Table 1. Inflammation-Associated Cancers in Digestive Organs

Inflammation-associated cancer	Underlying inflammation
Barrett's cancer	Reflux esophagitis
Gastric cancer	*H pylori*–induced chronic gastritis
Colitic cancer	Inflammatory bowel disease
	Celiac disease
HCC	HCV and hepatitis B virus chronic hepatitis
	Primary biliary cirrhosis
Cholangiocarcinoma	Primary sclerosing cholangitis
Pancreatic cancer	Chronic pancreatitis
	Hereditary pancreatitis
Lymphoma	*H pylori*–induced mucosa-associated lymphatic tissue lymphoma
	HCV-associated lymphoma
	Celiac disease–associated lymphoma

aberrant DNA methylation is present even in normal-appearing tissues, being involved in field cancerization.[13,15,16]

Digestive organs are inhabited by many microorganisms and are infiltrated by many immune cells in physiological and pathologic conditions, and thus they are more or less accompanied by certain levels of inflammation. Here, we review mechanisms of how inflammation is involved in cancer development in digestive organs, particularly focusing on the role of chronic inflammation in inducing genetic and epigenetic changes.

Cancers in Digestive Organs Associated With Inflammation

Many cancers arise in digestive organs. Indeed, gastric cancer remains the third leading cause of cancer death in men and the fifth leading cause in women, and colorectal cancer is the third most commonly diagnosed cancer in men and the second most commonly diagnosed in women worldwide.[17] In addition, hepatocellular carcinoma (HCC) is one of the most frequent malignancies and its incidence is increasing not only in an endemic area for the hepatitis virus but also in the United States and other Western countries.[18] Digestive organs cover a large part of the body surface in contact with the outer environment. Accordingly, they are inhabited not only by many microorganisms but also exposed to ingested food or chemical agents, and therefore infiltrated by many immune cells in pathologic as well as normal conditions, supporting the perpetuation of chronic inflammation. Therefore, it is reasonable that many cancers in digestive organs are associated with inflammation.

The best examples of inflammation-associated cancer in human beings are gastric cancer and HCC. Since the discovery of *H pylori* by Warren and Marshall[19] in 1982, it has been well established that *H pylori*–positive patients with chronic gastritis have a significantly higher risk for gastric cancer than *H pylori*–negative subjects,[20] and, moreover, careful investigations have shown more than 95% positivity for *H pylori* infection in gastric cancer patients.[21] On the other hand, hepatitis B virus and HCV infections account for approximately 60% and 33% of the total HCC cases in developing countries and 23% and 20% in developed countries, respectively,[6,22] and the majority of HCCs develop in patients who have chronic hepatitis or cirrhosis. Other inflammation-associated cancers in digestive organs are colitic cancers developed in patients with inflammatory bowel disease (IBD) or celiac disease,[23–25] primary sclerosing cholangitis (PSC)-associated cholangiocarcinoma,[26] primary biliary cirrhosis–associated HCC,[27] and Barrett's cancer developed in patients with reflux esophagitis.[28] In addition, the incidence of pancreatic cancer in patients with chronic pancreatitis is reported to be 4–8 times higher than in the general population,[29] and, more strikingly, the incidence of pancreatic cancer in patients with hereditary pancreatitis is 53 times higher than in the normal population,[30] indicating that chronic pancreatitis is a risk for pancreatic cancer.

In addition to cancers, inflammation is also a risk for developing various lymphomas in digestive organs. These include *H pylori*–induced mucosa-associated lymphatic tissue lymphoma or plasmacytoma,[31,32] HCV-related lymphoma,[33] and lymphoma related to celiac disease.[34]

Mechanisms for Inflammation-Associated Cancer Development

The inflammatory response is coordinated by a large range of mediators, which are released from immune cells, mesenchymal cells, and epithelial cells; these mediators exert various functions in maintaining or resolving inflammation, and at the same time are involved in cancer development. Among the mediators, cytokines play central roles in diversifying the inflammatory process, and interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 are known to be the major cytokines important for inflammation and cancer development.[35–37]

IL-1β and TNF-α act directly on epithelial cells to induce activation of transcription factor nuclear factor-κB (NF-κB), a key transcription factor mediating inflammation and cancer development.[36,37] NF-κB activation not only promotes growth or suppresses apoptosis of epithelial cells but also stimulates the production of growth factors and cytokines such as epidermal growth factor and IL-6, enhances cyclooxygenase (COX)-2 induction, and increases ROS production.[38] The induced COX-2 subsequently has many functions, including enhancement of cell growth and angiogenesis.[39] ROS modifies protein function.[40] IL-6 activates signal transducer and activator of transcription 3 (STAT3) and thereby enhances cell growth and stimulates growth factor production, including the Reg protein.[41] Interestingly, TNF-α and IL-6 often create a positive-feedback loop during cancer development.[42]

At the same time, these cytokines also activate mitogen-activated protein kinase (MAPK) cascades. For instance, TNF-α and IL-6 have been shown to activate the extracellular signal-regulated kinase/MAPK cascade, an impor-

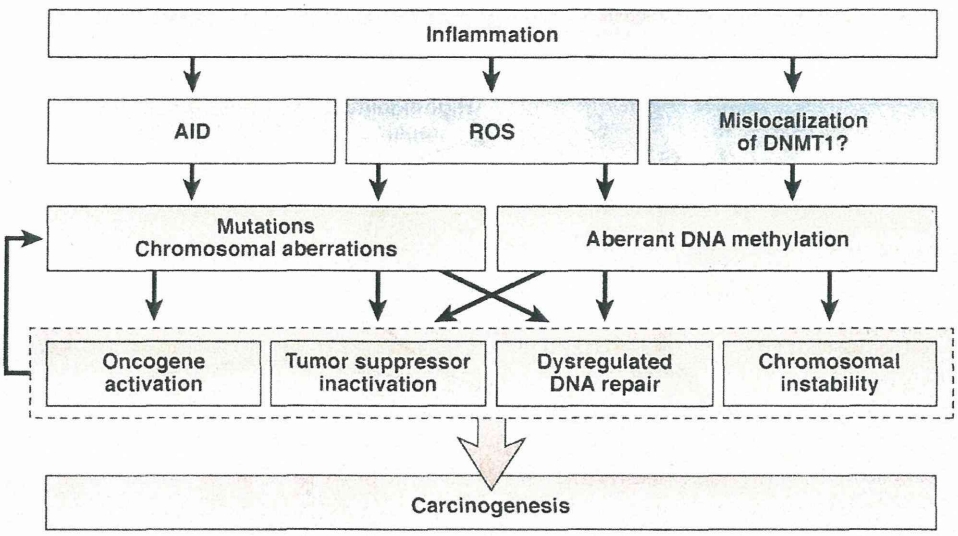

Figure 1. Molecular link between inflammation, genetic and epigenetic alterations, and carcinogenesis. Inflammation contributes to ROS production and transcriptional up-regulation of the DNA mutator enzyme, AID. These 2 factors were capable of inducing somatic mutations and chromosomal aberrations in tumor-related genes. On the other hand, inflammation results in mislocalization of DNMTs, inducing aberrant DNA methylation. The resulting genetic and epigenetic changes, including the activation of oncogenes, inactivation of tumor-suppressor genes, and dysregulation of DNA repair genes, could enhance genetic instability further, finally leading to carcinogenesis.

tant signaling pathway involved in many processes in carcinogenesis including cell proliferation, migration, and angiogenesis.[43,44] Similarly, IL1-β, TNF-α, and IL-6 all activate c-Jun N-terminal kinase (JNK). Although JNKs are attributed primarily to proapoptotic cell death or tumor suppression in response to inflammation or various stressors,[45] JNK activation, particularly JNK1, by proinflammatory cytokines has been reported to contribute to inflammation-associated cancer development through cell death–induced compensatory proliferation.[45-48] In this regard, an interesting thing to note is that *H pylori* directly activates extracellular signal-regulated kinase/MAPK and JNK in human gastric cells via a type IV secretion system–dependent mechanism.[49,50]

Thus, these mediators of inflammation form a complex of regulatory networks, and appear to work in concert to enhance cancer development. However, for normal cells eventually to be transformed and become cancer cells with clonal expansion, inflammation has to damage cellular DNA, either genetically or epigenetically, leading to permanent alteration within the genome.

Inflammation and Genetic Modulation

Cancer is a genetic disease resulting from stepwise accumulation of genetic and epigenetic alterations that drives the progressive transformation of normal cells into malignant derivatives.[51] Inactivation of tumor-suppressor genes and/or activation of oncogenes caused by somatic mutations, DNA copy number changes, or chromosomal aberrations are widely detectable in human cancer cells. Among them, the tumor suppressor *TP53* gene is one of the most frequent targets for genetic alterations in many human cancers.[52] An important point to note is that *TP53* mutations frequently are present also in noncancerous tissues with chronic inflammation before cancer development. Indeed, multiple genetic changes in the *TP53* gene have been detected in various inflammatory tissues such as IBD,[53,54] Barrett's esophagus,[55] and HCV-associated chronic hepatitis.[56] For example, by analyzing the individual crypt mutation burden across plaques of the dysplasia, it was shown that mutations in *TP53* genes could be identified in the majority of inflamed crypts of patients with ulcerative colitis.[57] Moreover, *TP53* mutations are detectable at the frequencies of 4–15 nucleotides of 10^4 nucleotides in the hepatocytes of the patients with chronic HCV infection.[56] Normal mutation rates cannot account for such abundant genetic changes that accumulate in inflamed epithelial cells, suggesting that certain molecular mechanisms underlie such a large number of genetic alterations. Therefore, to understand the mechanisms of inflammation-associated tumorigenesis, several possible intrinsic mutagens responsible for genetic aberrations in the inflammatory condition have been proposed. Among them, free radicals and intrinsic DNA mutator enzymes appear to be important candidates in the setting of chronic inflammation (Figure 1).

