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Reactivation of CDX2 in Gastric Cancer as Mark for Gene Silencing Memory

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To explore the epigenetic mechanism that reactivates CDX2 (a homeobox transcription factor that serves as a tumor-suppressor gene) in intestinal-type gastric cancer during cancer progression, we examined the methylation status of the CDX2 gene promoter and the expression pattern of methyl-CpG binding protein-2 (MeCP2). From archives of the pathology records of surgically excised advanced stomach cancer cases in the Department of Molecular Pathology, Ehime University in a past decate (n=265), 10 cases of intestinal-type tubular adenocarcinoma, well-differentiated type (wel) with minor poorly-differentiated adenocarcinoma (por) components were selected. The expression pattern of CDX2, MUC2 and MeCP2 in these 10 cases was analyzed by immunohistochemistry. The cancerous and non-cancerous areas were selectively obtained by microdissection, and the methylation status of the CDX2 promoter of each area was assessed by methylation-specific polymerase chain reaction (MSP). In all 10 cases, CDX2 expression was clearly observed in the nucleus of the non-cancerous background of the intestinal metaplasic area, where the unmethylation pattern of the CDX2 gene promoter prevailed with reduced MeCP2 expression. In this metaplastic area, CDX2 expression was co-localized with its target gene, MUC2. CDX2 expression then disappeared from the deep invasive wel area. Reflecting the reduced CDX2 expression, microdissected samples from all the wel areas showed hypermethylation of the CDX2 gene promoter by MSP, with prominent MeCP2 expression. Interestingly, while hypermethylation of the CDX2 gene promoter was maintained in the por area in 8 of the 10 cases, CDX2 expression was restored in por areas where MeCP2 expression was markedly and selectively reduced. The other two cases, however, showed a constant MeCP2 expression level comparable to the surrounding deep invasive wel area with negative CDX2 expression. Therefore, gene silencing by hypermethylation may be overcome by the reduction of methyl-CpG binding proteins, resulting in apparent but non-functional reactivation of CDX2 as a mere molecular mark for gene silencing memory.

Key words: gastric cancer, DNA methylation, CDX2, MeCP2, immunohistochemistry

I. Introduction

CDX2 is a homeobox transcription factor essential for embryogenesis and differentiation of intestines [36]. Ectopic CDX2 expression is frequently observed in gastric mucosa with intestinal metaplasia related to chronic inflammation or aging [3, 27, 35]; it is also implicated in the pathogenesis of Barrett's esophagus [25, 31] where gastro-esophageal reflux such as bile acids are able to induce the ectopic intestinal differentiation program through the up-regulation of NF-κB and CDX2 [15]. While CDX2 expression is maintained in normal colonic enterocytes as an essential transcription factor for differentiation [11] and is

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Table 1. *Clinicopathlogical background, CDX2 and MeCP2 expression, and CDX2 promoter methylation status of 10 selected cases*

No.	Age	Sex	CDX2 methylation (U/M)					TNM, Ope
			PGM	IM	Wel-s	Wel-d	Por	
1	58	M	U=M	U>M	U=M	U<M	U<M	pT3pN1pM0, eSTG
2	76	M	U=M	U>M	U<M	U<M	U<M	pT4apN1pM0, STG
3	72	F	U=M	U>M	U=M	M	M	pT3pN0pM0, eSTG
4	75	M	U=M	U	U=M	U<M	M	pT3pN1pM0, TG
5	75	F	U=M	U>M	U>M	M	M	pT4apN1pM0, eSTG
6	66	M	U=M	U>M	M	M	M	pT4apN0pM0, TG
7	77	M	U=M	U>M	U>M	M	M	pT3pN1pM0, eSTG
8	72	M	U=M	U	U>M	M	M	pT4apN1pM0, eSTG
9	78	F	U=M	U>M	U>M	U<M	M	pT4apN0pM0, STG
10	69	M	U=M	U	U=M	M	M	pT3pN0pM0, eSTG

□ CDX2−/MeCP2−, ▒ CDX2−/MeCP2+, ▒ CDX2+/MeCP2−, ■ CDX2+/MeCP2+

usually retained during the early stages of carcinogenesis as a tumor suppressor gene [5], its loss has been implicated in high tumor grade, advanced stage, and poor prognosis of colorectal cancers [1, 2]. Similarly, CDX2 expression is usually retained in the early stages of carcinogenesis in a subset of adenocarcinoma arising from intestinal-type epithelium in the stomach [17, 42], Barrett esophagus [31, 40] and the ovaries [12]; the expression is recurrently lost as the tumor progresses to more advanced stages [30, 44]. While the tumor-suppressive role of CDX2 is apparent, whether its aberrant expression observed in some advanced gastric cancers plays an oncogenic role is still unclear.

Gene silencing by DNA methylation is, on the other hand, one of the important epigenetic gene silencing mechanisms during X-chromosome inactivation [32], genome imprinting [22], and cell- and tissue-specific gene repression during embryogenesis and organogenesis [22, 41]. Alterations of DNA methylation, known as the epigenetic pathway [38, 39], also play critical roles in carcinogenesis and tumor progression, and genome-wide hypomethylation and CpG-island hypermethylation [29, 34] are general characteristics of cancer cells. As one of the representative target genes of the epigenetic pathway, silencing of CDX2 gene expression in advanced stages of cancer deriving from intestinal type epithelium is typically achieved by hypermethylation of the CpG-island located in its gene promoter [9, 16].

