phenotypic markers. Thus, EBV (+) carcinomas appeared to lose phenotypic markers during progression from differentiated to undifferentiated structure ($P<0.02$).

Relations between EBV infection and grading of gastritis surrounding non-neoplastic mucosa

Data for comparisons between EBV (+) and EBV (-) cases regarding the grade of gastritis surrounding non-neoplastic mucosa using Updated Sydney System are summarized in Table 5. The grades of mononuclear cell and neutrophil infiltration, mucosal glandular atrophy, and intestinal metaplasia showed no significant difference between the two groups.

Relations between EBV infection and expression of gastric and intestinal phenotypic markers, and Cdx2 in intestinal metaplastic glands

Data for comparisons between EBV (+) and EBV (-) cases regarding phenotypic marker and Cdx2 expression in intestinal metaplastic glands are summarized in Table 6 (Fig. 3). The average score for Cdx2 expression was significantly lower in EBV (+) than in EBV (-) cases ($P=0.016$). Regarding the other phenotypic markers, there were no significant differences between the two groups.

Discussion

Cdx2 is important for the maintenance of intestinal phenotypic expression not only in the normal small and large intestine (Silberg et al., 2000), but also in intestinal metaplasia (Mizoshita et al., 2001; Almeida et al., 2003; Tsukamoto et al., 2004) and carcinomas of the stomach (Almeida et al., 2003; Mizoshita et al., 2003). Cdx2 nuclear expression can be detected in approximately half of advanced stomach cancers (Mizoshita et al., 2003) and about 80% of early lesions (Mizoshita et al., 2004a,b). Many stomach cancers have expression of genes associated with induction and maintenance of the differentiation of small and large intestine, such as Cdx2 and Cdx1 (Chen et al., 2003). However, our present data provide clear evidence that Cdx2 expression is less frequent in EBV (+) than in EBV (-) stomach cancers.

Table 5. Correlation between EBV infection and status of surrounding non-neoplastic mucosa.

	The average grades in surrounding mucosa[a]			
	Neutrophils	Mononuclear Cells	Atrophy	Intestinal Metaplasia
EBV (+) stomach cancer (n=26)	1.154±0.107	1.692±0.173	1.154±0.120	0.577±0.173
EBV (-) stomach cancer (n=57)	1.175±0.087	1.474±0.118	1.140±0.088	0.720±0.120
P-values[b]	P=0.914	P=0.324	P=0.879	P=0.504

[a]: Each score is average±standard error (SE) for Updated Sydney System; [b]: Each *P*-value is analyzed by Mann-Whitney U test.

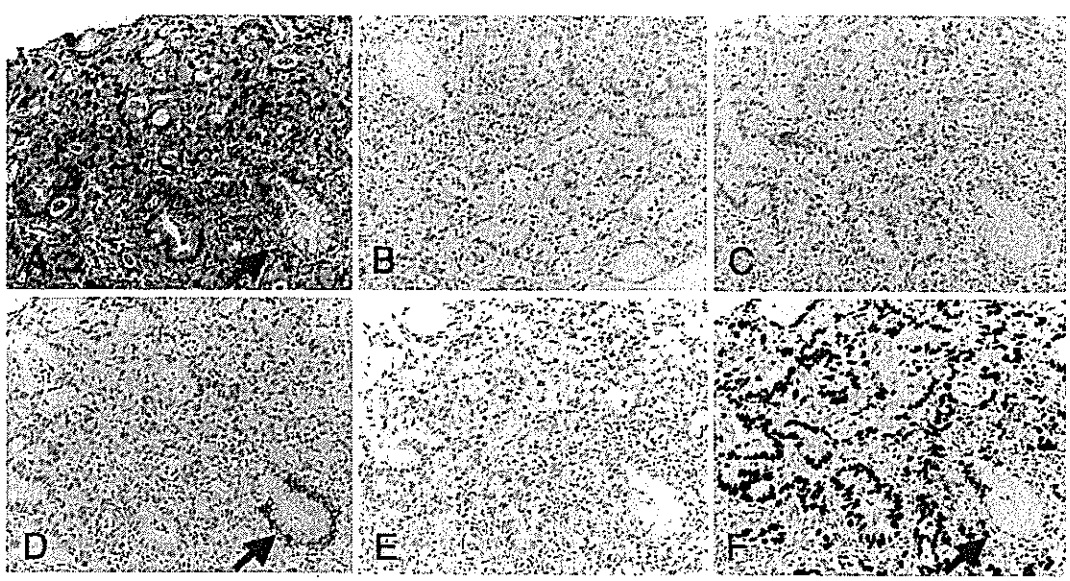

Fig. 1. An EBV (+) stomach cancer. A. HE staining. B. Note the lack of Cdx2 nuclear staining in the cancer cells. C. No MUC2 expression is detected in the cytoplasm of tumor cells. D. MUC5AC is present in the cytoplasm of normal gastric foveolar epithelium (red arrow), but not cancer cells. E. No MUC6 expression is apparent in the cytoplasm of tumor cells. F. EBER-1 is positive in the nuclei of cancer cells, but not normal gastric foveolar epithelium (arrow). x 200; EBER-1, EBV-encoded small RNA-1.

Chen et al. (2003) similarly found expression of intestinal specific genes to be lower in EBV (+) stomach cancers, as compared with EBV (-) lesions. Regarding the regulation of MUC2 expression, Yamamoto et al. (2003) have demonstrated that Cdx2 interacts with the MUC2 promoter and activates MUC2 transcription. Lee et al. (2004) have previously shown that there is negative association between EBV infection and expression of MUC2 in stomach cancers, again in line with the our present data (Table 2). Therefore, we consider that the absence of Cdx2 and MUC2 is linked in EBV (+) stomach cancers.

We also here demonstrated that stomach cancers are more likely to be of N type in the EBV (+) group, in line with the previous report that EBV (+) stomach cancers have lower MUC5AC and MUC2 expression than their EBV (-) counterparts (Lee et al., 2004). EBV associated stomach carcinomas are reported to lack intestinal phenotypic expression (Chen et al., 2003) and most EBV (+) stomach cancers were here classified phenotypically as N or G types (Table 3). Nakamura et al. (2005) also previously showed the G type to be more common in EBV (+) cases.

Several reports have shown that EBV (+) stomach

Table 6. Comparison of phenotypic markers in differentiated and undifferentiated regions in EBV (+) and EBV (-) stomach cancer cases.

Case No.	EBER-ISH	Histology	Phenotypes in total area	Phenotypical marker expression in each region		Ratio of N types in U region[a]
				D region	U region	
1	+	D>U	G	G	N	N=3/6 (50%)
2	+	D>U	I	I	I	
3	+	U>D	G	G	G	
4	+	U>D	G	G	G	
5	+	U>D	G	G	N	
6	+	U>D	I	I	N	
1	–	D>U	GI	GI	GI	N=0/9 (0%)
2	–	D>U	I	I	I	
3	–	U>D	G	G	G	
4	–	U>D	G	G	G	
5	–	U>D	G	G	G	
6	–	U>D	GI	GI	GI	
7	–	U>D	GI	GI	I	
8	–	U>D	GI	GI	I	
9	–	U>D	I	I	I	

[a]: P<0.02 (Fisher's exact test). Abbr.: D, differentiated; U, undifferentiated; G, gastric; I, intestinal; GI, gastric-and-intestinal-mixed; N, null.