Free radicals refer to any molecular species with one or more unpaired electron(s), including ROS and reactive nitrogen species.[38] Interestingly, increases in *TP53* gene mutations at codons 247 and 248 are paralleled by an enhanced expression of nitric oxide synthase (NOS) in the inflamed lesions of the colonic tissues of patients with ulcerative colitis.[54] HCV infection also induces inducible NOS messenger RNA (mRNA) expression, thereby enhancing NO production, which in turn results in DNA breaks and enhanced mutation frequencies.[58] Moreover, an increased level of NO accelerated spontaneous tumor

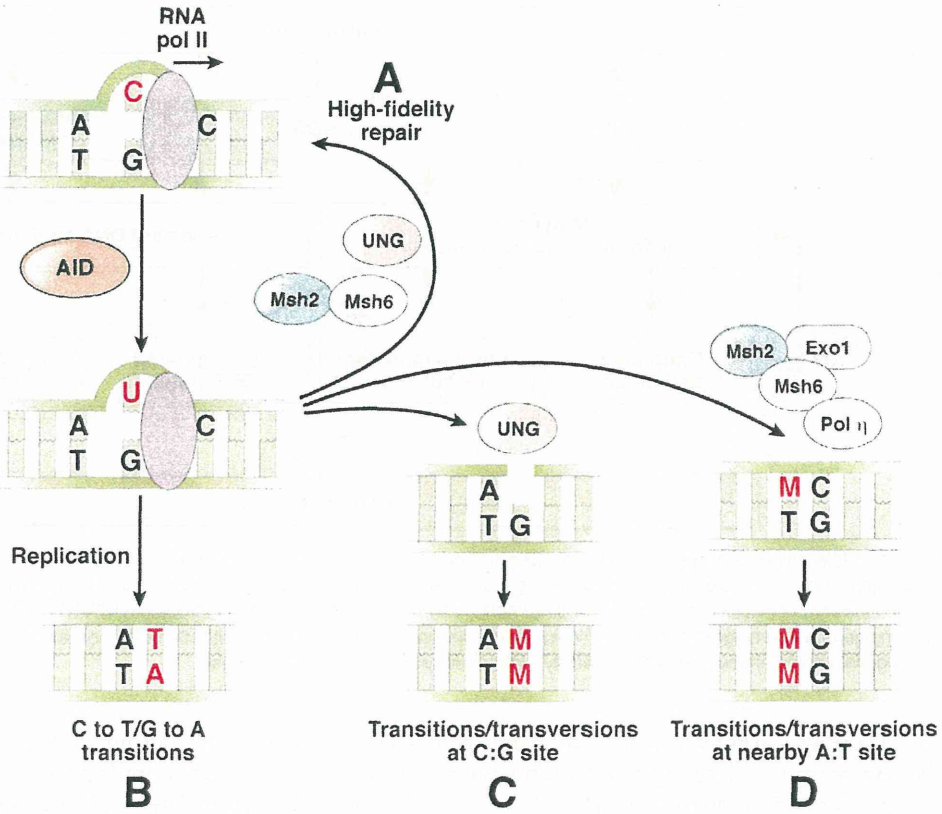

Figure 2. Mechanism of mutation induction by AID activity. AID deaminates cytosine (C), resulting in the generation of a uracil (U), and therefore can transform a DNA C:G pair into a U:G mismatch. (*A*) The AID-generated U:G mismatch can be recognized by uracil-DNA-glycosylase (UNG) or MSH2/MSH6 heterodimer and repaired correctly. (*B*) If DNA replication starts before recognition by the repair system, a U:G mismatch gives rise to C/G to T/A transition. Alternatively, (*C*) generation of an abasic site by UNG or (*D*) recognition of the U:G mismatch by the MSH2/MSH6 heterodimer induces any mutations in the AID-generated U:G mismatch or at a nearby A:T site, respectively, in an error-prone manner (indicated as M).

development, mostly lymphomas, in a *Trp53*-deficient mouse model infected with *Cryptosporidium parvum*.[59]

In the inflammatory condition, cellular ROS levels are increased substantially, and nucleic acids exposed to ROS generate various modified bases such as oxidatively altered purines and pyrimidines.[60] These modified nucleic acids could induce the putative DNA damage, including single- or double-stranded DNA breaks, DNA intrastrand adducts, and DNA protein cross-links.[61] In addition, ROS alters the mismatch repair function and allows mutations to accumulate in microsatellite sequences.[62] It has been well recognized that oncogene activation is capable of inducing genomic instability in precancerous lesions as well as cancer cells.[63] In this regard, ROS is also a putative mediator that links excessive activity of oncogene products and DNA damage. For example, oncogene *c-MYC* overexpression results in DNA damage before the S phase in association with ROS induction in normal human fibroblasts.[64] These findings suggested that the cumulative situation of ROS production, a condition of so-called *oxidative stress*, is involved in both the initiation and progression of inflammation-associated cancers through the induction of genetic instability.

Importantly, the typical mutation pattern induced by oxidative stress cannot account for a mutation signature observed in many human cancer tissues, particularly in inflammation-associated cancers. Among the oxidized nucleosides, one of the common products of free radical attack on DNA is 8-hydroxydeoxyguanine, which is considered to be a biomarker of oxidative stress.[65] The typical pattern of nucleotide alterations induced by 8-hydroxydeoxyguanine is guanine (G)/cytosine (C) to thymine (T)/adenine (A) transversions, which have been observed in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-RAS*) and *TP53* tumor-suppressor gene in lung and liver cancers.[66,67] However, recent genomewide analyses clearly showed that G/C to T/A transversions account for a minor proportion of the total mutations identified in human cancer cells, and instead C/G to T/A transitions are the most prevalent mutation pattern in various cancer tissues, including inflammation-associated cancers.[68] Thus, it appears reasonable to assume that there is an alternative mechanism that accounts for the most frequent mutational pattern, C/G to T/A transitions, detected in many human cancer tissues.

Recently, several human enzymes that are capable of inducing nucleotide alterations have been identified, providing a new avenue for understanding mutagenesis mechanisms.[69] Among them, activation-induced cytidine deaminase (AID) is a well-defined molecule involved in DNA mutations in the human genome. Through its enzymatic activity, AID can deaminate C on target DNA to produce a uracil (U), and therefore turns a DNA C:G pair into a U:G mismatch. When DNA replication starts before recognition by the repair system, a U:G mismatch gives rise to C/G to T/A transition. Alternatively, recognition of a U:G mismatch by uracil-DNA-glycosylase or mutS homolog 2 (MSH2)/mutS homolog 6 (MSH6) heterodimer

induces mutations in the U:G mismatch or at the nearby A:T site (Figure 2). As a result, AID can induce any type of mutations.[70] Under physiological conditions, AID contributes to generating antibody gene diversification in activated B lymphocytes by inducing somatic hypermutation and class switch recombination of immunoglobulin gene.[71] In sharp contrast to the favorable function of AID in the immune system, the role of AID in tumorigenesis through induction of genetic instability was first suggested in hematopoietic malignancies. A number of studies have shown that increased AID expression in various neoplasms of the B lymphocytic lineage was associated with unfavorable mutations and chromosomal translocations.[72,73] For instance, AID has been shown to be responsible for the chromosomal breaks in *c-MYC*, leading to a *c-MYC/IgH* translocation in B-cell lymphoma.[74] Moreover, AID induces *breakpoint cluster region–Abelson murine leukemia viral oncogene homolog 1* mutations leading to imatinib resistance in chronic myeloid leukemia cells.[75] Because the target of AID-mediated genotoxic effects was not restricted to immunoglobulin genes and a variety of other genes also received the AID-mediated mutations in B cells,[70] it was not surprising that aberrant up-regulation of AID induced genetic alterations in various tumor-related genes, leading to the transformation of hematopoietic cells.

As described, activation of NF-κB is induced in response to various inflammatory stimulations, and is deeply involved in multiple processes of cancer initiation and progression.[36] Interestingly, NF-κB is a major transcription factor for AID in B cells that is activated through cluster of differentiation 40-TNF receptor superfamily member 5 ligation by T cells (CD40),[76] suggesting that AID might link NF-κB activation and genetic instability in nonlymphoid cells in the setting of inflammation. In agreement with this hypothesis, AID expression is induced in response to proinflammatory cytokine stimulation via the NF-κB–dependent pathway in various epithelial cells (Figure 3). In hepatocytes, AID expression is induced by TNF-α through the I-κB kinase–dependent NF-κB signaling pathway.[77] Consistent with a previous finding that the HCV core protein triggers the activation of NF-κB in hepatocytes,[78] the HCV core protein itself also up-regulates endogenous AID in cultured hepatocytes.[77] NF-κB–mediated induction of AID expression is not limited to hepatocytes. In human gastric epithelial cells, AID expression is induced by TNF-α stimulation via activation of NF-κB, but is not detected in nonstimulated cells.[79] More interestingly, aberrant AID expression is induced by the infection of a pathogenic *H pylori* strain, the cytotoxin-associated gene pathogenicity island–positive strain that is capable of introducing bacterial virulence factors into the host cells through a type IV secretion system and activating NF-κB, indicating that both bacterial factors introduced into epithelial cells and the inflammatory mediators such as TNF-α and IL-1β induced by *H pylori* infection cooperatively promote aberrant AID expression in *H pylori*–infected gastric mucosal cells. Similar

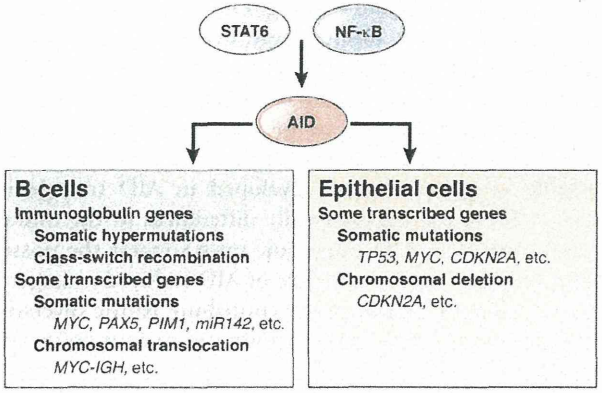

Figure 3. AID exerts both favorable and unfavorable effects. AID is a molecule that is indispensable for the diversification of immunoglobulin genes by inducing both somatic hypermutation and class-switch recombination in activated B lymphocytes. The genotoxic activity of AID, however, can be aimed to trigger the genetic alterations at both the nucleotide and chromosomal levels not only in B lymphocytes but also epithelial cells in the inflammatory conditions.