In this study, to address the issue of the pathological significance of the aberrant expression of CDX2 in advanced gastric carcinogenesis, archival samples of surgically resected gastric cancer arising from intestinal metaplasia with heterogeneous histopathological populations were selected and examined for CDX2 expression together with its methylation status and expression level of methyl-CpG binding protein-2 (MeCP2) [21].

II. Materials and Methods

Subjects

From archives of surgically excised advanced stomach cancer cases exmanined in the Department of Molecular Pathology, Ehime University in a past decate (n=265, M:F=2.1, mean age=72.5), 10 cases of advanced intestinal-type tubular adenocarcinoma, well-differentiated type (wel) with poorly-differentiated adenocarcinoma (por) components were selected (Table 1).

Immunohistochemistry

The expression pattern of CDX2, MUC2 and methyl-CpG binding protein-2 (MeCP2) in the 10 cases were analyzed by immunohistochemistry. Paraffin-embedded tissues were cut and dewaxed through a series of graded alcohol. After antigen retrieval by microwave (Citrate, pH 6) for 10 min, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min. The specimens were then incubated with 2% non-fat dry milk in phosphate-buffered saline (PBS) for 10 min and with primary antibodies against CDX2 (M3636, DAKO, Tokyo, Japan), MUC2 (NBP1-31231, Novus Biologicals, USA, CO) and MeCP2 (D4F3, Cell Signaling Technology, Inc., USA, MA) for 15 min. After three 10-min washes with PBS, the specimens were incubated with rabbit anti-mouse IgG antibody preabsorbed with non-immunized serum. Finally, the antigen-antibody complex was immunolocalized by the streptavidine-biotin peroxidase complex method.

Microdissection and methylation-specific PCR of CDX2 promoter

The paraffin-embedded samples were deparaffinized in xylene and stained with hematoxylin and eosin (HE). The slides were microdissected under a light microscope (Leica Microsystems LMD7000) with the aid of both HE staining and CDX2 immunostaining of the serial sections. The microdissected samples were embedded in liquefied low-melting agarose to a final concentration of 1.6%. Agarose beads were formed by chilling the samples on ice and then treated with proteinase K, followed by bisulfite conversion, as previously described [19]. Finally, bead fragments were analyzed by methylation-specific PCR (MSP) using sets of primers for accessing the methylation status of the *CDX2* gene. The Promoter region of human *CDX2* genomic sequence (GenBank accession no. AL591024)

Table 2. *Primer sequences used in polymerase chain reaction (PCR)-based assays, with product size (bp) and annealing temperature*

RT-PCR	Primer sequence	Size	Temp (°C)
CDX2 MS-PCR	(F) 5′-GTTAAGGGGTTTAGGGTTGGA (R) 5′-CAAAAACTTATATTAAAAAATAAAC	183	60
Methylated	(F) 5′-GGAGTTGTTTCGATAGGAGCGC (R) 5′-TTACTAAAACCGAACTAAACGCG	71	60
Unmethylated	(F) 5′-GGAGTTGTTTTGATAGGAGTGT (R) 5′-TTACTAAAACCAAACTAAACACA	71	60