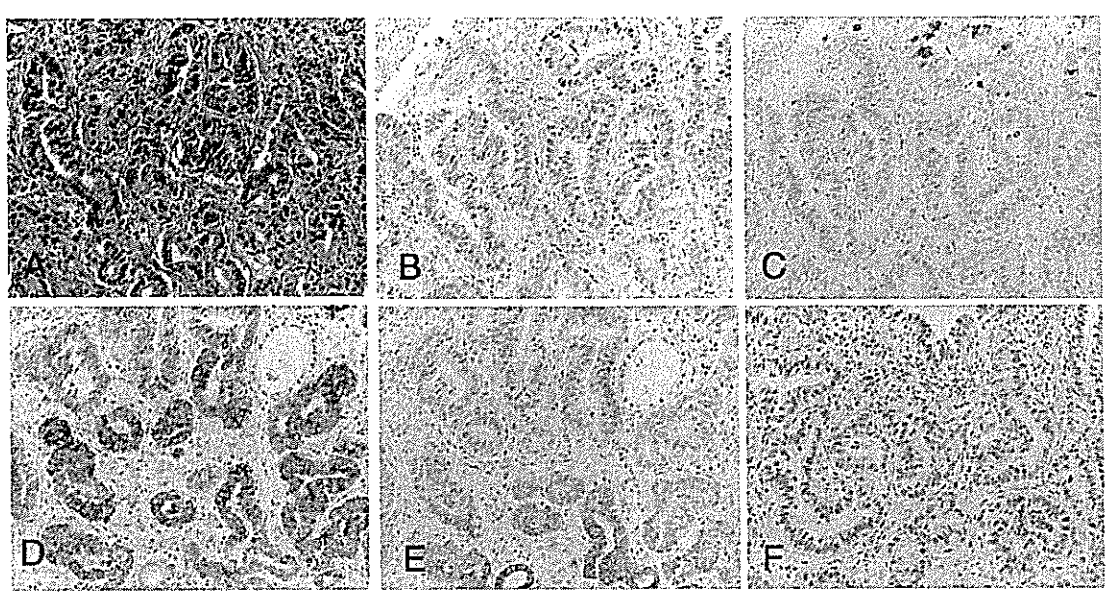

Fig. 2. An EBV (-) stomach cancer. A. HE staining. B. Cdx2 nuclear staining is positive in some cancer cells. C. MUC2 expression is detected in the cytoplasm of some tumor cells. D. MUC5AC is present in the cytoplasm of the cancer cells. E MUC6 is apparent in the cytoplasm of some tumor cells. F. EBER-1 is negative in the nuclei of the cancer cells. x 200; EBER-1, EBV-encoded small RNA-1.

cancers are most often undifferentiated histopathologically, according to the Japanese Classification of Gastric Carcinomas (Yanai et al., 1997; Wu et al., 2000; Lee et al., 2004). EBV (+) stomach cancers are more frequently moderately differentiated tubular adenocarcinomas (tub2), and solid poorly differentiated adenocarcinomas (por1) as compared with other histological types (Carrascal et al., 2003). To avoid bias, phenotypic expression was here evaluated in morphologically matched samples for EBV (+) and EBV (-) cases.

Regarding the histogenesis of EBV associated stomach cancers, Fukayama et al. (2001) previously suggested the hypothesis that they develop by clonal expansion of rare EBV-infected epithelial cells within stomach mucosa. EBV infection of intestinal metaplastic cells is unlikely (Fukayama et al., 2001). We have argued that the origin of stomach cancers is from progenitor cells specializing towards mucous differentiation in the fundic/pyloric glands, rather than intestinal metaplastic glands (Tatematsu et al., 2005). With EBV infection the histogenesis may be from cells that are specialized towards mucous differentiation in the fundic/pyloric glands, harboring neither typical gastric nor intestinal phenotypic expression.

In the present study, inflammatory response in the surrounding non-neoplastic mucosa was not statistically different between EBV (+) and EBV (-) cases. So EBV may not have significantly induced inflammatory cell infiltration in our Columbia cases. The Cdx2 expression in the intestinal metaplastic glands was also lower in non-neoplastic mucosa of EBV (+) cases, despite no EBV infection being observed by in situ hybridization. However, the presence of EBV in non-carcinomatous surrounding mucosa of EBV (+) stomach cancers has been detected by immunostaining of EBNA-1 and latent membrane protein 1 (LMP-1) (Yanai et al., 1997a,b). Hayashi et al. (1996) detected EBV in gastric glands with IM. Yanai et al. (1999) reported the evidence that all eight lesions of EBER-1-positive gastric carcinomas had intestinal metaplasia in the background among 8 EBER-1-positive stomach carcinomas. In contrast, Kaizaki et al. (1999) reported that only 13% of EBV (+) stomach cancers were surrounded by intestinal metaplasia, in contrast to 41% of EBV (-) ones. Zur Hausen et al. (2004) concluded that EBER-1/2 transcripts were restricted to the carcinoma cells in accordance with exclusive positivity of EBNA-1 immunohistochemistry (IHC) to the tumor cells. Negative LMP-1 IHC in all cases tested and absence of EBER-1/2 transcripts in preneoplastic gastric lesions (intestinal metaplasia and dysplasia) strongly suggested that EBV could only infect neoplastic gastric cells, indicating it as a late event in gastric carcinogenesis.

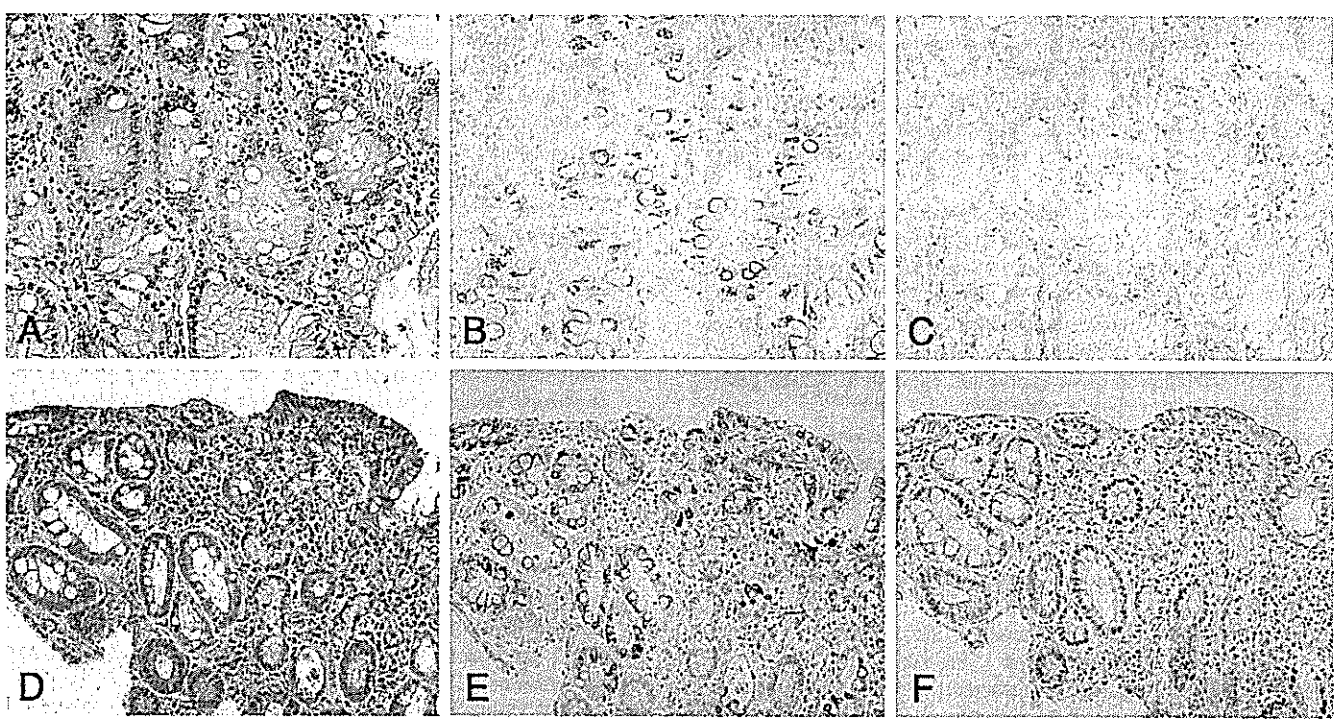

Fig. 3. Expression of MUC2 and Cdx2 in intestinal metaplastic glands in tissue surrounding adenocarcinomas. EBV (+) (A-C) and EBV (-) (D-F) stomach cancers. A and D. HE staining. B and E. MUC2 is detectable in the cytoplasm of intestinal metaplastic glands. C and F. No Cdx2 nuclear staining in intestinal metaplastic glands in an EBV (+) case (C) in contrast to apparent nuclear staining in an EBV (-) case. x 200.