to hepatocytes and gastric mucosal cells, TNF-α stimulation resulted in up-regulation of endogenous AID in human colonic cells via the I-κB kinase–dependent NF-κB signaling pathway.[80] In addition, IL-4 and IL-13, which are involved in T helper 2 cell-type immune response in IBD, induced aberrant AID expression in a STAT6-dependent manner in human colonic epithelial cells.[80] Of note, IL-4 is known to induce AID also in B cells.[71]

Consistent with the in vitro analyses, aberrant AID expression is widely detectable in not only various inflammation-associated cancer tissues but also in a variety of inflamed epithelial tissues in which tumorigenic risk is high, including chronic hepatitis and cirrhosis caused by HCV infection,[56] chronic gastritis caused by *H pylori* infection,[79] IBD,[80] PSC,[81] and the columnar cell-lined Barrett's esophagus.[82]

The impact of AID expression in nonlymphoid epithelial cells was clarified using both in vivo and in vitro systems with aberrant AID expression. Constitutive and ubiquitous AID expression in transgenic mice induced lymphoma development via the accumulation of somatic mutations in various nonimmunoglobulin genes, including the proto-oncogene *c-Myc*.[83] More importantly, further phenotypic analyses revealed that AID transgenic mice also develop neoplasia in epithelial tissues, including lung, liver, and stomach, accompanied by the emergence of *Trp53* mutations, indicating that aberrant AID expression in epithelial cells can induce genetic instability, leading to cancer development.[83,84] It is widely recognized that the frequently mutated tumor-related genes differ among different cancers. For instance, nucleotide alterations in the *K-RAS* are detectable in almost all human pancreatic cancers,[85] whereas it is relatively low in other human tumors. Similarly, the *c-MYC* is a frequent target for genetic alterations in human lung cancers, whereas its nucleotide alterations are rare in hepatocellular carcinoma.[86] However, the mechanisms underlying the accumulation

of organ-specific genomic changes in oncogenic pathways are not well known. Interestingly, organ-specific changes in mutational profiles were observed in the epithelial tissues of the AID transgenic mice. Indeed, the *c-Myc* gene was mutated frequently in noncancerous tissue of the lung, whereas *K-ras* gene mutations frequently were detectable in gastric cancer developed in AID transgenic mice.[84] Thus, the organ-specific differences in the mutational profiles in AID transgenic mice suggest the possibility that the target preference of AID-induced mutagenesis in different tissues might contribute to the diversity of tissue-specific oncogenic pathways in various epithelial organs.

In vitro analyses using human cultured cells with constitutive AID expression revealed that *TP53* mutations were induced frequently by AID genotoxic activity in hepatocytes, and gastric, colonic, and bile duct epithelial cells.[77,79-81] Similar to the *TP53* gene, the cyclin-dependent kinase inhibitor (*CDKN*)-*2B-CDKN2A* locus was identified as a target for AID-mediated genotoxic activity. The *CDKN2B-CDKN2A* locus encodes the potent suppressor proteins $p16^{INK4a}$, $p15^{INK4b}$, and $p14^{ARF}$, which regulate the activities of the retinoblastoma protein and the TP53 transcription factor. Aberrant AID expression preferentially induces somatic mutations at the *CDKN2B-CDKN2A* locus in gastric epithelial cells and biliary cells.[81,87] Moreover, comparative genomic hybridization analysis clearly showed that constitutive AID activation in cultured gastric epithelial cells caused submicroscopic deletions as represented by copy number losses of various chromosomal loci, especially at the *CDKN2B-CDKN2A* locus at 9p21. Copy number reduction of *Cdkn2b-Cdkn2a* also was seen in the gastric mucosa of AID transgenic mice.[87] In agreement with the preferential deletions at the *CDKN2B-CDKN2A* locus in gastric epithelial cells by AID introduction, AID expression was required for inducing DNA single-strand breaks in the *CDKN2B* gene in leukemia cells,[88] and, furthermore, the deletion of the *CDKN2B-CDKN2A* locus frequently is detectable in AID-expressing lymphoid blast crisis leukemia cells.[75] These findings suggest that AID can induce both mutations and deletions at the same gene locus, and, moreover, that the representative tumor-suppressor genes, *TP53* and *CDKN2B-CDKN2A*, may be common targets for AID-mediated genotoxic effects in various human tissues in the setting of inflammation.

Finally, a recent finding that a deficiency of endogenous AID reduced the incidence of both accumulation of somatic mutations in the *Trp53* gene and the development of colitis-associated colorectal cancers further supports the critical role of AID in inflammation-associated cancer development via its ability to induce genetic alterations in tumor-related genes.[89]

Inflammation and Epigenetic Modulation

Epigenetic modifications are DNA-associated modifications that are inherited upon somatic cell replication, which include DNA methylation and histone modifications.[90] Coordinated changes of epigenetic modifications control development and tissue differentiation, and erasure of epigenetic modifications is involved in reprogramming. In somatic cells, DNA methylation is present in repetitive elements, CpG-sparse regions, and in a very limited number of CpG islands.[91,92] DNA methylation of a CpG island in a promoter region causes silencing of its downstream gene, whether it is a protein-coding gene or a microRNA (miRNA) gene, by forming nucleosomes and thus possibly blocking access of RNA polymerase II to the promoter.[93,94] In contrast, DNA methylation of a gene body often is associated with increased gene expression.[91,95]

Histone modifications denote chemical modifications, such as acetylation, methylation, and ubiquitination of lysine and arginine residues of histones, mainly H3 and H4, but also H2A and H2B.[93] Specific histone modifications, such as acetylation of histones H3 and H4 (H3Ac and H4Ac) and trimethylation of lysine 4 of histone H3 (H3K4me3), are associated with active gene transcription. In contrast, dimethylation and trimethylation of H3 lysine 9 (H3K9me2 and H3K9me3) and trimethylation of H3 lysine 27 (H3K27me3) are associated with gene repression. H3K9me2 represses gene transcription in concert with DNA methylation, whereas H3K27me3 works independently of DNA methylation.[96] Trimethylation of H3 lysine 36 (H3K36me3) is considered to mark exonic regions of active genes. However, the mechanisms of how histone modifications are inherited upon somatic cell replication remains unclear.[97]

In cancer cells, the presence of regional hypermethylation and global hypomethylation has been described.[98,99] Regional hypermethylation refers to aberrant DNA methylation of promoter CpG islands physiologically kept unmethylated.[95,100] If aberrant methylation is induced in a promoter CpG island it consistently induces silencing of its downstream gene.[90] Many tumor-suppressor genes that have promoter CpG islands, such as *CDKN2A*, mutL homolog 1 (*MLH1*), cadherin-1, and RAS-association domain family 1, isoform A, can be inactivated permanently by aberrant DNA methylation as drivers, which have significant roles in cancer development. At the same time, most of the aberrant DNA methylation of promoter CpG islands are considered to be passengers that play no role in carcinogenesis.[14] Several hundreds to thousands of promoter CpG islands are methylated aberrantly in a cancer, and the number is too large for all of them to be drivers. Moreover, most of the genes methylated in cancers are not expressed in normal tissues,[101,102] and such genes are considered not to be involved in carcinogenesis. Global hypomethylation was shown to be causally involved in carcinogenesis by inducing genomic instability.[103] In addition, induction of H3K27me3 is considered to be an alternative mechanism to induce gene silencing,[96] and aberrant H3K27me3 was observed in promoter regions consisting of 200-600 genes.[96,104] Again, the number is very large, and most are expected to be passengers.

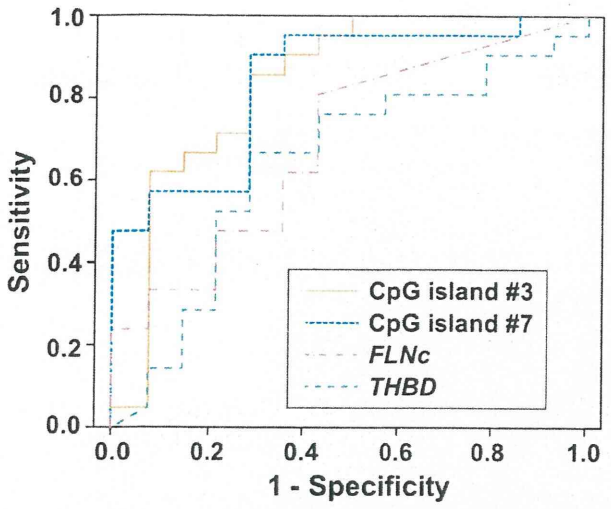

Figure 4. The degree of epigenetic field defects can be assessed using methylation levels of appropriate marker CpG islands, mostly passengers. Receiver-operating characteristic curves were drawn to distinguish gastric mucosae of gastric cancer patients and those of healthy individuals with past infections by *H pylori*. The receiver-operating characteristic curves of newly isolated methylation risk markers, CpG islands #3 and #7, had a much larger area under the curve values than those of 2 previously isolated markers, filamin C (FLNc) and thrombomodulin (THBD), reaching 0.78–0.84. Modified from Nanjo et al.[169]

As inducers of aberrant DNA methylation, aging was first indicated,[105] and chronic inflammation then was suggested by the presence of aberrant DNA methylation of specific tumor-suppressor genes in noncancerous colonic mucosae of patients with IBD.[106,107] Aberrant DNA methylation was present more frequently in liver tissues of patients with HCC than in those with metastatic liver tumors.[108] By measuring methylation levels of passenger genes in gastric mucosae of *H pylori*-infected individuals, a very close association between *H pylori* infection and high methylation levels in gastric mucosa was shown.[15] Aberrant DNA methylation is particularly prominent in chronic inflammation-associated cancers, such as gastric cancer, HCCs, colitic cancer, cholangiocarcinoma, Barrett's cancer, and pancreatic cancer.[13] These findings strongly indicated that the major inducer of aberrant DNA methylation is chronic inflammation.