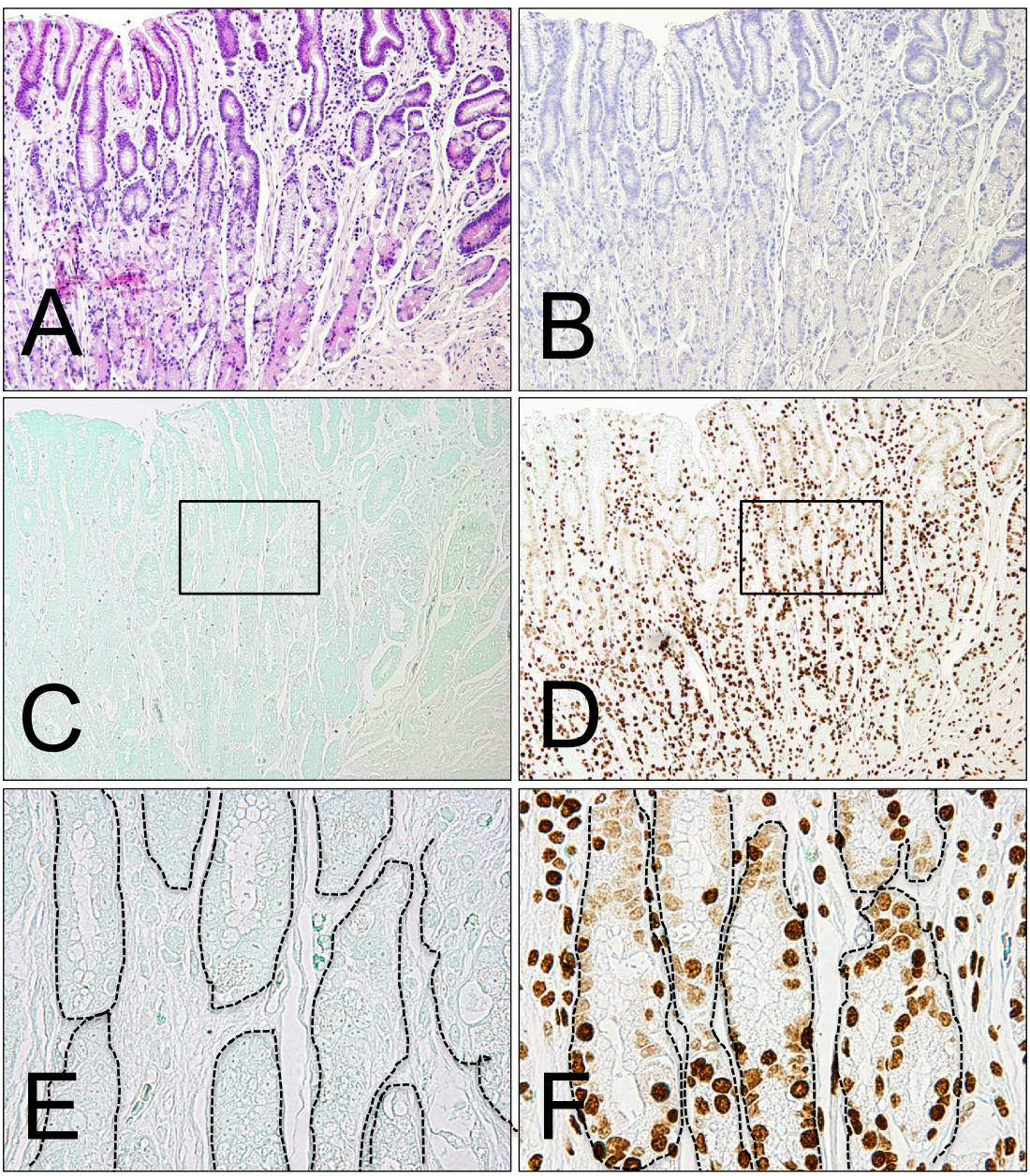

Fig. 1. Proper gastric mucosae of fundic gland area (**A**, HE, ×100) show negative MUC2 (**B**, ×100) and CDX2 (**C**, ×100 and **E**, ×400, hatched areas of **C**) expression. MeCP2 expression is, albeit less extensive than surrounding inflammatory cells, positive in the nuclei of proper gastric glands (**D**, ×100 and **F**, ×400, hatched areas in **D**).