Thus down regulation of Cdx2 might not be due to infection of EBV to the surrounding mucosa. EBV (+) stomach cancer and surrounding intestinal metaplasia were similar to down regulation of Cdx2. We considered EBV might have infected the progenitor cell or stem cell after late event in gastric carcinogenesis and intestinal metaplasia, and the down regulation of Cdx2 were similar mechanism to EBV (+) stomach cancer and surrounding intestinal metaplasia. Further studies of EBV infection in non-neoplastic stomach epithelia appear warranted.

In conclusion, EBV (+) stomach cancers are characterized by a relative lack of intestinal phenotypic expression, including Cdx2, and only occasional presence of gastric phenotypic expression. The progenitor cell may thus be specialized towards mucous differentiation in the fundic/pyloric glands.

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Review

Colorectal cancer: genetics of development and metastasis

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It has been well documented that there are two major pathways in colorectal carcinogenesis. One is the chromosomal instability pathway (adenoma–carcinoma sequence), which is characterized by allelic losses on chromosome 5q (*APC*), 17p (*p53*), and 18q (*DCC/SMAD4*), and the other is a pathway that involves microsatellite instability. Recent progress in molecular biology, however, has shown that colorectal carcinogenesis is not necessarily clearly divided into these two pathways, but is in fact more complicated. Other routes, including the transforming growth factor-β/SMAD pathway, the serrated pathway, and the epigenetic pathway, have been reported. Cross talk among these pathways has also been reported. In the invasion and metastasis steps of colorectal cancers, many more genes have now been identified as being involved in proteolysis, adhesion, angiogenesis, and cell growth. Recently accumulated evidence indicates that colorectal cancer is a genetically heterogeneous and complicated disease.

Key words: colorectal cancer, chromosomal instability, microsatellite instability, metastasis

Introduction

It is now generally accepted that colorectal cancer develops by genetic alterations. Analysis of the molecular mechanism makes it possible to develop a more targeted approach to prevention and treatment of this cancer. There are two major pathways in colorectal carcinogenesis. One is the chromosomal instability pathway (adenoma–carcinoma sequence), which is characterized by allelic losses, and the other is a pathway involving microsatellite instability (MSI). However, recent studies have suggested that colorectal carcinogenesis is not necessarily clearly divided into these two pathways. Other routes, including the serrated and epigenetic pathways, have been reported. There is also some cross talk among these pathways. Moreover, in the progression and metastasis steps of colorectal cancers, many more gene alterations are involved.

In this review, we describe the latest molecular biology of carcinogenesis and metastasis in colorectal cancers.

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Chromosomal instability pathway

Fearon and Vogelstein[1] proposed a multistep model of colorectal carcinogenesis, in which mutations in the adenomatous polyposis coli (*APC*) gene occur early during the development of polyps, *K-ras* mutations arise during the adenomatous stage, and mutations of *p53* and deletions on chromosome 18q occur concurrently with the transition to malignancy. This pathway is characterized by allelic losses on chromosome 5q (*APC*), 7p (*p53*), and 18q (*DCC/SMAD4*), and is therefore called the chromosomal instability (CIN) pathway. One of the cornerstones of the CIN pathway is the model represented by familial adenomatous polyposis (FAP), in which multiple small adenomas form as a result of initiation of tumorigenesis, namely, two hits in the *APC* gene, followed by mutations of *K-ras*, and subsequently mutations of *p53* and deletion on chromosome 18q. It has been surmised that this same mechanism is also applicable to sporadic colorectal carcinogenesis. The genes that have been reported to be involved in this pathway are listed in Table 1.

APC/β-catenin

APC is a tumor suppressor gene on chromosome 5q whose germline mutation is responsible for FAP.[2,3] APC

Table 1. Oncogene genes and tumor suppressor genes in colorectal cancers

Gene	Chromosomal location	Prevalence of mutation (%)	Function of Gene product	References
Tumor suppressor genes				
APC	5q21	34–72	Inhibition of cell growth via β-catenin degradation	8–11
P53	17p12	40–50	G1 cell-cycle arrest, apoptosis induction	18–21
SMAD	18q21	16–25	Growth arrest via p15 and p21 induction	53–55
DCC	18q21	6	Binding to netrin	34
Oncogenes				
K-ras	12p12	40–65	Growth promotion via RAF/MAPK, JNK, and PI3-K	15, 23–24
β-catenin	31q21	5	Transcription of growth promoting genes (*cyclin D1*, *c-myc*, etc.)	12

APC, adenomatous polyposis coli; DCC, deleted in colorectal cancer

protein, a member of the Wnt signal pathway, normally binds to β-catenin to form a complex with axin and GSK-3β, which is degraded through ubiquitylation.[4–6] When it is inactivated, accumulated β-catenin translocates from the lateral cell membrane to the nucleus, where it drives the transcription of multiple genes implicated in tumor growth and invasion. The large majority of *APC* mutations result in a premature stop codon and thus a truncated protein.[7] *APC* mutations are identified in approximately 30%–70% of sporadic adenomas and in 34%–72% of sporadic cancers.[8–11] About 50% of sporadic tumors with intact *APC* are reported to show mutations of β-catenin itself, resulting in the accumulation of β-catenin.[12] Thus, the APC/β-catenin pathway is considered to play a major role in colorectal carcinogenesis.

However, recent studies have claimed that the APC/β-catenin pathway is not necessarily invaluable first genetic alterations in colorectal cancer. For example, Umetani et al.[13] reported that the frequency of the *APC* mutation is 7% in flat tubular adenomas and 36% in polypoid tubular adenomas. We analyzed APC mutations in aberrant crypt foci (ACF), putative precursors of adenoma and cancer, by an in vitro synthesized protein (IVSP) assay and found no APC mutations in dysplastic or nondysplastic ACF.[14,15] Moreover, no β-catenin accumulation was observed in these lesions. Although one study reports APC mutations in dysplastic ACF, the positive rate was low.[16]

p53

p53 is the most commonly mutated tumor suppressor gene in various kinds of malignant tumors. p53 protein normally induces G1 cell-cycle arrest to facilitate DNA repair during replication, or it induces apoptosis. *p53* mutations are generally considered to occur at the time of the transition from adenoma to cancer.[1] Most mutations occur in highly conserved areas of exons 5 to 8.[17] Moreover, the majority (approximately 80%) are missense mutations (GC to AT), which occur principally in five hotspot codons (175, 245, 248, 273, and 282).[18] *p53* mutations have been identified in 40%–50% of sporadic colorectal cancers.[19] The frequency of *p53* mutations is higher in distal colon and rectal cancers than in proximal colon cancers.[20] Patients with cancers involving a *p53* mutation have a worse outcome and shorter survival time than patients whose cancers do not have a mutation in this gene.[21]

K-ras

K-ras mutations have been detected in various kinds of cancers, particularly in gastroenterological cancers, including colorectal, pancreatic, and bile duct cancers. They have been found in 15%–68% of sporadic colorectal adenomas and in 40%–65% of cancers.[15,22–24] The majority of *K-ras* mutations occur as an activating point mutation in codons 12, 13, and 64.[25–27] Mutated K-ras protein activates a variety of effector pathways, including RAF/MAPK, JNK, and phosphatidylinositol 3-kinase (PI3-K), leading to constitutive growth promotion. Some of the downstream gene targets of K-ras include the cyclin D1, DNA methyltransferase, and vascular endothelial growth factor (*VEGF*) genes.[28–30]

DCC/SMAD

As noted above, allelic losses on chromosome 18q have been identified in approximately 70% of primary colorectal cancers, particularly in advanced colorectal cancers with hepatic metastasis, implying that an 18q deletion may contribute to the progression of colorectal cancers.[1] The *DCC* (deleted in colorectal cancer) gene was long ago proposed as a candidate tumor suppressor gene on 18q.[31] Point mutations of the *DCC* gene have been identified in approximately 6% of sporadic colorectal cancers.[32] However, the candidacy of this gene has recently been called into question. Mice het-

erozygous for *DCC* have been reported to lack the tumor predisposition phenotype.[33] Moreover, other tumor suppressor genes, including *SMAD4/2,* have been reported on 18q.[34,35] In particular, *SMAD4* is currently a candidate gene because the inactivation of SMAD4 has been causally associated with progression of cancers. The detailed role of *DCC* in colorectal cancers needs further study.