Levels of aberrant DNA methylation accumulated in normal-appearing tissues correlate with the risk of gastric, colon, breast, and renal cancers.[15,109–112] Such accumulation mainly involves passenger genes and driver genes to some extent, and is considered to form an epigenetic field for cancerization (epigenetic field defect) (Figure 4).[113] Chronic inflammation-associated cancers are known to show multiple events, which can be explained by the presence of a field defect in normal-appearing tissues. Along with the accumulation of genetic alterations, an epigenetic field defect is deeply involved in the development of inflammation-associated cancers. The degree of epigenetic field defects can be measured easily using methylation levels of marker genes,[114] which are passenger genes in most cases and show relatively high methylation levels in predisposed tissues.[113]

Mechanistic studies, including cause and effect of accumulated aberrant DNA methylation and chronic inflammation, were conducted using animal models. When *H pylori*-induced inflammation was suppressed by cyclosporine A in Mongolian gerbils, induction of aberrant DNA methylation markedly was suppressed, although the number of *H pylori* in gastric mucosae was unaffected.[16] This indicated that inflammation, not *H pylori* itself, is critical for induction of aberrant DNA methylation. Expression analysis of inflammation-related genes showed that expression levels of *Il-1b*, *Nos*, *Tnf*, and chemokine (C-X-C motif) ligand 2 correlated with methylation levels in gastric mucosae. *H pylori*-induced inflammation was capable of inducing aberrant DNA methylation, but not repeated induction of acute inflammation by ethanol or a high sodium concentration.[115] *Il-1β*, *Nos2*, and *Tnf* were specifically up-regulated by the *H pylori*-induced inflammation. Notably, in human beings, a polymorphism of the *IL-1B* promoter was associated with not only gastric cancer susceptibility,[35] but also the presence of the CpG island methylation phenotype in gastric cancers.[116]

Another animal model for methylation induction by chronic inflammation is mouse colitis induced by administration of dextran sulfate sodium (DSS).[117] Aberrant DNA methylation of multiple genes occurred in DSS-induced colitis mucosae before induction of colon tumors, showing an epigenetic field.[118] The induction of aberrant DNA methylation was unaffected even in severe combined immunodeficiency mice that lacked T and B cells, suggesting that infiltrated macrophages might be critical for methylation induction. Gene expression analysis in colonic mucosae in wild-type and severe combined immunodeficiency mice showed that expression levels of *Il-1b*, *Nos*, and *Ifn-g* were associated with methylation induction in colonic mucosae. Taken together with the finding in the *H pylori*-infected gerbils, infiltration of macrophages and resulting secretion of Il-1β and Tnf-α, as well as production of active oxygen species, are believed to be involved in induction of aberrant DNA methylation in epithelial cells (Figure 1).

Several in vitro studies have been conducted to examine inflammatory signals that lead to methylation induction in target cells. Treatment of insulinoma or blood cells with IL-1β or a NO donor induced methylation of endogenous genes by increasing activity of DNA methyltransferase(s) (DNMTs).[119] IL-6 induces DNMT1 transcription by increasing its promoter activity and suppressing microRNA (miR)-148a and miR-152, both of which target DNMT1.[120,121] Although some studies suggested that DNA methylation is induced by IL-1β or IL-6, the changes were marginal, possibly because identification of appropriate target CpG islands was difficult and the levels of increase were too small to be detected by ordinary methods. Prostaglandin E2 treatment of cancer cell lines increased DNMT1 and DNMT3B expression, and induced

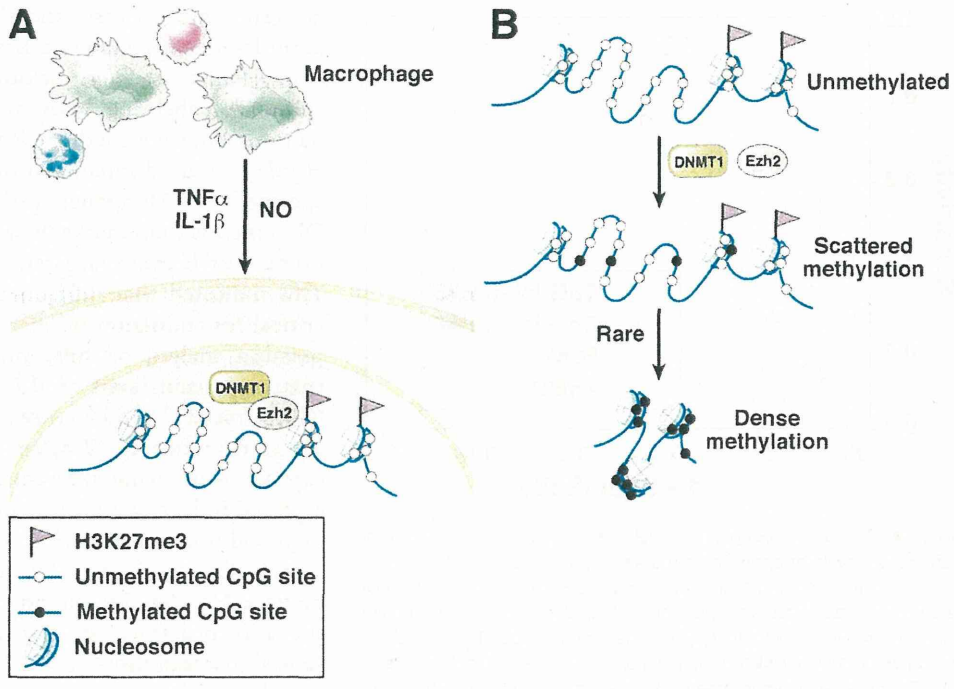

Figure 5. Current model of aberrant DNA methylation induction by chronic inflammation. (*A*) Cytokines, such as IL-1 and TNF-a from macrophages, and oxidative stress, such as NO, are associated with methylation induction in epithelial cells. EZH2 and DNMT1 are reported to be recruited to a promoter CpG island of a damaged gene, and mark it with a flag of H3K27me3. (*B*) Scattered methylation, introduced by DNMT1, leads to dense methylation although the frequency is low.

DNA methylation of specific genes, which also was observed in vivo.[122]

In contrast to in vitro studies, mRNA expression levels of Dnmt1, Dnmt3A, and Dnmt3B were not increased in vivo, such as colonic mucosae with DSS-induced colitis,[16] and human gastric tissues with *H pylori* infection.[123] In line with these in vivo findings, O'Hagan et al[124] recently showed in vitro that oxidative damage recruits complexes containing DNMTs, a histone deacetylase (sirtuin 1), and histone methyltransferase (enhancer of zeste homolog 2 [EZH2]) to damaged chromatin, and induces DNA methylation. They also showed that in Apc^{Min} mice infected with an inflammation-inducing bacterium, Dnmt1 and Ezh2 are recruited to promoter CpG islands of untranscribed or minimally transcribed genes. Promoter CpG islands with H3K27 me3 and without RNA polymerase II are susceptible to DNA methylation induction.[101,102]

Taken together, we can hypothesize a model for aberrant DNA methylation induction in vivo (Figure 5). Inflammatory signals mainly from macrophages, such as IL-1β, TNF-α, and IL-6, and oxidative stress, possibly produced by NO synthase, are likely to recruit a complex with DNMT1 and EZH2 to promoter CpG islands with H3K27 me3 flag and without protection by RNA polymerase II. Because DNA methylation is harmful to a gene, aberrant DNA methylation is likely to be induced only rarely and at scattered CpG sites within a CpG island (seeds of methylation).[123] Most seeds of methylation are erased during cell replication, but can lead to dense methylation of a CpG island at very low frequencies.[125,126] If such dense methylation is induced in a promoter CpG island of a tumor-suppressor gene, the tissue becomes predisposed to carcinogenesis, and forms an epigenetic field defect.

In addition to aberrant DNA methylation of promoter CpG islands, cancer cells are characterized by global DNA hypomethylation as well as aberrant hypomethylation of oncogenes.[99,127] Gastric mucosa infected by *H pylori* displays global hypomethylation.[128] In this regard, it is interesting to note that AID recently was shown to be involved in active DNA demethylation during fetal development.[129] Mechanistically, AID deaminates 5-methyl cytosine to yield T. This T subsequently would be removed by either of the T:G mismatch-specific glycosylases, thymidine DNA glycosylase, or methyl-CpG binding domain protein 4. The resulting abasic site then would be replaced by an unmethylated C via base excision repair processes, resulting in DNA demethylation. Notably, AID participates in active demethylation by 5-methyl cytosine hydroxylase, ten-eleven translocation 1, and subsequent gene expression in the dentate gyrus of adult mouse brain.[130] Thus, whether AID is involved in DNA demethylation during cancer development is an interesting topic for future studies.[131] The fact that AID targets the chromatin marked by H3K4 me3 histone modification,[132] in contrast to preferential DNA methylation at promoter CpG islands with H3K27 me3 histone modification,[101,102] might suggest opposing mechanisms for induction of DNA methylation and demethylation.

Inflammation and miRNA Modulation

miRNAs are short noncoding RNAs that regulate the expression of many target genes post-transcriptionally, and thus are involved in a variety of cellular functions. Recent studies have revealed that miRNAs have important roles in cancer development as either oncogenes or tumor-suppressor genes by regulating various cancer-related proteins or mRNA expressions.[133,134] In-

deed, cancer cells are associated with dysregulation of many miRNA expressions, which occurs through a variety of mechanisms, such as genetic changes, epigenetic regulation, or altered expression of transcription factors.[135] On the other hand, miRNA expression also is altered in inflammatory conditions, and such alterations in miRNA expression appear to play roles not only in controlling chronic inflammation, but also in promoting cancer development.[136,137] Many of the changes in miRNA expressions observed in inflammatory tissues are derived from immune cells that may participate in hematopoietic tumorigenesis.[138] However, recent reports have shown that inflammation also induces changes in cancer-related miRNAs in epithelial cells, suggesting a direct link between alteration of miRNA expressions and inflammation-associated cancer development.[139,140]