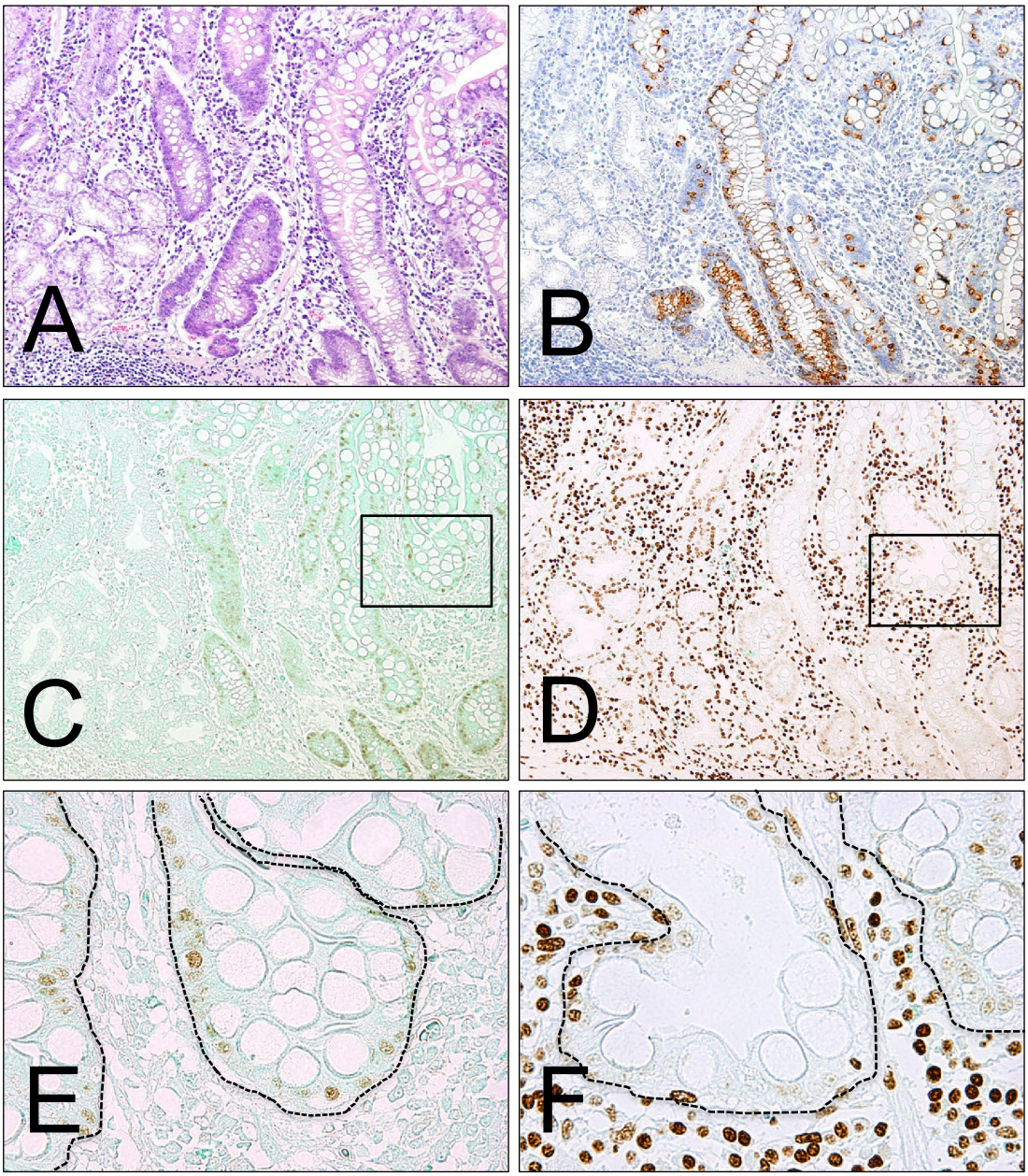

Fig. 2. In the intestinal metaplastic area (**A**, HE, ×100), MUC2 (**B**, ×100) expression is colocalized with that of CDX2 (**C**, ×100 and **E**, hatched areas of **C**, ×400). Compared with the non-metaplastic glands (Fig. 1D and F), and surrounding inflammatory cells, MeCP2 expression is positive in nuclei of the glands in intestinal metaplastic area, but the extent is much less than that in proper gastric mucosa.

was searched for CpG islands through an online search engine (www.ebi.ac.uk/emboss/cpgplot). One of the CpG islands (AL591024 nt 28391–28683) was selected for detailed analysis by MSP. The primers for the first-step PCR (forward) 5′-gccaaggggcctagggctgga-3′, and (reverse) 5′-gttcacctcctaatacaagcctttg-3′, are designed to form a 183-bp amplicon containing 71-bp CpG sites. PCR was conducted under the following conditions: 98°C 2 min, 30 cycles (98°C 10 s, 50°C 15 s, 68°C 39 s). The primers used for second-step PCR were amplified with two primers, (forward) 5′-ggagctgccccgacaggagcg-3′, and (reverse) 5′-cgcgcccagctcggtttcagcaa-3′ (Table 2), under the following conditions: 98°C 2 min, 25 cycles (98°C 10 sec, 60°C 15 sec, 68°C 30 sec) [24]. The PCR mixture contained Mighty AMP® DNA polymerase (Takara, Tokyo, Japan) and bead fragments in a final volume of 25 µl. The PCR products were electrophoresed in a 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

III. Results

Immunohistochemistry

The non-cancerous background of the 10 cases showed both proper (non-metaplastic) gastric mucosa and