Microsatellite instability pathway

Microsatellite instability (MSI) is characterized by expansions or contractions in the number of tandem repeats of simple DNA sequences (microsatellites). MSI has been identified in colorectal cancer associated with hereditary nonpolyposis colorectal cancer (HNPCC) syndrome,[36–38] and DNA mismatch repair (MMR) enzymes, including hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6, have since been shown to be responsible for MSI.[39–43] Moreover, mutations in microsatellites of target genes such as the transforming growth factor-β (*TGF-β*) gene (Table 2) have also been identified in MSI positive tumors.[44–50] Interestingly, MSI is also present in sporadic colorectal cancers: MSI-H (high-frequency MSI) is present in 10%–20% and MSI-L (low-frequency MSI) in 5%–50% of such cancers. In approximately 80% of MSI-H sporadic colorectal cancers, hypermethylation of the *hMLHI* promoter is observed.[51] Sporadic cancers with the MSI-H phenotype demonstrate distinct clinicopathological features compared with MSS (microsatellite stable) or MSI-L tumors, occurring predominantly in the proximal colon and more frequently in women than in men.[52] These cancers are also characterized by distinct histopathological features, including mucinous or signet-ring cell differentiation, medullary features, and excess lymphocyte infiltrations. However, neither MSI-L nor MSS tumors demonstrate such characteristic features. Moreover, MSI-L and MSS tumors more frequently have *K-ras* and *p53* mutations and loss of heterozygosity (LOH) at 5q, 19p, and 18q. Therefore, it is still controversial whether MSI-L and MSS tumors are really different from each other. There is a possibility that MSI-L tumors develop and progress in association with both the MSI and CIN pathways. In addition, approximately 30%–40% of sporadic MSI-H cancers have *APC* mutations. Similarly, approximately 36% of sporadic MSI-H cancers have *p53* mutations. Thus, a subset of colorectal cancers develop in association with both MSI and *APC* or *p53* mutations.

TGF-β/SMAD signaling pathway

The TGF-β/SMAD signaling pathway is composed of TGF-β receptor type I (TGFβRI) and type II (TGFβRII) and SMAD proteins. When TGF-β binds to TGFβRII, which then complexes with TGFβRI, TGFβRI phosphorylates SMAD2, which binds to SMAD4. This complex translocates into the nucleus and induces the Cdk inhibitors, p15 and p21, leading to growth arrest. Mutations leading to the inactivation of the *SMAD4* gene have been found in 16%–25% of colorectal cancer cases.[53–55] Alterations of SMAD2 have been identified in 6% of cases.[34] In contrast, a *TGFβRII* mutation is frequently identified in the 10-bp polyA tract in MSI-positive tumors.[44] In MSI-positive tumors without the *TGFβRII* mutation, mutations of the IGF-II receptor have been frequently identified.[45] Both *TGFβRII* mutation and *SMAD* mutation are reported to occur with the same timing during carcinogenesis, at the transition from adenoma to carcinoma.

Recently, studies suggesting cross talk between the TGF-β and Wnt signal pathways have attracted much attention. A direct physical interaction between TGF-β and Wnt pathway components has been reported. That is, the TGF-β and Wnt pathways synergistically promote carcinogenesis of colorectal cancers through direct interaction of SMAD proteins and LEF-1.[56] Moreover, in heterozygote mice of both *APC* and

Table 2. Target genes of MSI in colorectal cancer

Gene	DNA repetitive sequence (nucleotides)	Incidence of mutations (%)		Function of the gene products	References
		HNPCC	Sporadic MSI positive tumor		
TGFβRII	$(A)_{10}$ (709–718)	75–83	80–90	Inhibition of cell growth	44
BAX	$(G)_8$ (114–121)	50–55	11–50	Induction of apoptosis	45
IGFIIR	$(G)_8$ (4089–4096)	13	9	Growth promotion	46
hMSH6	$(C)_8$ (3049–3056)	33	25–36	Mismatch repair enzyme	47
hMSH3	$(A)_8$ (1141–1148)	50–58	35–46	Mismatch repair enzyme	48
PTEN	$(A)_6$ (795–800)	—	18.8	Inhibition of cell growth	49
E2F-4	$(CAG)_{13}$ (918–956)	71	42–57	Progression of cell-cycle	50

TGFβRII, TGF-β receptor II; IGFIIR, IGF-II receptor; MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer

Table 3. Gene alterations in serrated and classical adenoma

Gene	Serrated adenomas (%)	Classical adenomas (%)	References
K-ras	15–20	15–68	58, 60
APC	0–20	30–70	58
p53	5–8	0–25	58, 60
MSI			
MSI-H	17–21	5	58, 59
MSI-L	5–50	11	58, 59, 62

MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability

SMAD4 genes, intestinal polyps develop into more malignant tumors compared with those of *APC* alone.[57] Thus, one signaling pathway is coordinately associated with another signaling pathway in the process of carcinogenesis.

Serrated pathway

Recently, the potential role of serrated adenomas and hyperplastic polyps in the genesis of colorectal cancer has gained considerable attention. Serrated adenomas are histologically defined as adenomas that have the morphological features of hyperplastic polyps but which also contain cytological features of conventional adenomas. It has been reported that 30%–50% of serrated adenomas display MSI, mostly at a low level (MSI-L), whereas they show a low rate of *K-ras* and *APC* mutations (Table 3).[58–60] In serrated adenomas with the MSI-H phenotype, aberrant methylation [CpG island methylator phenotype (CIMP)] of the *hMLH-1* gene, and loss of its expression have been frequently noted.[61] Moreover, mutations of the same target genes as those in MSI-H cancers, for example, *TGFβRII*, *BAX*, and *IGFIIR*, have also been reported.[45–47] The genetic alterations of *p53* are still controversial. However, several recent studies have shown low incidences of *p53* mutations, similar to those in classical adenoma.[58,60] Thus, evidence of the MSI-H serrated pathway in colorectal cancers has accumulated. Regarding serrated adenoma with the MSI-L phenotype, expression of the DNA repair gene *O*-6-methylguanine DNA methyltransferase (*MGMT*) has been reported to be lost by methylation.[62,63] However, details remain unclear.

Epigenetic mechanism of colon carcinogenesis

Recent molecular biology studies have revealed that an epigenetic mechanism plays an important role in colorectal carcinogenesis. CpG-rich regions located at the 5′ end of coding sequences can undergo hypermethylation, leading to the silencing of the gene. Cancers demonstrating methylation and silencing of multiple genes are described as CIMP positive. The *hMLH1* gene is a frequent target of hypermethylation, which leads to the MSI-H phenotype, as described above. Many genes other than *hMLH1*, including $p16^{INK4A}$, *MGMT*, estrogen receptor (*ER*), *APC*, and *COX-2*, have been reported to undergo hypermethylation and silencing in human colorectal cancers.[64] Recently colorectal adenomas, in particular, villous and tubulovillous adenomas as well as cancers, have been reported to show CIMP.

Genes related to invasion and metastasis

The conventional paradigm of invasion and metastasis of colorectal cancer consists of a complex series of steps, including proteolysis of the local extracellular matrix (ECM), adhesion, angiogenesis, dissemination, and cell growth. Many gene alterations are complexly involved in these processes (Table 4).