miRNA expressions in epithelial cells can be altered during inflammation through various mechanisms such as NF-κB activation by Toll-like receptors or cytokine stimulation and STAT3 phosphorylation by IL-6 or other cytokines.[139-143] Among those, several miRNAs are identified as tumor-suppressor miRNAs. *miR-7* targets not only epidermal growth factor receptor (*Egfr*) but also latrophilin 2, brain abundant, membrane-attached signal protein 1, and musculoaponeurotic fibrosarcoma oncogene homolog, and thus is considered to be a tumor-suppressor miRNA.[142] In a mouse model of inflammation-associated cancer development, expression of *miR-7* was shown to be inhibited by activated macrophages in *Helicobacter*-infected gastritis mucosa and was shown to be involved in gastric cancer development, although it was increased in germ-free conditions.[142] Lethal-7 (*Let-7*), consisting of 12 members, targets the *RAS* family and *c-MYC*,[144,145] and genomic locations of *let-7* family members frequently are deleted in colon cancers and other solid cancers.[146] NF-κB activation enhances *Lin28B* transcription, which causes posttranscriptional inhibition of *let7* family member expression, and *let-7* directly inhibits IL-6 expression, a cytokine often produced in cancer cells. Thus, reduction of *let-7* expression by NF-κB activation appears to play a role in a positive feedback loop for NF-κB activation through an increase of IL-6 in cancer cells.[147]

miR-155, a possible oncogenic miRNA, is involved in blood cell maturation, immune responses, and autoimmune disorders, and high expression of *miR-155* is associated with the development of myeloproliferative disorders.[148] Recent studies have revealed a direct link between increase of *miR-155* and tumor formation and development in gastric and colon cancers.[148,149] *miR-155* expression is induced by NF-κB, interferon-β, and TLR stimulation,[150] and thus enhanced by *H pylori* and lipopolysaccharide (LPS) treatment.[151] Recently, Tilli et al[143] reported that TNF-α/LPS stimulation enhances *miR-155* expression in association with an increased mutation rate. They also showed that *miR-155* targets mitosis inhibitor protein kinase 1, which blocks cell-cycle progression, and therefore reasoned that reduction of mitosis inhibitor protein kinase by *miR-155* allowed cell division to continue even in the presence of DNA damage, leading to enhanced mutation induction. In another study, they also showed that *miR-155* promotes gene mutations by down-regulating the core mismatch repair proteins, hMSH2, hMSH6, and hMLH1.[152] Of particular interest are the recent reports showing that *miR-155* negatively regulates AID in B cells. Teng et al[153] showed that *miR-155* is up-regulated in B cells undergoing class-switch recombination, and regulates the germinal center reaction by modulating AID. Moreover, *miR-155* has been suggested to inhibit *MYC-IGH* translocation by reducing AID mRNA and protein in B cells.[154] Thus, although an inhibitory effect of *miR-155* on AID has not been examined in non–B cells, *miR-155* also may have a tumor-suppressor function in epithelial cells by inhibiting AID production.

A miRNA expression pattern distinct from normal colonic mucosa has been found in the colonic mucosa and in colitic tumor of patients with IBD as well as mice with colonic inflammation, including up-regulation of *miR-21* and *miR-3*.[155] *miR-21* is one of the most highly expressed miRNAs in colonic tissues of patients with ulcerative colitis,[155] and its expression is enhanced by LPS and IL-6 through STAT3 activation, targeting key regulators of cell proliferation and apoptosis such as phosphatase and tensin homolog and programmed cell death 4.[156] Olaru et al[157] recently showed that in colitic cancer development *miR-31* expression increases in a stepwise fashion from IBD to cancer, and that *miR-31* directly targets regulating factor inhibiting hypoxia-inducible factor 1, decreasing its repressor activity for hypoxia-inducible factor 1.

It is now evident that miRNAs exert various functions in inflammation-associated cancer development. However, alterations of miRNA expression observed in inflammatory tissues occur in both immune cells and epithelial cells. Accordingly, it is important to dissect miRNA changes in the 2 cell types because the patterns of the miRNA changes are different between immune cells and epithelial cells. Further elucidation of the changes of miRNA expression, particularly in epithelial cells, will facilitate our understanding of the role of tumor-related miRNAs in inflammation-associated cancer development.

Application to Cancer Prevention, Diagnostics, and Therapeutics

To prevent inflammation-associated cancer development, it is crucial to cure or control inflammation. Indeed, it has been shown repeatedly that long-term therapy with anti-inflammatory drugs resulted in fewer appearances of tumors.[158] The best way to control chronic inflammation is, of course, to eliminate causative infections. In other cases unrelated to infection such as IBD and PSC, one approach is to block the action of key regulators of inflammation. In this regard, NF-κB or STAT3, and their activators TNF-α or IL-6, respectively, may be good targets for suppressing the inflammatory response. However, because treatment usually needs to be continued for long periods to control chronic inflamma-

tion, agents without serious side effects with lower costs should be developed. For this purpose, many natural agents derived from vegetables, fruits, spices, and their components have been tested. Among them, curcumin, derived from yellow spice turmeric (Curcuma longa) has been used for centuries, and has been shown to suppress NF-κB- as well as STAT3-regulated inflammation,[159] and thus can be administered safely over the long term.[160] Indeed, a recent study showed that curcumin reduced TNF-α expression, prevented cancer-associated weight loss, and induced apoptosis of tumors in patients with colorectal cancer.[161] Resveratrol, a natural polyphenolic, nonflavonoid antioxidant found in grapes and other berries has been shown to have generalized inhibitory effects on inflammation-related molecules such as NF-κB, COX-2, and tyrosine kinases.[162] Recently, resveratrol was found to alter the expression of many tumor-related miRNAs.[163] Similar types of agents may have the potential to both prevent and treat cancers.[164]

In contrast to controlling inflammatory mediators, blocking genetic modulation appears to be difficult. One might consider inhibiting AID. However, because AID plays a critical role in immunoglobulin maturation in B cells, specific targeting for AID in the epithelial cells without affecting AID in B cells is critical. Control of epigenetic modulation can be considered from 2 aspects: suppression of methylation induction and reversal of induced methylation. Because induction of methylation is not essential in adult somatic cells, control of this process is a promising approach to prevent chronic inflammation-associated cancers. On the other hand, reversal of aberrant DNA methylation is an attractive idea to repair an epigenetic field defect, but targeting only aberrant DNA methylation without affecting physiological DNA methylation is currently very difficult.

H pylori eradication ameliorates chronic inflammation, and reduces the risk for gastric cancer. However, it is apparent that eradication cannot completely resolve chronic inflammation because some patients develop gastric cancer even after successful eradication.[165] Likewise, some patients with chronic hepatitis or cirrhosis as a result of HCV infection also develop HCC after obtaining a sustained virologic response.[166] As such, when inflammation is not appropriately controlled or even when inflammation is resolved after long-standing inflammation, accurate prediction for the risk of developing cancers in the inflammatory tissues becomes important. As was discussed, carcinogenesis is characterized by a stepwise accumulation of both genetic and epigenetic changes. Importantly, previous data suggested that the extent of those genetic and epigenetic modulations is paralleled with the duration or severity of inflammation,[15,167] and the degree of epigenetic field defect can be measured relatively easily and accurately. Thus, both qualitative and quantitative detection of these genetic and epigenetic changes in inflammatory tissues or tissues previously exposed to inflammation may provide a good risk marker for inflammation-associated cancer development. Indeed, epigenetic risk markers that can differentiate gastric mucosae of cancer patients from those of healthy individuals with odds ratios between 12.7 and 36.0 have been isolated,[168,169] and a prospective study is now being conducted.

Conclusions

Many cancers in digestive organs develop in the background of chronic inflammation. During chronic inflammation, a variety of mediators for inflammation such as cytokines, growth factors, eicosanoids, ROS, and NOS form complex networks not only for maintaining or reducing inflammation but also promoting cell growth, angiogenesis, and inhibiting apoptosis. These events eventually merge into and result in both genetic and epigenetic changes of the cellular genome, leading to inflammation-associated cancer development. In particular, AID plays a crucial role in inducing not only mutations, but also chromosomal aberrations during inflammation. Moreover, signals from macrophages with resulting mislocalization of DNMTs appear to be involved in the induction of epigenetic alterations.

Interestingly, epigenetic inactivation of *MLH1* leads to accumulation of genetic alterations.[170] At the same time, recent studies have shown that AID induces DNA demethylation through its deaminating activity on methylated cytosines.[131] Thus, genetic and epigenetic events are mutually related and work in concert in the development of inflammation-associated cancers.

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Conflicts of interest

The authors disclose no conflicts.

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Development of gastric cancer in nonatrophic stomach with highly active inflammation identified by serum levels of pepsinogen and *Helicobacter pylori* antibody together with endoscopic rugal hyperplastic gastritis

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This study aimed to elucidate groups at high risk of developing cancer among patients with serologically identified *Helicobacter pylori* infection and nonatrophic stomach. Annual endoscopy was performed for a mean of 5.4 years in 496 asymptomatic middle-aged men who were *H. pylori* antibody-positive and pepsinogen (PG) test-negative. Subjects were stratified according to the activity of *H. pylori*-associated gastritis measured by serum levels of PG and *H. pylori* antibody, and/or by endoscopic findings of rugal hyperplastic gastritis (RHG), and cancer development was investigated. During the study period, seven cases of cancer developed in the cohort (incidence rate, 261/100,000 person-years), with 85.7% developing in the group showing a PGI/II ratio ≤3.0, reflecting active inflammation-based high PGII levels. Cancer incidence was significantly higher in this group (750/100,000 person-years) than in groups with less active gastritis. Furthermore, cancer incidence for this group was significantly higher in the subgroup with high *H. pylori* antibody titers than in the low-titer subgroup. Meanwhile, endoscopic findings revealed that 11.7% of subjects showed RHG reflecting localized highly active inflammation, and cancer risk was significantly higher in patients with RHG than in patients without. Combining the two serum tests and endoscopic examination for RHG allowed identification of subjects with more active gastritis and higher cancer risk. No cancer development was observed in these high-risk subjects after *H. pylori* eradication. Subjects with highly active gastritis identified by the two serological tests and endoscopic RHG constitute a group at high risk of cancer development with *H. pylori*-infected nonatrophic stomach.