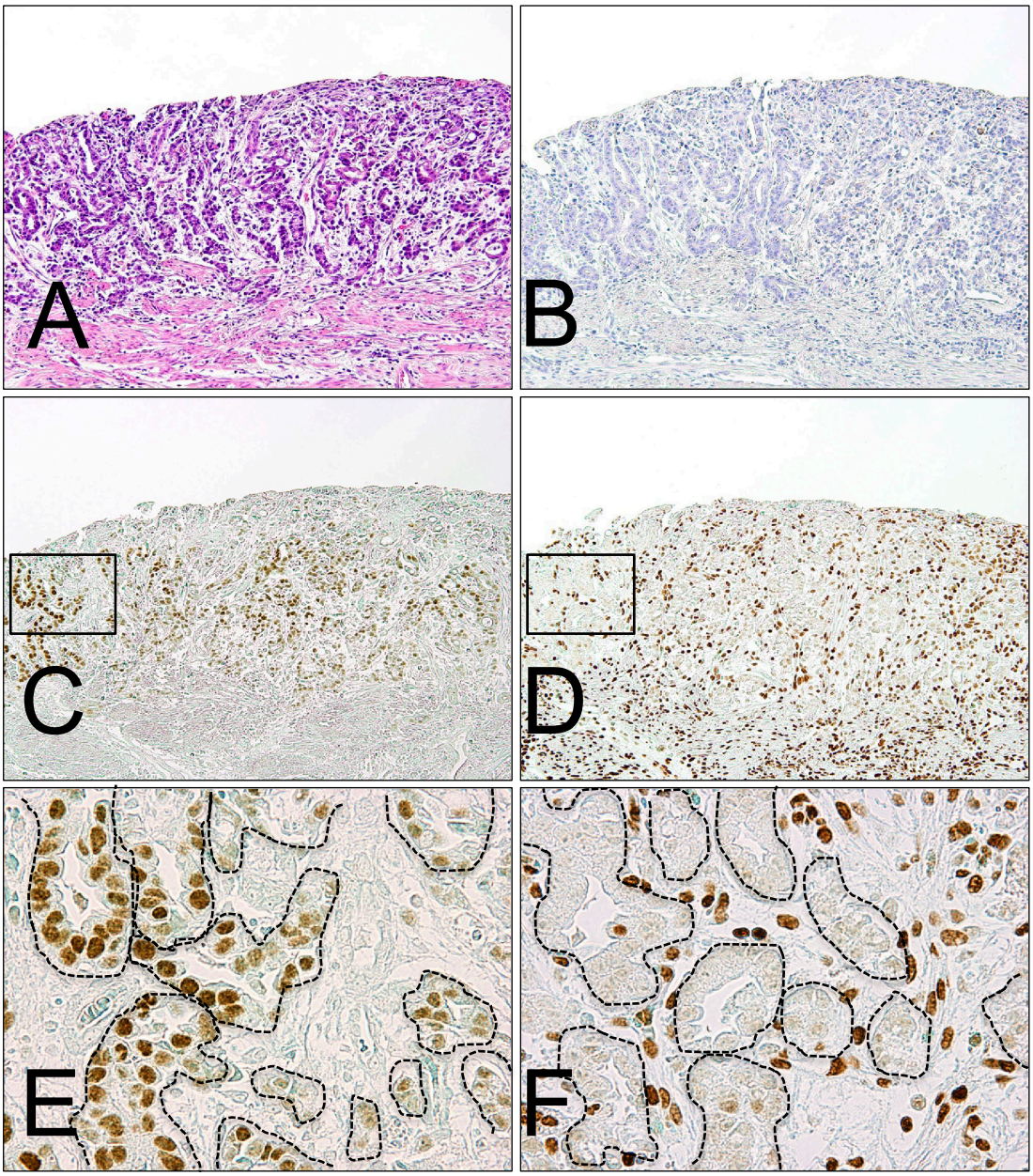

Fig. 3. In the superficial wel area (**A**, HE, ×100), while MUC2 expression is almost negative (**B**, ×100), CDX2 (**C**, ×100 and **E**, ×400, hatched areas of **C**) expression is partly but clearly preserved. MeCP2 expression (**D**, ×100 and **F**, ×400, hatched areas in **D**) is negative in most of the wel glands.

intestinal metaplastic areas. In the proper gastric mucosa of the fundic gland area (Fig. 1A, HE, case 5 in Table 1), MUC2 (Fig. 1B) and CDX2 (Fig. 1C and E, hatched area of C) expression was totally negative, while MeCP2 expression was, albeit less extensive than surrounding inflammatory cells, positive in the nuclei of proper gastric glands (Fig. 1D and F, hatched areas of D). In the intestinal metaplastic area (Fig. 2A, HE, case 5 in Table 1), on the other hand, MUC2 (Fig. 2B) and CDX2 (Fig. 2C and E, hatched areas of C) expression was clearly and selectively observed among metaplastic glands. While some nuclei of glands in the intestinal metaplastic area were positive for MeCP2 expression, the extent was much less than that in proper gastric mucosa (Fig. 2D and F, hatched areas of D) and surrounding inflammatory cells. In the superficial wel area (Fig. 3A, HE), while MUC2 expression was almost negative (Fig. 3B), CDX2 (Fig. 3C and E, hatched areas of C) expression was partly but clearly preserved in six cases (cases 4 to 10), where MeCP2 expression (Fig. 3D and F, hatched areas of D) was almost negative in the wel glands (F, hatched area of Fig. 3D). On the other hand, glands in the deep invasive area (Fig. 4A, HE) showed negative CDX2 and MUC2 immunoreactivity (Fig. 4B, C and E, hatched areas of C), where MeCP2 was strongly positive in