Genes for proteolysis

In the proteolysis step, proteinases, which are produced by cancer cells or fibroblasts, degrade ECM components and enable cancer cells to detach from the primary site. Of the many kinds of proteinases, matrix metalloproteinases (MMPs), which currently are known to comprise more than 25 enzymes, appear to exert a dominant effect. MMPs are collectively able to degrade virtually all ECM components, that is, collagens, laminin, fibronectin, vitronectin, enactin, and proteoglycans. In particular, MMP-7 (matrylysin) is reported to play an important role in the degradation of ECM. Matrylysin is overexpressed in the majority of colorectal cancers, and its expression is positively correlated with metastatic potential.[65] MMPs other than matrylysin, including collagenases, gelatinases, and stromelysin, are also reported to be involved in proteolysis of ECM.[66–71] On the other hand, tissue inhibitors of metalloproteinase (TIMP) in colorectal cancer tissues protect against ECM degradation.[72] Urokinase

Table 4. Genes related to invasion and metastasis in colorectal cancers

Genes	Characters of gene products	References
Genes for proteolysis		
MMP-7 (matrylisin)	Digestion of fibronectin, laminin, collagen IV, and protoglycans	65, 71
MMP-2, -9 (gelatinases)	Digestion of gelatins and collagen IV	66, 68
MMP-1, -8, -13 (collagenases)	Digestion of collagens I, II, III, IV, VI, IX, X, and XI	66, 67
MMP-3 (stromelysin-1)	Digestion of fibronectin and laminin	70
TIMP-1	Tissue inhibitors of MMP	72
uPAR	Activation of plasmin-plasminogen system	73
Genes for adhesion		
Integrins	Binding to laminin, collagen, fibronectin, and vitronectin	74, 75
Cadherins	Cell–cell adhesion	76, 77
CD44	Binding to hyaluronan	78
CEA	Binding to a receptor on Kupffer cells	79–81
Genes for angiogenesis		
VEGF	Angiogenesis, MMP-9 induction	82, 84–88
PD-ECGF	Angiogenesis	83
Genes for cell survival and others		
TRAIL-R	Binding to TRAIL to induce apoptosis	89–93
CXCR4	Binding to SDF-1 to enhance migration and invasiveness	94, 95
Drg-1	Cell differentiation	96
c-Met	Binding to HGF to enhance motility and invasiveness	97, 98

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; uPAR, urokinase plasminogen activator receptor; CEA, carcinoembryonic antigen; VEGF, vascular endothelial growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; SDF, stromal cell-derived factor; HGF, hepatocyte growth factor

plasminogen activator receptor (uPAR) is another factor that has been implicated in this process.[73]

Genes for adhesion

Many adhesion molecules, including integrins, cadherins, selectins, CD44, ICAM-1, VCAM-1, and carcinoembryonic antigen (CEA), have been identified in colorectal cancers.[74–82] For example, integrins can bind many ECM molecules, such as laminin, collagen, fibronectin, and vitronectin. Cancer cells expressing these adhesion molecules are more likely to adhere to the ECM, leading to subsequent invasion and metastasis.

E-cadherin is a cell–cell adhesion molecule that participates in a homotypic, calcium-dependent interaction to form an epithelial adherens junction. The invasiveness and metastatic potential of a broad range of cancers are often associated with downregulation of E-cadherin expression.[76] Previously, we also reported that expression of E-cadherin is inversely correlated with tumor aggressiveness.[77] CEA, a well-known human tumor marker, has also been reported to function as an intercellular adhesion molecule.[79–81] It is well documented that CEA expression is positively correlated with liver metastasis. However, the CEA receptor molecule and the mechanism of their binding are not yet clarified.

Genes for angiogenesis

Angiogenesis is an important step in the outgrowth of a primary tumor and also provides a source for hematogenous dissemination, progression, and metastasis. Many potential angiogenic factors have been characterized, including VEGF and platelet-derived endothelial cell growth factor (PD-ECGF).[82,83] Of these factors, VEGF is the most important, and it has been well examined for its role in the invasion and metastasis of cancer cells. Currently, six VEGF molecules (VEGF A–F), are known, and they induce angiogenesis by acting as highly specific mitogens for endothelial cells. Signal transduction involves binding to tyrosine kinase receptors (VEGF receptors; VEGFR) and results in endothelial cell proliferation, migration, and new vessel formation, as well as increased vascular permeability.[84,85] This process is also closely associated with other signal transductions such as mitogen-activated protein kinase.[86] Colorectal cancers with increased *VEGF* expression are well known to be associated with a poor prognosis.[87,88]

Genes related to cell growth and evasion of the immune system

Only a small population of tumor cells lodged in distant organs have the ability to survive, grow, and evade the immune system. Many molecular factors are involved in this process (Table 4). For instance, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a mem-

ber of the TNF family, is known to be expressed in human hepatic NK cells.[89,90] Recent studies have revealed that a tumor cell that expresses TRAIL receptor 1 (TRAIL-R1) or 2 (TRAIL-R2) is destroyed by NK cells through apoptosis in the liver, whereas a tumor cell expressing TRAIL receptor 3 (TRAIL-R3) or 4 (TRAIL-R4) can survive by resisting apoptosis.[91,92] Patients with colorectal cancers with high TRAIL-R1 expression have been reported show a significantly poorer prognosis than those with low TRAIL-R1 expression.[93]

The gene for chemokine receptor CXCR4, whose ligand is the chemokine stromal cell-derived factor (SDF-1) has also been reported to be involved in metastasis of colorectal cancers.[94] There is growing evidence that CXCR4 and SDF-1 regulate migration and metastasis of cancer cells. Zeelenberg et al.[95] reported that CXCR4-deficient colon cancer cells did not proliferate but stayed as single cells in the liver, although the control cells grew there, indicating that CXCR4 plays an important role after the cancer cells colonize the liver. It has also been reported that other genes such as *Drg-1* and *c-Met* play a role in this process of metastasis.[96–98]

The gene alterations involved in invasion and metastasis occur by various mechanisms, including chromosomal instability, MSI, and promoter methylation.

Epilogue

Rapid advances are being achieved in the understanding of the molecular genetics and epigenetics of colorectal cancers. Accumulating evidence has shown that colorectal cancer is heterogeneous and complex. However, we believe that detailed genetic and molecular biological analyses of colorectal cancer will contribute to its prevention and diagnosis and to effective therapeutics in the future.

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Aberrant Crypt Foci: Detection, Gene Abnormalities, and Clinical Usefulness

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Human aberrant crypt foci (ACF) were first identified as lesions consisting of large thick crypts in colonic mucosa of surgical specimens after staining with methylene blue. Previously we succeeded in identifying ACF by using magnifying endoscopy and analyzed the number, size, and dysplastic features of ACF in normal controls and patients with adenoma or cancer patients. On the basis of these analyses, we strongly suggested that ACF, particularly dysplastic ACF, are precursor lesions of the adenoma-carcinoma sequence in humans. In most sporadic ACF, K-ras mutations were positive, but APC mutations were negative irrespective of nondysplastic or dysplastic features. Conversely, in most ACF from familial adenomatous polyposis patients, APC mutations were positive but K-ras mutations were negative. These results may suggest that the molecular mechanism of sporadic colon carcinogenesis is not necessarily the same as that of familial adenomatous polyposis. It was shown that ACF acquired resistance to apoptosis induced by bile salts, whereas normal colonic epithelial cells are turning over consistently by apoptosis. This apoptosis resistance was closely associated with glutathione S-transferase P1-1 expression. One of the most important clinical applications of ACF observation with magnifying endoscopy is its use as a target lesion for chemoprevention. Because ACF are tiny lesions, they should be eradicated during a short time by administration of chemopreventive agents. In fact, we performed an open chemopreventive trial of sulindac and found that the number of ACF was reduced markedly in 2 months. We currently are proceeding with a randomized double-blind trial targeting ACF.