Epidemiology

Key words: pepsinogen, *Helicobacter pylori*, diffuse-type gastric cancer, giant-fold, rugal hyperplastic gastritis

Abbreviations: CAG: chronic atrophic gastritis; CI: confidence interval; EGD: esophagogastroduodenoscopy; ELISA: enzyme-linked immunosorbent assay; HR: hazard ratio; IL: interleukin; NNT: number needed to treat; NSAIDs: nonsteroidal antiinflammatory drugs; PG: pepsinogen; RHG: rugal hyperplastic gastritis; SD: standard deviation

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Gastric cancer is one of the leading causes of cancer-related deaths in Japan, with 50,017 deaths attributed to this cancer in 2009.[1] *Helicobacter pylori* infection is now widely accepted as playing a major role in the development of gastric cancer in areas showing high-risk for this cancer, including Japan.[2–5] *H. pylori* triggers chronic inflammation in the gastric mucosa, leading to a series of molecular and morphological events known as the atrophy-metaplasia-dysplasia-cancer sequence.[3–5] This carcinogenic sequence is considered to represent a major route of stomach carcinogenesis, and previous studies, including our own, have identified positive correlations between extent of chronic atrophic gastritis (CAG) and risk of gastric cancer.[6–13] Our longitudinal cohort study clearly indicated that progression of *H. pylori*-associated chronic gastritis increases cancer risk in a stepwise manner, and that patients with metaplastic gastritis, an end result of chronic *H. pylori* infection, show the highest annual cancer incidence rate of around 1% among asymptomatic middle-aged Japanese men.[11] Other

studies have indicated a different subroute, in which incomplete and unstable CAG is directly associated with cancer development.[14] In fact, clinicopathological studies have indicated that 20–30% of all stomach cancers in Western countries develop from nonatrophic stomach,[5,15] and seroepidemiological studies using serum pepsinogen (PG) in Japan have revealed that the background stomach does not appear to have extensive CAG in 20–40% of cancer cases.[12,16] The bacterial load in *H. pylori*-associated chronic gastritis is generally reported to be larger in stomachs with mild to moderate atrophy than in stomachs with extensive atrophy, and the bacterium is spontaneously eradicated with the establishment of metaplastic gastritis.[17–19] Thus, if *H. pylori* infection itself plays a pivotal role in the development of gastric cancer, a high-risk group should be identifiable among subjects without extensive CAG. Indeed, our previous longitudinal cohort study revealed a small group of subjects at high-risk of cancer among CAG-negative subjects identified by serum PG levels.[12] This group of subjects developed cancer at an annual incidence rate of more than 0.2%, comparable to that in CAG-positive subjects identified by PG levels. Characteristics of this high-risk group with nonatrophic stomach are low serum PGI/II ratio (due to a high PGII level), reflecting a process of highly active gastric inflammation, and more importantly, frequent development of diffuse-type cancer as the histopathological-type, representing cancer with a higher malignant potential than cancer developing from the aforementioned main route of stomach carcinogenesis, *i.e.*, intestinal-type cancer.[12] The incidence rate of diffuse-type cancer in this high-risk group is 120/100,000 person-years, considerably higher than the reported rate of <5/100,000 person-years in Western countries,[20] or even the rate of around 40/100,000 person-years among middle-aged Japanese men.[11,21]

Highly active *H. pylori*-induced inflammation in the nonatrophic stomach body has also been reported to induce enlargement of the rugal folds,[22–25] appearing endoscopically as rugal hyperplastic gastritis (RHG).[26] This specific type of gastritis is characterized by enhancements of inflammatory cell infiltration and proinflammatory cytokine production, including interleukin (IL)-1β, which leads to the proliferation of mucosal epithelia and foveolar hyperplasia, and finally to the establishment of enlarged folds or rugal hyperplasia.[23,27] Subjects with RHG are suggested to be at a higher risk for cancer, particularly diffuse-type cancer, than subjects with CAG.[28–31] However, no long-term follow-up data analyzing cancer development from RHG have been reported. Furthermore, the relationship between this serologically identified high-risk group without CAG and the group with RHG is unclear. The present longitudinal cohort study was initiated to clarify these issues and to investigate cancer development from highly active inflammation in the nonatrophic stomach as identified by serum tests and/or endoscopy.

Subjects and Methods

Study subjects

Between January 1999 and December 2000, a total of 36,762 middle-aged factory workers (34,610 men, 2,152 women) between 40- and 60-years old participated in an annual multiphasic health-screening program in a factory in Wakayama City, Wakayama Prefecture, Japan. This type of screening program is generally performed by various workplaces throughout Japan to detect incident diseases in the early stages, and includes an interview to ascertain general state of health, physical examination, chest radiography, electrocardiography, blood laboratory tests, urinalysis, fecal occult blood testing and gastric cancer screening by either barium X-ray or esophagogastroduodenoscopy (EGD) as selected by the individual. All the participating subjects were essentially asymptomatic and individuals presenting with symptoms requiring prompt medical care had been excluded from the screening program. The subjects could thus be considered to represent the healthy Japanese population. Of these subjects, those who had undergone barium X-ray as a part of an annual health check-up in the same workplace were investigated in the previous cohort studies for gastric cancer risk with special reference to CAG as diagnosed by serum PG levels.[11–13] In the present study, 3,334 workers who selected EGD for cancer screening were candidates for inclusion, and *H. pylori*-infected subjects with nonatrophic stomach were selected for the study based on the two serological tests, PG and *H. pylori* antibody titer, as described in the following section. Figure 1 shows the schema for the selection of study subjects.

Follow-up by endoscopy

As described above, the 3,334 subjects underwent annual check-up by panendoscopy (Type XQ200; Olympus, Tokyo, Japan). The day of gastric cancer identification was defined as the day of the health check-up when cancer was detected. The observation period was calculated for each subject from the time of the baseline survey to the diagnosis of gastric cancer. The location of cancer detected in the stomach was classified as cardia or noncardia based on clinical or histopathological records. Resected specimens of gastric cancer obtained by surgery were histopathologically assessed and classified as intestinal or diffuse-type, according to the classification described by Lauren.[32] RHG was diagnosed based on endoscopic findings of severely enlarged tortuous folds in the gastric body that did not disappear despite adequate insufflation, according to the classification of the Sydney system.[26]

Serological analysis

Aliquots of separated sera from fasting blood samples collected as routine laboratory tests for the aforementioned general health check-up were stored at -20°C and used for the measurement of serum levels of PG and *H. pylori* antibody. Serum PGI and PGII levels were measured using a modification (RIA-beads Kit; Dainabott, Tokyo, Japan) of our previously reported

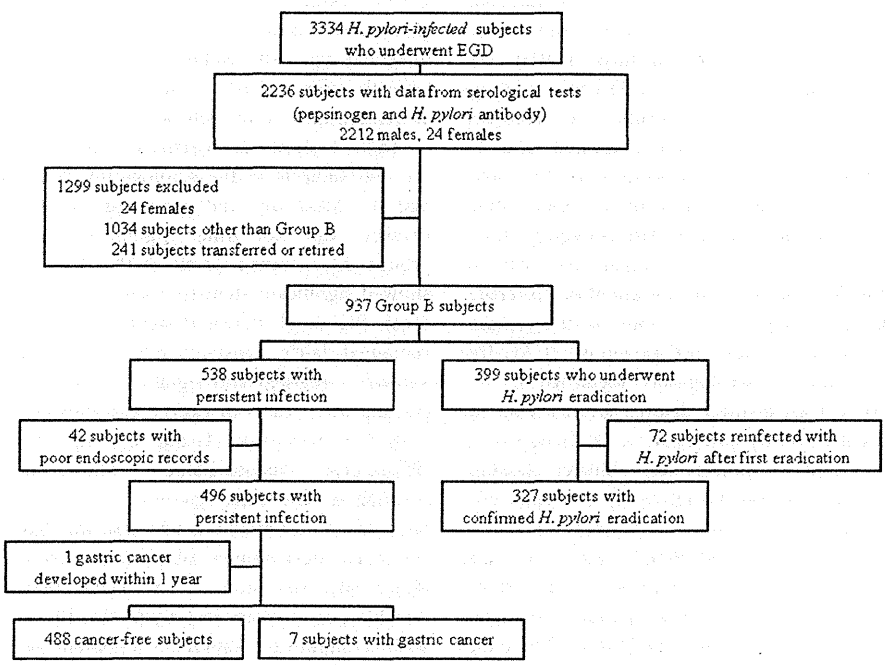

Figure 1. Schematic flow of the study subjects.

radioimmunoassay.[33] Subjects with extensive CAG were diagnosed based on PG test-positive criteria of PGI $\leq$50 ng ml^{-1} and PGI/II ratio $\leq$3.0.[16] These criteria offer 69% sensitivity and 80% specificity for the diagnosis of extensive CAG.[16] Serum *H. pylori* antibody levels were measured using enzyme-linked immunosorbent assay (ELISA) (MBL, Nagoya, Japan).[34] Subjects with antibody titers >50 U ml^{-1} were classified as *H. pylori*-infected. Sensitivity and specificity of the ELISA used in the present study were 93.5 and 92.5%, respectively.[34] Based on the difference in cancer risk according to the antibody titer level as described in our previous study,[13] *H. pylori*-infected subjects were further divided into two groups depending on the antibody titer: a high-titer group comprising subjects with titers >500 U ml^{-1}; and a low-titer group.

Subjects with a previous history of gastric cancer or adenoma, surgical resection of the stomach, *H. pylori* eradication or renal failure, and those who had been prescribed medications that might affect gastrointestinal function, such as proton pump inhibitors, adrenocortical steroids or nonsteroidal antiinflammatory drugs, were excluded from the study. As a result, among the 3,334 subjects who selected EGD for cancer screening, a total of 2,236 subjects (2,212 men, 24 women) opted to pay the extra charge for measurement of serum levels of PG and *H. pylori* antibody, in addition to the ordinary screening program (Fig. 1). Female subjects were subsequently excluded from analysis in this study because of their small number.

The remaining 2,212 male subjects were classified into one of the following four groups based on the results of the two serum tests for PG and *H. pylori* antibody: Group A, *H. pylori*-negative and CAG-negative; Group B, *H. pylori*-positive and CAG-negative; Group C, *H. pylori*-positive and CAG-positive; and Group D, *H. pylori*-negative and CAG-positive. This classification reflects each stage of serial changes in the stomach mucosa induced by the progression of *H. pylori*-associated chronic gastritis, with Group A including subjects with *H. pylori* infection-free healthy stomach, Group B including *H. pylori*-infected subjects without CAG, Group C including subjects with *H. pylori*-induced CAG, and Group D including subjects with extensive CAG together with widespread intestinal metaplasia.[11]

In the present cohort, 937 subjects were classified into Group B, and were advised to attend the clinic at their workplace. At the clinic, they were told about the possible roles of *H. pylori*-infection in peptic ulcer disease or gastric cancer, and also about the possible effects of *H. pylori* eradication, including costs and potential adverse effects. Thereafter, during the period of enrollment 399 subjects underwent *H. pylori* eradication with dual therapy consisting of the proton pump inhibitor omeprazole at 20 mg twice a day and amoxicillin at 750 mg or 500 mg twice a day for 2 weeks or with triple therapy consisting of omeprazole at 20 mg twice a day, amoxicillin at 750 mg twice a day and clarithromycin at 200 mg twice a day for 1 week. These patients were followed separately for analysis of the effects of eradication. In study subjects, *H. pylori* status including the completeness of eradication was assessed by serum *H. pylori* antibody level at all annual health check-ups during the study period.