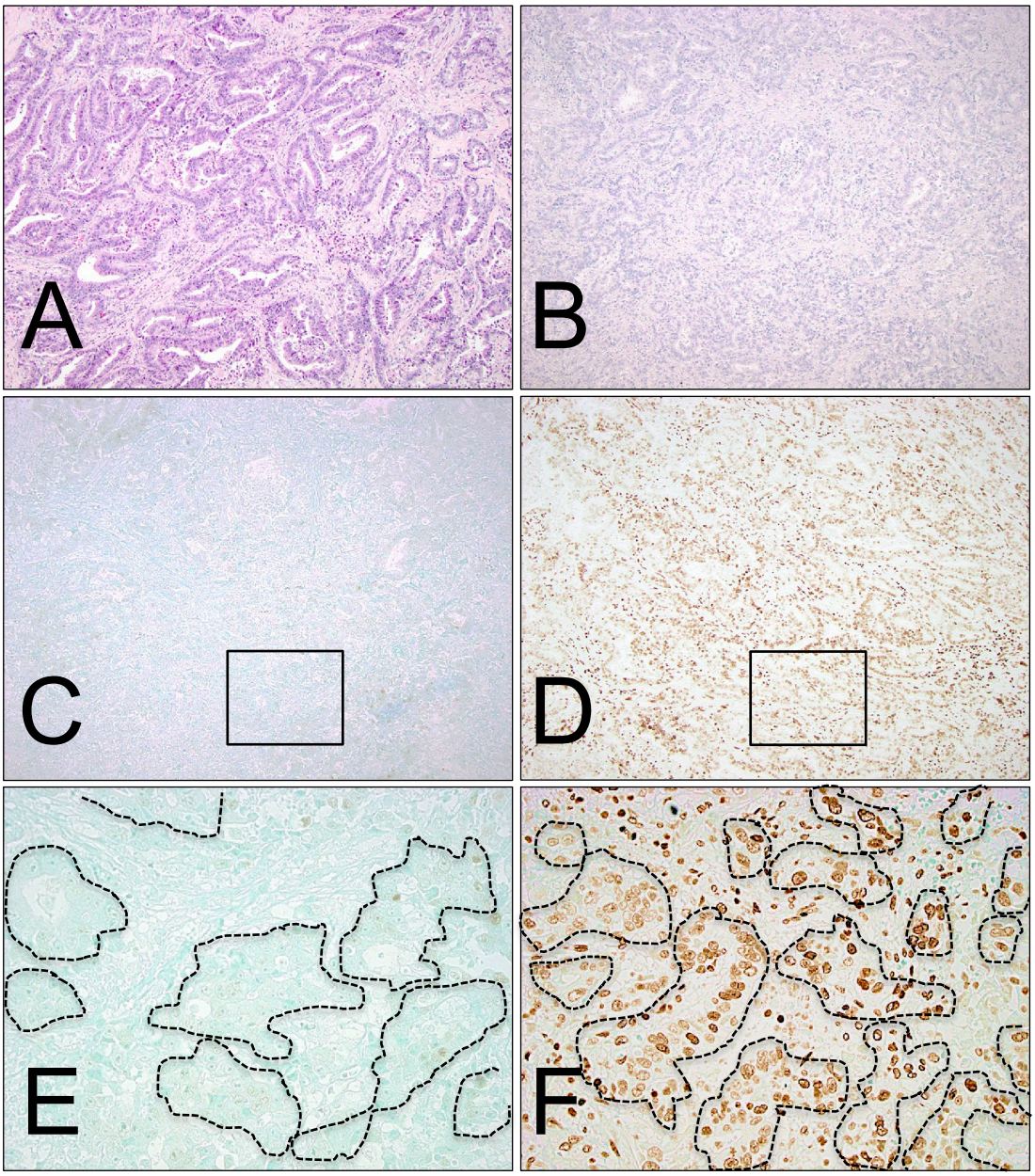

Fig. 4. Wel glands in the deep invasive area (**A**, HE, ×100) show negative for CDX2 (**B**, ×100) and MUC2 (**C**, ×100, and **E**, ×400, hatched area of **C**) immunoreactivity, where MeCP2 is strongly positive in the nuclei of the invasive wel glands (**D**, ×100, and **F**, ×400, hatched area of **D**).

the nuclei of the invasive wel glands (Fig. 4D and F, hatched area of D) in all 10 cases. Among the 10 cases selected solely on the basis of morphological features involving por elements adjacent to the wel area, immunohistochemical analysis showed, suprisingly, as many as nine cases of the reactivation of CDX2 expression. Figure 5A (case 5 in Table 1) illustrates HE staining of the por area with distorted cancer nests and increased cellularity. While MUC2 expression remained negative (Fig. 5B), CDX2 expresson was evident in the nuclei of the por elements (Fig. 5C and E, hatched areas of C) where MeCP2 expression became negative (Fig. 5D and F, hatched areas of D).

Changes in methylation status of CDX2 gene promoter

In proper stomach glands, both unmethylated and methylated bands for CDX2 gene promoter was equally detected in all ten cases by MSP (Fig. 6A, case 5 in Table 1). Similarily, in intestinal metaplasia, both unmethylated and methylated bands for CDX2 gene promoter was detected in all ten cases by MSP (Fig. 6B, case 5 in Table 1).

In metaplastic glands with goblet cells, albeit conventional MSP is not an accurate quntitative assay, unmethylated populations were more prevalant than methylated ones. In the superficial wel area where CDX2 expression was still maintained, hypermethylation of the CDX2 gene promoter was noted (Fig. 6C) in three of six cases exam-