Aberrant crypt foci (ACF) were first described by Bird[1] as lesions consisting of large thick crypts in methylene blue–stained specimens of colon from mice or rats treated with a carcinogen. Features of ACF were described in Bird's[1] report as follows: (1) grossly normal-appearing mucosa, (2) large crypts densely stained with methylene blue, and (3) often, a wide pericryptal space. Many studies have shown that ACF are precancerous lesions in models of colon chemical carcinogenesis in animals. In humans, ACF were first identified in whole-mount preparations of normal-appearing colonic mucosa with methylene blue staining obtained from surgical resection from patients with colon cancer.[2] Subsequently, oncogenic abnormalities such as K-ras mutations have been reported in ACF. Increased proliferative activities such as proliferating cell nuclear antigen and bromodeoxyuridine in ACF also were reported. However, these studies were performed mainly on surgical specimens. Data from normal controls and patients with adenomas, which could provide essential information about the relation of ACF to cancer, were lacking until recently.

Identification of Aberrant Crypt Foci Using Magnifying Endoscopy

ACF can be identified in situ by using magnifying endoscopy with the aid of methylene blue staining, as previously reported.[3] First, the colorectum should be cleaned thoroughly by pretreatment. A magnifying endoscope (models EC-490ZW and EC485-ZW; Fujinon-Toshiba ES System, Tokyo, Japan) is used throughout the examination, and all patients undergo complete colonoscopy. As shown in Figure 1, the colorectal mucosa was washed with water, sprayed thoroughly with .25% methylene blue, left to stand for about 2 minutes, and washed again with water. ACF are classified histologically as nondysplastic-nonhyperplastic, nondysplastic-hyperplastic, or dysplastic. These types of ACF can be identified distinctly by magnifying endoscopy (Figure 2). In general, nondysplastic-nonhyperplastic ACF consist of crypts with round or oval lumens. Nondysplastic-hyper-

Abbreviations used in this paper: FAP, familial adenomatous polyposis; GST P1-1, glutathione S-transferase P1-1; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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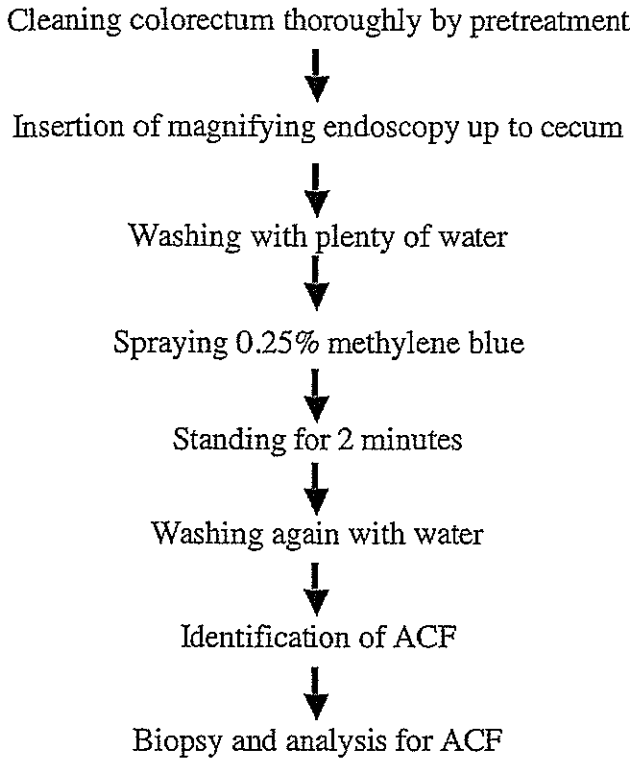

Figure 1. Identification of ACF by magnifying endoscopy. The panel shows ACF identified by magnifying endoscopy (EC-485-ZW; Fujinon-Toshiba ES system).

plastic ACF show the crypts with asteroid or dendritic (or slit-like) lumens. Dysplastic ACF consist of crypts in which the epithelial lining is thicker and each lumen is compressed or not distinct. Generally, dysplastic ACF show a darker staining with methylene blue.[3] Similar ACF also are identified in patients with familial adenomatous polyposis (FAP). ACF from FAP patients show a similar appearance to dysplastic ACF in sporadic cases. However, they show much weaker staining and each crypt lining may be obscure. Histologically, ACF from FAP patients consist of large crypts in which there are slight nuclear stratifications and nuclear disorientation, suggesting dysplastic features.[4]

We first examined ACF in the entire colorectal mucosa and found that ACF was highly prevalent in the rectosigmoidal colon. To quantify the number of ACF and to minimize the time needed, we confined further examinations to the lower rectal region from the middle Houston valve to the dentate line.

Analysis of Aberrant Crypt Foci in Normal Patients, Patients With Adenoma, and Patients With Cancer

To clarify the significance of ACF in human colonic carcinogenesis, it is important to analyze ACF in normal people with adenoma and patients with cancer by using a magnifying endoscope. We found that the number of ACF, in particular dysplastic ACF, progressively increased in normal controls, adenoma patients, and cancer patients.[3] We also found a significant correlation between the number of ACF and the number of adenomas. When ACF were classified into small, medium, or large according to the number of crypts, there was a clear correlation between the size of ACF and the number of adenomas. Moreover, in some cases, ACF were superimposed by small polyps. These results strongly suggested that ACF, particularly dysplastic ACF, are precursor lesions of adenomas and subsequent cancers (Figure 3).[3]

It still is controversial whether nondysplastic ACF precede dysplastic ACF or not. However, there is a report that some ACF contained dysplastic and nondysplastic

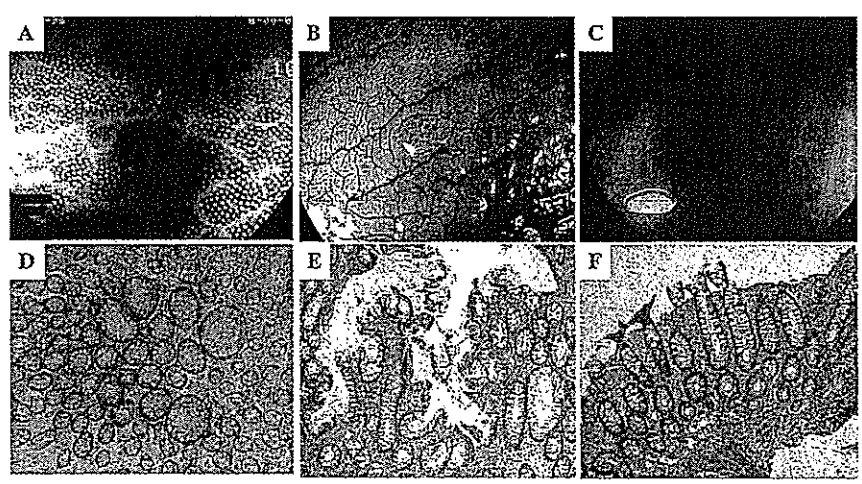

Figure 2. Three different types of ACF. Endoscopic appearances of (*A*) nondysplastic nonhyperplastic ACF, (*B*) nondysplastic hyperplastic ACF, and (*C*) dysplastic ACF. Histologic findings of (*D*) nondysplastic nonhyperplastic ACF, (*E*) nondysplastic hyperplastic ACF, and (*F*) dysplastic ACF.

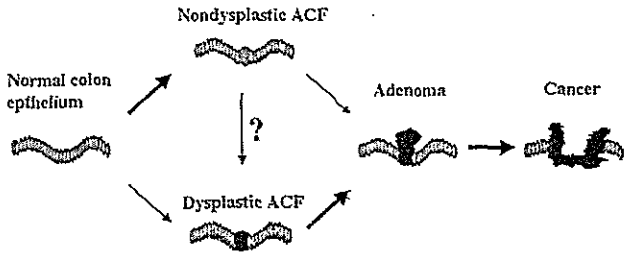

Figure 3. Colon carcinogenesis through ACF. Accumulating data on ACF strongly suggest that ACF, particularly dysplastic ACF, are precursor lesions of adenoma and subsequent cancer.

features in the same lesions.[5] We also found and confirmed the existence of these kinds of ACF lesions.

