took a biopsy. Similar numbers of endoscopists never carried out EMR. During discontinuation of anticoagulants or antiplatelet agents for biopsy and EMR, three (23%) and three (23%) endoscopists, respectively, experienced patients with thromboembolic events.
Conclusions: There is still a wide variation and confusion among endoscopists after establishment of our national guideline. A robust national guideline with clearer description based on the scientific evidence is needed.

Introduction

Increasing use of anticoagulants and antiplatelet agents in primary and secondary prophylaxis of cardiovascular, cerebrovascular and venous thromboembolic diseases, and in the status after implantation of a mechanical stent or valve/aortic prostheses, has raised a big management issue for patients with those agents during diagnostic and therapeutic endoscopy. From the standpoint of endoscopists, they should be stopped to lessen the risk of hemorrhage. However, the doctor who initially prescribed them would like to continue them because of a fear of thromboembolic events. Ideally, changes of treatment should be made with estimation of the risks and the benefits and be tailored to individual patients on a case-by-case basis. However, in fact, it is very difficult to estimate the risks of hemorrhage and thromboembolic events for each case. The dilemma has made a movement to establish a Japanese guideline on the management of anticoagulation and antiplatelet therapy for endoscopic procedures from Japan Gastroenterological Endoscopy Society in 2005 as well as in other countries (Tables 1,2).[1] Permeation of the guideline throughout our endoscopy society and the usefulness in daily practice should be validated after a few years of establishment and the guideline should be reformed according to the current inconsistency every few years. So, we conducted this pilot study using a survey questionnaire in the Tokyo area in order to determine the current daily practice regarding the

Table 1 Management of anticoagulant (warfarin) for endoscopic procedures

	Low-condition risk of thromboembolic event	High-condition risk of thromboembolic event
Low procedural risk	Discontinue 3–4 days before procedure Start at little rebleeding risk Check of INR ≤ 1.5 before procedure is desirable	Discontinue 3–4 days before procedure Start at little rebleeding risk Check of INR ≤ 1.5 before procedure is desirable Consider heparin
High procedural risk	Discontinue 3–4 days before procedure Start at little rebleeding risk Check of INR ≤ 1.5 is mandatory	Discontinue 3–4 days before procedure Start at little rebleeding risk Check of INR ≤ 1.5 before procedure is mandatory Consider heparin

INR, international normalized ratio.

Table 2 Management of antiplatelet agents for endoscopic procedures

	Low-condition risk of thromboembolic event	High-condition risk of thromboembolic event
Low and high procedural risk	Discontinue 3 days before procedure for aspirin alone Discontinue 5 days before procedure for ticlopidine alone Discontinue 7 days before procedure for aspirin and ticlopidine No description for start	Discontinue 3 days before procedure for aspirin alone Discontinue 5 days before procedure for ticlopidine alone Discontinue 7 days before procedure for aspirin and ticlopidine Consider heparin and hydration No description for start
Extremely high procedural risk	Discontinue 7 days before procedure for aspirin Discontinue 10–14 days before procedure for ticlopidine No description for start	Discontinue 7 days before procedure for aspirin Discontinue 10–14 days before procedure for ticlopidine Consider heparin and hydration No description for start

management of anticoagulation and antiplatelet therapy for endoscopic procedures.

Methods

From the participants' records of Tokyo Gastrology Clinical Diagnosis Conference (TGCDC) meetings held in Tokyo three times a year, survey questionnaires were sent between November 2007 and December 2007 to 13 endoscopists who identified themselves as representative endoscopists in middle or high-volume hospitals in the Tokyo area. Initial approval to collect the data was obtained from all the board members of TGCDC by email beforehand and final official approval to have collected the data and announce the results including publication was obtained at the board meeting held on 11 January, 2008.

The following 13 hospitals participated in the present survey: The University of Tokyo, Tokyo; National Cancer Center Hospital, Tokyo; Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo; International Medical Center of Japan, Tokyo; St Luke's International Hospital, Tokyo; Saitama Medical University International Medical Center, Saitama; Tokyo KoseiNenkin Hospital, Tokyo; Chofu Touzan Hospital, Tokyo; Tokyo Metropolitan Toshima Hospital, Tokyo; Tokyo Teishin Hospital, Tokyo; Mitsui Memorial Hospital, Tokyo; Toranomon Hospital, Tokyo; Tochigi Cancer Center, Tochigi, Japan.

Several questions about management of patients on anticoagulants and antiplatelet agents undergoing esophagogastroduodenoscopy (EGD) with biopsy and endoscopic mucosal resection (EMR) including endoscopic submucosal dissection (ESD) for stomach neoplasms were asked of the endoscopists as follows: (i) Do you have a standard protocol at your hospital on the management of anticoagulation and antiplatelet therapy for endoscopic procedures? (ii) Do you feel the necessity for solid evidence on the management of anticoagulation and antiplatelet therapy for endoscopic procedures? (iii) How many days before and after the procedure is each agent stopped when it can be stopped? The agents asked about were warfarin, aspirin, ticlopidine, clopidogrel, cilostazol, ethyl icosapentate, beraprost sodium, sarpogrelate hydrochloride, dipyridamole, ozagrel sodium, trapidil and dilazep hydrochloride. (iv) How do you manage the patients with a major risk of thromboembolism? (v) Have you had patients with a bleeding complication due to insufficient discontinuation of anticoagulation and antiplatelet therapy? (vi) Have you had patients with a thromboembolic event during discontinuation of anticoagulation and antiplatelet therapy?