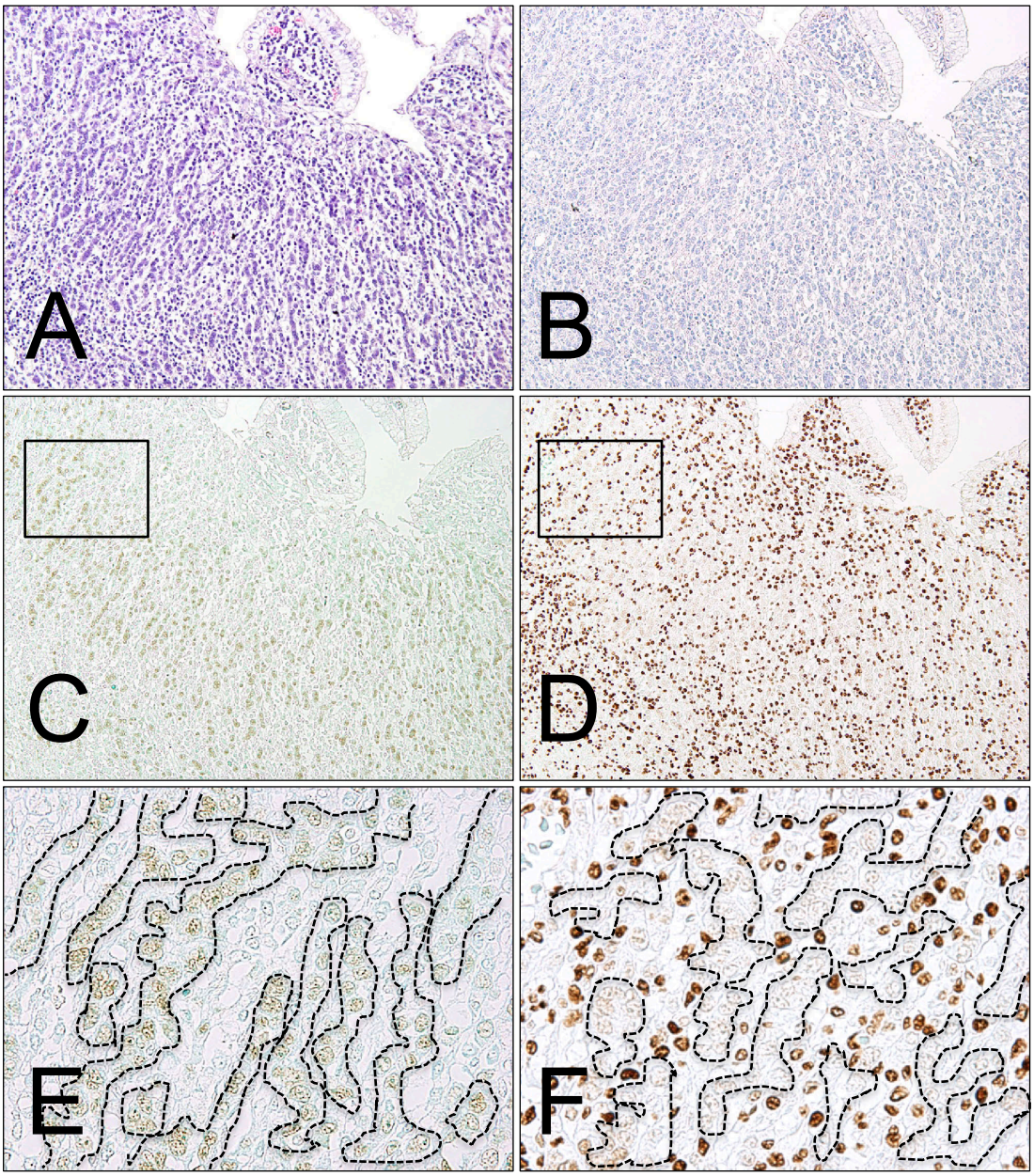

Fig. 5. In the area where por elements are seen in a portion of the wel area (**A**, ×100), without its target gene, MUC2 expression (**B**, ×100), strong CDX2 expression (reactivation, **C**, ×100, **E**, ×400, hatched area of **C**) is evident in the nuclei of the por elements. MeCP2 expression is almost negative in the por areas (**D**, ×100 and **F**, ×400, hatched areas of **D**).

ined (cases 4, 5, and 6 in Table 1); all three cases showed negative MeCP2 immunostaining in the nuclei of the glands in the wel area (Fig. 3F). CDX2 gene expression then disappeared from the deep invasive wel areas of all 10 cases. Reflecting the reduced CDX2 gene expression, microdissected samples from all the wel areas showed hypermethylation of the CDX2 gene promoter (Fig. 6D) with prominent nuclear MeCP2 immunostaining. Interestingly, except for cases 1 and 2, while hypermethylation of the CDX2 gene promoter was maintained in the por area, CDX2 expression was restored in the por area in eight cases, where MeCP2 expression was markedly and selectively reduced. The two other cases, however, showed negative CDX2 expression with a constant MeCP2 expression level compared with the surrounding deep invasive wel area.

IV. Discussion

In this study, examination of CDX2 expression, the methylation status of the CDX2 gene promoter, and MeCP2 expression with the use of microdissection in 10 selected cases of advanced gastric adenocarcinoma involving heterogeneous histological components at the cellular level

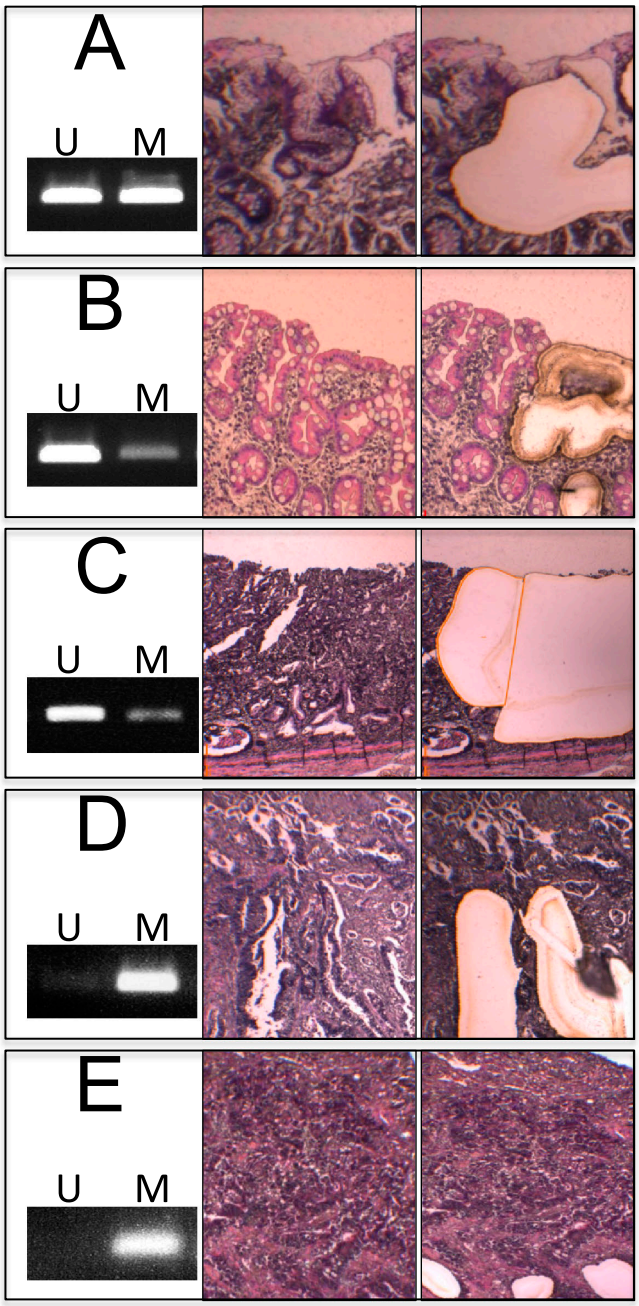

Fig. 6. Microdissected samples were bisulfite converted and subjected to MSP to analyze the methylation status of CDX2 gene promoter. Methylation is seen in proper gastric mucosa (**A**), and then became hypomethylated in glands with intestinal metaplasia (**B**). Hypermethylation of the CDX2-gene promoter is evident in wel in both superficial (**C**) and deep invasive (**D**) areas. Hypermethylation is evident at por area (**E**) where CDX2 expression is reactivated.