The remaining 538 subjects did not undergo eradication therapy, and the prevalence of RHG on endoscopy at the time of enrollment was analyzed. The diagnosis of RHG was made by each endoscopist based on endoscopic findings according to the Sydney system.[26] In addition, the recorded endoscopic images of each subject were examined, and the diagnosis of RHG was confirmed retrospectively by two other endoscopists, who were blinded to information about study subjects, including cancer development. After excluding those with poor-quality recorded endoscopic images or with an inappropriately distended stomach on examination, interpretation of the endoscopic images was possible in the remaining 496 Group B subjects. As described previously, CAG-free subjects (Groups A and B) serologically identified by PG test-negative criteria consist of three distinct groups from the perspectives of gastritis activity and cancer risk: Groups α, β and γ.[12] The 496 Group B subjects were further classified into the following three groups: Group Bα (n = 111), with PGI ≤50 ng ml^{-1} and PGI/II ratio >3.0; Group Bβ (n = 235), with PGI >50 ng ml^{-1} and PGI/II ratio >3.0; and Group Bγ (n = 150), with PGI >50 ng ml^{-1} and PGI/II ratio ≤3.0. These subjects were followed annually to detect endoscopically incident cancer, as described above. The ethics committee of Wakayama Medical University approved the study protocols, and informed consent was obtained from all subjects prior to participation.

Statistical analysis

Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL) and STATA software (STATA, College Station, TX). Differences in continuous values were tested for significance using the t test for comparisons between two groups. The χ^2 test was used to compare categorical variables. Long-term effects of endoscopic findings of RHG and/or serum levels of PG and *H. pylori* antibody on gastric cancer development were analyzed using the Kaplan–Meier method and statistical differences between curves were tested using the log-rank test. Hazard ratios (HRs) were calculated using Cox proportional hazards modeling. For all comparisons, probability values <5% ($p < 0.05$) were considered statistically significant.

Results

A total of 496 *H. pylori*-infected subjects without CAG (*i.e.*, Group B subjects) were followed for a mean [standard deviation (SD)] period of 5.4 (4.0) years (Table 1). During the study period, eight cases of gastric cancer developed in study subjects. Among these was one case of cancer that developed within the first year of follow-up. That case was excluded from the study, since the cancer might have been present from the start of the study. The overall cancer incidence rate was thus 261/100,000 person-years. All seven cancers detected in the present study were localized in the middle- or upper-third of the stomach, and none involved the gastric cardia. Histopathologically, all were early-stage cancers confined to the mucosa or submucosa, and six cases (85.7%) were diffuse-type (three mucosal cancers, three submucosal cancers), while the remaining case (14.3%) was intestinal-type with submucosal invasion. All cancer cases were therefore treated by surgical resection and the patients all remain alive under follow-up at our clinic.

Table 1 shows the details of cancer development among the 496 subjects in the serologically defined Groups Bα, Bβ and Bγ. Mean age and percentages of smokers and alcohol drinkers did not differ significantly among these three groups. Mean serum levels of PG II and *H. pylori* antibody showed significant stepwise increases from Bα to Bγ, whereas mean PGI level increased significantly from Bα to Bβ, then remained fairly constant in Bγ. As a result, PGI/II ratio showed a marked and significant reduction from Bβ to Bγ. Among the 7 cases of cancer examined in the present study, 6 (85.7%) were from Group Bγ, with 83.3% (5/6) showing diffuse-type histopathology. These developed steadily throughout the study period after 1.5 years of follow-up (Fig. 2*a*). The remaining one case was from Group Bβ, with no cancer development observed in Group Bα. Cancer incidence rates were thus 0, 78/100,000 person-years and 750/100,000 person-years in Groups Bα, Bβ and Bγ, respectively, with a significant difference apparent between Groups Bβ and Bγ [HR, 10.00; 95% confidence interval (CI), 1.19–84.05]. High *H. pylori* antibody titer (>500 U ml^{-1}) was seen in 32.1% (159/496) of study subjects, and the percentage of the high-titer group increased in a stepwise manner from Group Bα (13.5%) to Group Bβ (33.6%) to Group Bγ (43.3%). No significant differences in mean age or alcohol-drinking status were seen between the high- and low-titer groups, but presence of a smoking habit was significantly less frequent in the high-titer group, reflecting mainly smoking status in Group Bβ rather than that in Group Bγ. Cancer incidence was significantly higher in the high-titer group (577/100,000 person-years) than in the low-titer group (110/100,000 person-years) (HR, 6.51; 95%CI, 1.24–34.17), and 71.4% (5/7) of cancers developed in the high-titer group. All cancers that developed in the high-titer group belonged to Group Bγ, and 80% (4/5) showed diffuse-type histopathology. A marked increase in cancer development was apparent in Group Bγ with an increase in antibody titer ($p < 0.05$, Fig. 2*b*); cancer incidence rates in Group Bγ with high and low titers were 1,524/100,000 person-years and 212/100,000 person-years, respectively (Table 1). Meanwhile, endoscopic findings revealed RHG in 11.7% (58/496) of study subjects, and the percentage of RHG increased in a stepwise manner with the transition of serologically classified groups from Bα to Bγ, with 0% (0/111) in Group Bα, 7.7% (18/235) in Group Bβ and 26.7% (40/150) in Group Bγ (Table 1). In both Groups Bβ and Bγ, RHG was more prevalent in that subgroup of individuals with a high titer of *H. pylori* antibody. The percentage of RHG was thus highest in Group Bγ with high titer (35.4%).

As shown in Table 2, mean age, alcohol drinking and smoking habits did not differ significantly between subjects

Table 1. Baseline characteristics of study subjects stratified by two serologic tests (pepsinogen and *H. pylori* antibody titer) and gastric cancer development

				Groups based on pepsinogen level								
		HP antibody titer level		Bα			Bβ			Bγ		
Screening criteria	**Total**	**HP low-titer**	**HP high-titer**	**Total**	**HP low-titer**	**HP high-titer**	**Total**	**HP low-titer**	**HP high-titer**	**Total**	**HP low-titer**	**HP high-titer**
Subjects, *n* (%)	496 (100)	337 (67.9)	159 (32.1)	111 (22.3)	96 (19.3)	15 (3)	235 (47.4)	156 (31.5)	79 (15.9)	150 (30.2)	85 (17.1)	65 (13.1)
				(100)	(86.5)	(13.5)	(100)	(66.4)	(33.6)	(100)	(56.7)	(43.3)
Follow-up years [mean (SD)]	5.4 (4.0)	5.4 (4.0)	5.4 (4.0)	5.4 (3.9)	5.5 (3.9)	4.7 (4.4)	5.4 (4.1)	5.2 (4.2)	5.9 (4.1)	5.3 (4.0)	5.6 (4.1)	5.0 (3.9)
Person-years	2680	1814	866	600	529	71	1280	813	467	800	472	328
Age [mean (SD)]	52.2 (5.1)	52.1 (5.0)	52.5 (5.3)	52.0 (4.8)	51.9 (4.9)	53.1 (4.0)	52.1 (5.2)	52.0 (5.3)	52.6 (4.9)	52.5 (5.1)	52.7 (4.4)	52.4 (5.9)
Ever-smoking (%)	63.3	68.5	51.7^{+}	60.5	63.2	42.9	66.7	75.2	48.6^{+}	60.1	62.4	57.1
Alcohol drinking (%)	71.8	70.6	74.1	68.5	69.8	66.7	71.5	69.9	74.4	74	72.9	75.4
RHG, n (%)	58 (11.7)	26 (8.0)	32 (20.1)	0	0	0	18 (7.7)	9 (6)	9 (11.4)	40 (26.7)	17 (20)	23 (35.4)
HP antibody titer U ml^{-1} [mean (SD)]	434.3 (510.0)	185.8 (123.3)	964.2 (608.7)$^{+}$	211.3 (236.0)	133.9 (102.9)	706.7 (249.0)$^{+}$	438.8 (498.9)*	189.0 (122.9)	938.5 (586.0)$^{+}$	592.1 (608.0)$^{*,\#}$	238.5 (122.7)	1054 (676.5)$^{+}$
PGI ng ml^{-1} [mean (SD)]	71.3 (32.9)	67.4 (31.8)	79.7 (33.9)$^{+}$	38.6 (8.3)	38.2 (8.7)	41.4 (4.9)	80.9 (29.7)*	78.2 (30.3)	86.8 (28.0)$^{+}$	80.5 (33.6)*	81.0 (29.9)	80.2 (38.2)
PGII ng ml^{-1} [mean (SD)]	22.7 (16.1)	20.5 (15.6)	27.7 (16.4)$^{+}$	8.4 (2.9)	8.0 (2.9)	11.0 (1.8)$^{+}$	20.0 (8.8)*	18.0 (9.0)	21.7 (7.2)$^{-}$	38.9 (17.7)$^{*,\#}$	39.1 (16.8)	38.6 (19.0)
PGI/II [mean (SD)]	3.9 (1.7)	4.2 (1.8)	3.3 (1.2)$^{+}$	4.9 (1.6)	5.1 (1.7)	3.8 (0.6)$^{+}$	4.5 (1.3)*	4.8 (1.5)	4.1 (0.9)$^{+}$	2.2 (0.5)$^{*,\#}$	2.2 (0.5)	2.2 (0.5)
Total gastric cancer cases/incidence rate[1]	7/261	2/110	5/577	0/–	0/–	0/–	1/78	1/123	0/–	6/750	1/212	5/1524
HR (95%CI)		1	6.51 (1.24–34.17)[2]				1			10.00 (1.19–84.05)[3]	2.52 (0.16–40.60)[3]	25.41 (2.89–223.11)[3]
Intestinal gastric cancer cases/incidence rate[1]	1/37	0/–	1/115	0/–	0/–	0/–	0/–	0/–	0/–	1/125	0/–	1/305
Diffuse gastric cancer cases/incidence rate[1]	6/224	2/110	4/462	0/–	0/–	0/–	1/78	1/123	0/–	5/625	1/212	4/1220

Study subjects were classified into three groups (Bα, Bβ and Bγ) based on the following criteria:
Bα, PGI ≤50 ng ml^{-1} and PGI/II >3.0; Bβ, PGI > 50 ng ml^{-1} and PGI/II >3.0; Bγ, PGI >50 ng ml^{-1} and PGI/II ≤3.0
PG, pepsinogen; HR, hazard ratio; HP, *Helicobacter pylori*; RHG, rugal hyperplastic gastritis; HP low-titer, ≤500 U ml^{-1}; HP high-titer, >500 U ml^{-1}
HR adjusted for age, smoking habit and alcohol drinking was evaluated in each group according to Cox proportional hazards modeling.
*$p < 0.05$ compared with group Bα in total. #$p < 0.05$ compared with group Bβ in total. $^{+}p < 0.05$ compared with HP low-titer.
[1]per 100,000 person-years. [2]HR calculated in comparison with cancer incidence rate in the Hp low-titer group. [3]HR calculated in comparison with cancer incidence rate in group Bβ in total.