Results

Responses were obtained from all the endoscopists. In five (38%) and six (46%) hospitals, there were individual standard protocols for the management of anticoagulation and antiplatelet therapy for endoscopic biopsy and EMR, respectively, whereas, in the remainder of the hospitals, endoscopists themselves determined the protocol by referring to the Japanese guideline and/or the doctors who prescribed those agents. Twelve (92%) and 13 (100%) endoscopists desired solid evidence on the management of anticoagulation and antiplatelet therapy for EGD with biopsy and EMR, respectively.

Standard policy to stop and start agents

There were wide variations between endoscopists regarding consideration about discontinuation of anticoagulation and antiplatelet therapy to prevent bleeding complications from EGD with biopsy and EMR, although warfarin was stopped 4 days before the procedures, and aspirin, ticlopidine, and ethyl icosapentate were stopped 7 days before the procedures by most of the endoscopists (Fig. 1). The asked agents seemed to be divided into three groups, the agents with standard policy and consistency to some extent among endoscopists (warfarin, aspirin, ticlopidine, ethyl icosapentate), the agents with standard policy but inconsistency among endoscopists (clopidogrel, cilostazol, beraprost sodium, sarpogrelate hydrochloride, dipyridamole), and the agents without standard policy among endoscopists (ozagrel sodium, trapidil, dilazep hydrochloride).

No major differences were observed between EGD with biopsy and EMR regarding discontinuation before the procedures in any agents. In comparison with discontinuation before the procedures, more endoscopists had no standard policy regarding discontinuation after the procedures in any agents, especially after EMR.

Management of the patients with a major risk of thromboembolism

Seven (54%) endoscopists never took a biopsy and six (46%) endoscopists never performed EMR at their hospitals, if the patients had a major risk of thromboembolism with discontinuation of anticoagulants. Five (38%) endoscopists never took a biopsy and six (46%) endoscopists never performed EMR in their hospital, if the patients had major risk of thromboembolism with discontinuation of antiplatelet agents. The remainder of the endoscopists took a biopsy or carried out EMR after replacement by heparin for 3 to 7 days. The main protocol was withdrawal of the continuous perfusion of heparin 4–6 h before the procedures and resumption of heparin 6–8 h after the procedures with overlapping warfarin until adequate international normalized ratio (INR) was obtained.

Experience of bleeding complication and thromboembolic event

No endoscopists experienced patients with severe bleeding due to EGD with biopsy or EMR due to insufficient discontinuation of anticoagulation and antiplatelet therapy. On the contrary, two endoscopists (15%) and one endoscopist (8%) experienced a patient with cerebral infarction during discontinuation of aspirin and cilostazol, respectively, for EGD with biopsy. Two endoscopists (15%) and one endoscopist (8%) experienced a patient with cerebral infarction and mesenteric arterial thrombosis, respectively, during discontinuation of warfarin for EMR. The patient with mesenteric arterial thrombosis finally died after the event. Detail clinical course of each event was not obtained due to the nature of the survey with questionnaires.

Discussion

Several guidelines on the management of anticoagulation and antiplatelet therapy for endoscopic procedures including the Japanese guideline have gradually been established in the last decade.[1–7] A common feature of these guidelines is to classify the procedural risks into low and high and the condition risks for thromboembolism into low and high. In terms of the procedural risks, EGD with biopsy is considered to be a low-risk procedure and EMR including ESD is considered to be a high-risk procedure. However, how to manage the patients with different procedural risks is inconsistent between Western guidelines and the Japanese guideline. If the procedure is classified as a low-risk procedure, the risk of hemorrhage is very low and no change in medication may be acceptable in the Western guidelines. On the contrary, even when the endoscopic procedures are classified into a low-risk group, it is recommended to stop anticoagulants and antiplatelet agents for a considerable duration in the Japanese guideline. Although it is difficult to conclude the superiority of each standpoint, we have to keep in mind the facts that some endoscopists experienced major thromboembolic events during discontinuation of those agents and considerable percentages of endoscopists declared that they never took a biopsy at their hospital from patients with high-condition risk for thromboembolism.

In the literature, as far as we are aware, there are only a few reports on thromboembolic events during discontinuation of anticoagulants or antiplatelet agents.[8–11] However, this does not mean that the agents are safely ceased in any occasion as shown in this study. Recent small surveys from Korea and Japan revealed that six of 81 endoscopists 7.4%)[12] and seven of 81 endoscopists (8.6%),[13] respectively, experienced an embolism in their patients after cessation of these agents, similar to our survey. It should be stressed again that induced thromboembolic events during discontinuation of the agents may result in fatalities.

Racial differences in susceptibility to thromboembolism are sometimes used to establish different guidelines between countries, especially between the West and the East, because it is believed, without solid evidence, that Caucasians are potentially more susceptible to thromboembolism than Asians. A recent international survey conducted by Eastern and Western endoscopists on the issue supported a wide difference in the management between the two groups, which revealed that Eastern endoscopists were more concerned about an increased risk of bleeding.[14] However, we have to mention that this opinion is not obtained by the clinical data in the literature that compares the different rates of complications in gastrointestinal endoscopy between the East and the West. Furthermore, the survey also revealed, interestingly, that personal experience seemed to be a more powerful driver of practice than was the published literature even among Western endoscopists with the American Society for Gastrointestinal Endoscopy (ASGE) guidelines.[14]

Another large issue revealed by the present study is that there were several antiplatelet agents showing inconsistencies among endoscopists or without a standard policy regarding their managements. The major reason may be limited evidence on antiplatelet agents. However, in the Japanese guideline, the duration of discontinuation before procedures for aspirin and ticlopidine is only described from the data based on the quantitative bleeding time test and the platelet aggregation test in healthy Japanese.[15,16] Even when referring to Western guidelines, a clear description of the management of antiplatelet agents is also not made; the possibility of aspirin, ticlopidine, clopidogrel and dipyridamole interfering with platelet aggregation is only mentioned.[2–7] From this