revealed that CDX2 gene silencing was achieved only when hypermethylation of the CDX2 gene promoter occurred synchronously with a relatively high level of MeCP2 expression.

While CpG-dinucleotides within CpG-islands located at the promoter region of transcriptionally active genes tend to be hypomethylated [10], accumulation of methylated cytosine in CpG-islands of specific genes has been attributed to transcriptional inactivity, resulting in tumor suppressor gene inactivation during carcinogenesis and tumor progression [14]. Currently, the precise mechanism of the suppression of gene expression through hypermethylation of CpG-islands is categorized into two major ways: first, direct sequestration of methylation-sensitive transcriptional factors that must interact with GC-rich binding sites in their DNA recognition elements in the major grooves of the double helix [13], and second, achieved by recruitment of methylcytosine binding proteins (MBPs) that recognize methylated DNA [4]. Furthermore, the latter MBP-mediated suppressing mechanism consists of two auxiliary machineries: 1) methyl-CpGs included in or around the promoter region of genes recruit MBPs, resulting in direct blocking of transcriptional activators to their binding elements [4] and 2) mediation by MBPs in a sequence-independent process that involves global histone modifications by transcriptional repressors, leading to the formation of inactive (closed or condensed) heterochromatin [6]. Among these methyl-CpGs-mediated gene-silencing mechanisms, formation of heterochromatin by global histone modification is universal and fundamental in epigenetic gene regulation in higher organisms [6]. Among MBPs, since MeCP2 is the most abundantly expressed and central to recruitment of transcriptional repressors, a sufficient amount of residing MeCP2 expression is crucial for efficient epigenetic gene silencing by methyl-CpGs [21, 26]. Indeed, in our previous study, reduced MeCP2 expression also derepresses or reactivates the epigenetically silenced cyclin D1 gene during spermatogenesis [8] and the E-cadherin gene during tumor progression [7]. MeCP2, on the other hand, preferentially or with high affinity, binds to DNA sequences containing A/T-rich bases (more than four continuous A/T sequences) adjacent to methyl-CpG [20], and indeed, only a single methyl-CpG adjacent to TATA-box and a small amount of residing MeCP2 expression is sufficient to sequester TATA-box binding protein binding to the transcription start site in human sFRP-4 [28] and mouse RANKL genes [18]. Thus, reduced residing MeCP2 expression may evoke global but selective reactivation of genes silenced mainly by the formation of heterochromatin in a sequence-independent manner, while keeping silenced another certain set of specific genes with a sequence-dependent high affinity binding site for MeCP2 in their transcription start site. Further *in vitro* exosome examinations by knockdown or forced overexpression of MeCP2 are needed to prove this hypothesis.

CDX2 is a factor responsible for proliferation and differentiation of intestinal epithelial cells [37]. In many gastric cancers of the intestinal type, gradual decrease of CDX2 expression from gastric epithelial dysplasia to early and advanced gastric cancers (present in 64, 40 and 27% of the cases, respectively) has been noted [23]. Furthermore, a negative correlation has also been observed between CDX2

expression and the depth of tumor invasion [44]. Indeed in the present study, when focusing on the wel area, all 10 selected cases showed decrease of CDX2 expression from the superficial to the deep invasive area, and increase of methylation of CDX2 promoter. Nonetheless, in eight cases adjacent to the deep invasive wel area, while hypermethylation of the CDX2 promoter persevered, CDX2 expression reappeared in the por area, where MeCP2 expression was markedly reduced. It is not rational, however, that reactivation of the tumor suppressor gene occurs during progression to a further aggressive phenotype. Since reactivation of CDX2 is not followed by reactivation of its target gene, MUC2 expression [43] in these por areas, we speculate that apparent CDX2 expression in the late progression phase may have lost its cognate function as a tumor suppressor gene.

In conclusion, our data illustrate that gene silencing by hypermethylation can be overcome by the reduction of methyl-CpG binding proteins, resulting in a mere molecular mark for gene silencing memory [24, 33]. When characterizing cancer cells by immunophenotyping, since immunohistochemical results may simply reflect the result of reactivation as a mere gene silencing memory and have no biochemical significance, any apparent positive results need to be interpreted carefully with the help of the hypermethylation status of the gene promoter.

V. Author Contributions

Kameoka Y performed the majority of experiments and analyzed the data; Ariyasu K, Tachibana R and Haraguchi R carried out the molecular investigations; Mizuno Y and Kitazawa R participated in histopathological examinations and confirmation of diagnosis; Kitazawa S designed and coordinated the research; Kameoka Y, and Kitazawa S wrote the paper.

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