Gene Analysis of Aberrant Crypt Foci

In the adenoma-carcinoma sequence, it is well accepted that genetic alterations accumulate in the following order: APC, K-ras, and P53.[6] This is supported by many studies on FAP. Therefore, it is important to compare the genetic abnormalities in sporadic ACF with those of FAP.[7,8] We analyzed the mutation cluster region of APC genes by truncation assay and found that APC mutation was negative in all sporadic ACF irrespective of nondysplastic and dysplastic features.[4] Meanwhile, it was detected in all of the dysplastic ACF from FAP patients. These were somatic mutations because germline mutations were detected in other regions of the APC gene. Immunofluorescence showed β-catenin accumulation in FAP ACF, but not in sporadic ACF. The frequencies of K-ras mutations in sporadic ACF were 63%–82%, as shown by the 2-step polymerase chain reaction restriction fragment length polymorphism method,[4] which is consistent with other studies.[9] However, it hardly was detected in FAP ACF. Therefore, it is surmised that in sporadic colorectal carcinogenesis, K-ras mutation occurs during the formation of ACF, which then become adenomas wherein APC mutations occur. On the other hand, in FAP, somatic mutation of APC predominantly occurs during ACF formation, followed by K-ras mutation.

Resistance of Aberrant Crypt Foci to Apoptosis Induced by Bile Acids

It has been suggested that normal colonic epithelial cells undergo apoptosis induced by some cytotoxic substances such as bile salts. We found that ACF are resistant to apoptosis induced by deoxycholic acid, a secondary bile acid, as shown by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining in ACF specimens. Thus, some TUNEL-positive cells were detected in normal epithelial crypts, whereas much fewer TUNEL-positive cells were detected in ACF as well as in adenoma. When the specimens were treated with deoxycholic acid, the difference became more pronounced.[10]

It is well known that cyclooxygenase-2 plays an important role in colon carcinogenesis through its cytoprotective activity. Therefore, we examined the ACF for cyclooxygenase-2 expression by immunohistochemistry but found none or little, although it was detected in adenoma and cancers.[10] It also is known that glutathione S-transferase P1-1 (GST P1-1), a phase II detoxifying enzyme, is expressed in colonic adenomas and cancer. We found that GST P1-1 is expressed in ACF as well as in adenomas and cancer, and that it was induced by K-ras mutation.[11] Because GST P1-1 sequesters xenobiotics such as bile salts, it is plausible that GST P1-1 serves as a cytoprotecting factor in ACF.

Clinical Applications of Aberrant Crypt Foci Observation by Magnifying Endoscopy

One clinical application of ACF is as a marker to predict the risk for colorectal cancer. For example, on the basis of our data on ACF in normal patients, adenoma patients, and cancer patients, we can calculate odds ratios for adenoma and cancer. Odds ratios for adenoma and cancer in patients with dysplastic ACF are 4.2 and 10.2, respectively. Odds ratios for adenoma and cancer in patients with large ACF (≥20 crypts) are 5.3 and 24.6, respectively. Thus, the patients who have large and dysplastic ACF have a high risk for adenoma and subsequent cancer.

Perhaps the most important application of ACF is as a target lesion for chemoprevention. Currently, the most common target lesion for evaluation of chemopreventive agents has been the polyp. However, in using polyps as a target, the following problems have been raised: polyps are too large to be eradicated completely and the evaluation of chemopreventive effects on polyps requires a long period of time. In this context, ACF are the most appropriate lesions because they are the earliest and smallest detectable lesions with a simple genetic alteration (K-ras mutation). Therefore, the advantage in using ACF are as follows: (1) short-term treatment for evaluation; (2) complications are less frequent, as in the case with gastric ulcers caused by nonsteroidal anti-inflammatory drugs; and (3) there is good patient compliance because of the short-term treatment. In fact, we performed an open chemopreventive trial of sulindac and

found that the number of ACF was reduced markedly in only 2 months. We currently are proceeding with a randomized double-blind trial targeting ACF. The results will be obtained in the near future.

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トピックス

I. 疫学と病態
4. 大腸癌の前癌状態

高山　哲治　　勝木　伸一　　新津洋司郎

要　旨

大腸癌の発生母地として，メチレンブルーに濃染する微小病変であるaberrant crypt foci(以下ACF)が注目されている．われわれはこれまで，拡大内視鏡を用いて，ヒトACFを観察し，ACF(特にdysplastic ACF)が腺腫の前病変であることを指摘した．ACFではK-ras変異が高率に認められ，細胞増殖活性ならびにアポトーシス抵抗性が亢進している．ACFを内視鏡的に観察することは，大腸癌の高危険群の絞り込みや癌予防の臨床試験に有用である．　〔日内会誌　96：220～225，2007〕

Key words：aberrant crypt foci（ACF），大腸癌，前癌病変

はじめに

大腸癌の前癌病変として，古くからポリープ(腺腫)が良く知られている．ポリープは，通常の内視鏡検査で容易に観察し得る病変であり，診断されれば前癌病変として内視鏡的切除が行われている．しかし，ポリープより早期の病変についてはこれまで十分な検討は為されていなかった．一方，最近内視鏡技術の進歩により拡大内視鏡がルーチンに使用できるようになった．われわれは，拡大内視鏡を用いて，ヒトにおいて大腸の微小病変であるaberrant crypt foci(ACF)を観察し，ACFが腺腫，癌の前病変である可能性を指摘してきた[1]．本稿では，ACFの前癌病変としての意義を中心に述べるとともに，潰瘍性大腸炎に合併する癌の前病変についても言及する．

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1. Aberrant crypt foci（ACF）とは

1987年，Birdは発癌剤（アゾキシメタン）を投与したマウスやラットの大腸粘膜を実体顕微鏡下に観察し，メチレンブルーに濃染する微小病変を見出しaberrant crypt foci（ACF）と命名した[2]．彼らは，ACFを以下のように定義している．すなわち，1)肉眼的には正常に見える大腸粘膜（ポリープのような明らかな隆起性病変を認めない），2）実体顕微鏡を用いて観察しうるメチレンブルーに濃染する大きな腺管の集まり，である．その後の研究により，ACFは1)発癌プロモーターとして知られるケノデオキシコール酸により，その数や大きさが増加するとともに，核異型やdysplasiaを伴うこと，2)発癌に抑制的に働くとされるアスピリンやドコサヘキサノイン酸によりその数が減少し，大きさも小さくなること，3）proliferating cell nuclear antigen(PCNA)などの細胞増殖活性の亢進や癌遺伝子の変化（K-ras，c-fosなど）が認められること，などが報告され，ACFは動物発癌の初期病変と

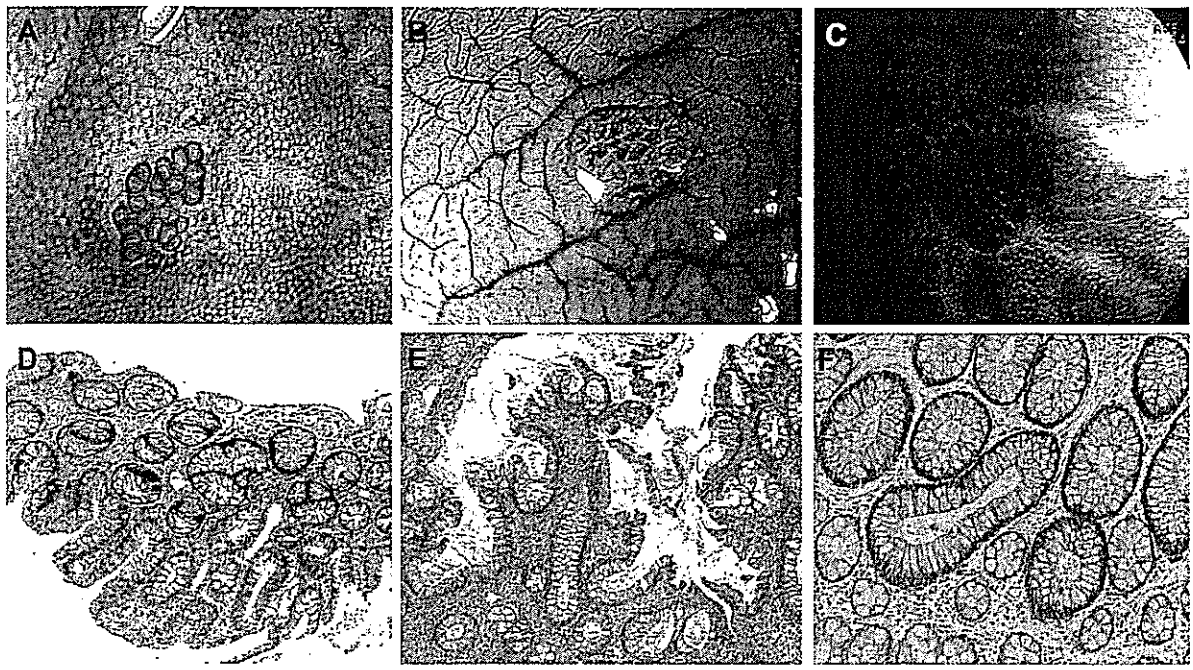

図1. ACFは，類円形の腺口形態を呈するNondysplastic-nonhyperplastic ACF（A.D），各々の腺管が星芒状を呈するHyperplastic ACF（B.E），各腺口形態が不明瞭（又はスリット状）のDysplastic ACF（C.F）に分類される．

考えられている．

一方，ヒトにおいては，Pretlowらが大腸切除標本を実体顕微鏡下に観察し，ヒトACFの存在を報告した．さらに，ヒトACFにおいても高率にK-ras変異が認められると報告された．しかし，ヒトACFの病的意義は十分には検討されていなかった．

2. 拡大内視鏡所見を用いたACFの観察

われわれは，ヒトACFを拡大内視鏡（フジノン東芝ESシステム社製 450CM）を用いて以下の方法により観察し得ることを報告した．すなわち，1）大腸粘膜を微温湯で良く洗浄する，2）0.2％メチレンブルーを十分に散布する，3）約2分間後に再度微温湯で良く洗浄する，4）拡大観察によりACFを観察する，という方法である．初めは全大腸のACFを観察したが，時間と労力を省くために代表的な場所として下部直腸領域（第2ヒューストン弁から歯状線まで）のACFを観察し，数を定量化するようにしている．このようにして観察したACFを図1に示す．ACFは内視鏡的に3つに分類することができる．全体の形が類円形で，各々の腺管も円形または類円形の腺口形態を呈するもの（図1A），各々の腺管が星芒状を呈するもの（図1B），全体の形がいびつで各腺口形態が不明瞭（またはスリット状）のもの（図1C）である．それぞれのACFは，病理組織学的にはnondysplastic-nonhyperplastic ACF（simple ACF），nondysplastic-hyperplastic type，dysplastic ACFに相当する（図1D～F）．

3. 腺腫の前病変としてのACF

ACFが腺腫の前病変であるかどうかを明らかにするためには，どうしても健常人と大腸腺腫患者，さらに大腸癌患者のACFを解析する必要がある．そこで，これらの症例におけるACFの数を検討したところ，健常人では1.7±2.2個，腺腫患者では7.6±6.3個，癌患者では31.4±15.7個であり，ACF数はこの群の順に有意に増加した．これらの群におけるACFのsubtypeを調べる

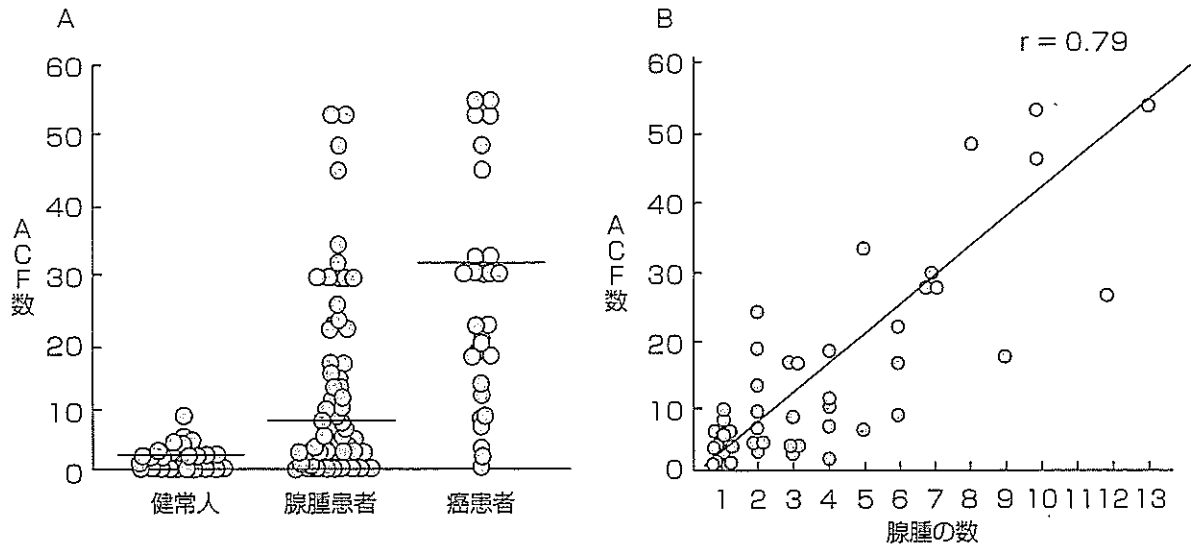

図2. A. 健常人，大腸腺腫及び癌患者におけるACF数の検討．B. 大腸腺腫患者におけるACF数と腺腫数の関係

と，特にdysplastic ACFの数が優先的に増加した．さらに，ACFを腺管の数により，large(20腺管以上)，medium(10～19腺管)，small(9腺管以下)に分けて検討すると，large ACFが優先的に増加することも判明した．また，腺腫患者におけるACFの数と腺腫の数を比較検討すると，両者の間には正の相関性が認められた．以上の結果より，ACFが腺腫の前病変であることが示唆され，とりわけdysplastic typeの大きいACFが腺腫の前病変であることが強く示唆される(図2)．

4. ACFの遺伝子異常と細胞増殖活性

Adenoma-carcinoma sequenceにおいては，古くからAPC変異により腺腫が発生し，K-ras変異が生じて異形度が増し，p53変異が加わり癌になり，DCC変異により転移・浸潤をきたす，と考えられてきた[3]．この説は，特に家族性大腸腺腫症（familial adenomatous polyposis；FAP）のポリープや癌の解析結果に裏付けされている．ヒトACFの遺伝子解析では，Pretlowらがその73%にK-ras変異が認められることを報告した．一方，OhtoriらはhyperplasticACFではAPC変異は認められないが，dysplastic ACFでは40%に変異が認められると報告した．われわれは，家族性大腸腺腫症（FAP）と散発性症例に分けてACFの遺伝子解析を行った．その結果，FAP患者のACFではAPC変異が全例に認められたが，K-ras変異は低率（17%）であった．逆に，散発性のACFではAPC変異は認められなかったが，K-ras変異は高率(87%)に認められた．このように，FAPでは，従来の説の通り，APC，K-ras，p53の順に変異が生じるのに対し，散発性症例ではK-ras，APC，p53の順に変異が生じる経路があることが示唆された．

ACFにおける細胞増殖活性に関する検討も為されている．Roncucciらは，正常腺管に比べてACFでは1腺管あたりの細胞数が有意に多いこと，さらに細胞増殖活性を表すBromodeoxyuridine（BrdUrd）の発現が高いことを報告している．また，SpitzらはACF，特にdysplastic ACFではPCNAの陽性率が高く，腺腫と同様に増殖帯が管腔側に移行していることを報告している．

5. ACFにおけるアポトーシス抵抗性の獲得

正常大腸上皮は，絶えず二次胆汁酸などの毒