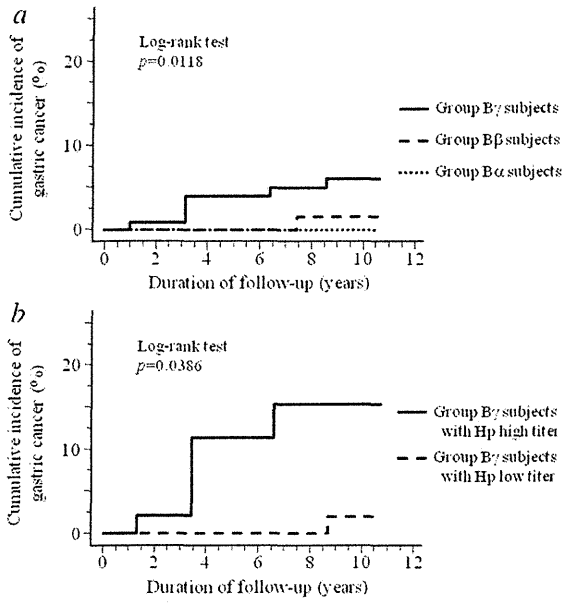

Figure 2. Gastric cancer development in groups of *H. pylori*-infected subjects with nonatrophic stomach classified by serum levels of pepsinogen (PG) and *H. pylori* antibody. (*a*) *H. pylori*-infected subjects with nonatrophic stomach were classified into three groups (Bα, Bβ and Bγ) based on serum PG levels, as described in the text. Cumulative incidences of gastric cancer in the three groups were plotted using Kaplan–Meier analysis; differences between groups were assessed using log-rank analysis. Cancer incidence rates for Groups Bα, Bβ and Bγ were 0, 78/100,000 person-years and 750/100,000 person-years, respectively, showing a significant difference between Groups Bβ and Bγ ($p = 0.0365$; log-rank test). (*b*) Group Bγ subjects were divided into two groups based on serum *H. pylori* antibody level (that is, high-titer and low-titer groups, as in the text) and cancer development in each group was analyzed by Kaplan–Meier analysis. Cancer incidence rates for high- and low-titer groups were 1,524/100,000 person-years and 212/100,000 person-years, respectively, showing a significant difference between the two groups ($p = 0.0386$; log-rank test).

with and without RHG. Serum levels of PGI, PGII and *H. pylori* antibody were significantly higher, and the PGI/II ratio was significantly lower in subjects with RHG. During the study period, continuous development of cancer was observed in subjects with RHG (Fig. 3), and a total of 85.7% (6/7) of the detected cancers derived from these subjects, 83.3% (5/6) of which were diffuse-type cancers. Cancer incidence was significantly higher in subjects with RHG (1,749/100,000 person-years) than in subjects without (43/100,000 person-years, HR, 43.32; 95% CI, 5.16–363.41, Table 2).

As described above and listed in Tables 1 and 2, inclusion in Group Bγ, having a high titer of *H. pylori* antibody or presence of RHG on endoscopy were revealed as independent risk factors for gastric cancer by Cox proportional hazards modeling after adjusting for age, smoking habit and alcohol drinking habit. As a result, 150 subjects from Group Bγ were stratified based on serum *H. pylori* antibody titers and endoscopic finding of RHG, and cancer development was investigated (Table 3). Among the four stratified groups, mean age and percentages of smokers and alcohol drinkers tended to be highest in subjects with high titer and RHG, but no significant differences were evident among the four groups. In both high- and low-titer groups, serum levels of PGI, PGII and *H. pylori* antibody were higher, and PGI/II ratio was lower in subjects with RHG than in those without, and the difference in PGII level was significant between high-titer groups with and without RHG. Cancer development was higher in the high-titer group with RHG (2,857/100,000 person-years) than in the high-titer group without RHG (532/100,000 person-years) or in the low-titer group with RHG (1,064/100,000 person-years) (Table 3). In the present cohort, 57.1% (4/7) of cancers developed from the *H. pylori* antibody high-titer group with RHG. Meanwhile, no cancer development was seen in the low-titer group without RHG.

During the study period, a separate group of 399 subjects underwent *H. pylori* eradication, with successful treatment in 327 subjects. Of these, 149 subjects belonged to Group Bγ, and baseline characteristics of the group did not differ significantly from the above-described 150 Group Bγ subjects without eradication, except for a significantly higher mean age in the latter group (Table 3). Serum levels of *H. pylori* antibody and PGI/II ratio were significantly higher in the eradication group. *H. pylori* eradication led to marked regression of endoscopic findings of severely enlarged folds in all subjects with RHG. In addition, reductions in the observed high serum levels of PG and *H. pylori* antibody, which were characteristic of Group Bγ subjects, were observed in all subjects (not shown). As a result, no cancer development was observed in these subjects by the end of the study period, regardless of *H. pylori* antibody level or endoscopic findings of RHG at baseline. The number needed to treat (NNT) for 1 year to prevent development of a single case of cancer in Group Bγ was thus estimated to be around 133. Furthermore, if the target of eradication is set for Group Bγ subjects with both high-titer *H. pylori* antibody and RHG, the 1-year NNT would be around 35.

Discussion

In addition to initiating a cascade of events leading mainly to intestinal-type cancer—that is, the atrophy-metaplasia-dysplasia-cancer sequence—the inflammatory process induced by *H. pylori* infection is postulated to directly induce cancer, particularly diffuse-type cancer, from the nonatrophic stomach without passing through this sequence.[4,5] Although *H. pylori*-induced chronic inflammation is recognized as a universal precursor condition, other precancerous or high-risk conditions for cancers developing from nonatrophic stomach have not been described in detail. The present long-

Table 2. Baseline characteristics of study subjects according to RHG and gastric cancer development

Screening criteria	Total	RHG(−)	RHG(+)
Subjects *n* (%)	496 (100)	438 (88.3)	58 (11.7)
Follow-up years [mean (SD)]	5.4 (4.0)	5.3 (4.0)	5.9 (4.0)
Person-years	2680	2337	343
Age [mean (SD)]	52.2 (5.1)	52.2 (5.1)	52.7 (5.1)
Ever-smoking (%)	64	64.2	62.1
Alcohol drinking (%)	71.8	70.8	79.3
HP antibody titer U ml^{-1} [mean (SD)]	434.3 (510.0)	384.2 (433.8)	811.2 (806.7)$^{+}$
PGI ng ml^{-1} [mean (SD)]	71.3 (32.9)	68.9 (30.6)	89.2 (43.0)$^{+}$
PGII ng ml^{-1} [mean (SD)]	22.7 (16.1)	20.8 (14.0)	37.8 (22.3)$^{+}$
PGI/II [mean (SD)]	3.9 (1.6)	4.1 (1.7)	2.7 (1.2)$^{+}$
Total gastric cancer cases/incidence rate[2]	7/261	1/43	6/1749
HR (95%CI)[1]		1	43.32 (5.16–363.41)
Intestinal gastric cancer cases/incidence rate[2]	1/37	0/–	1/292
Diffuse gastric cancer cases/incidence rate[2]	6/224	1/43	5/1458

PG, pepsinogen; HR, hazard ratio; RHG, rugal hyperplastic gastritis; HP, *Helicobacter pylori*.
$^{+}p < 0.05$ compared with RHG(−).
[1]HR adjusted for age, smoking habit and alcohol drinking was evaluated in each group according to Cox proportional hazards modeling.
[2]Per 100,000 person-years.

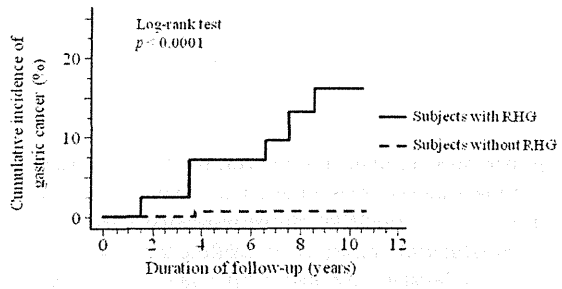

Figure 3. Gastric cancer development in endoscopic rugal hyperplastic gastritis (RHG). *H. pylori*-infected subjects with nonatrophic stomach were classified into groups with and without RHG diagnosed by endoscopy. Gastric cancer development was analyzed by Kaplan–Meier analysis. Cancer incidence rates were 1,749/100,000 person-years and 43/100,000 person-years, respectively, showing a significant difference between the two groups ($p < 0.0001$; log-rank test).

term follow-up study clearly demonstrated that a group of subjects in the cohort with highly active gastritis identified by the two serological tests of PG and *H. pylori* antibody are at high-risk for development of gastric cancer, particularly diffuse-type cancer with higher malignant potential. Moreover, among these serologically defined subjects at high risk of cancer, those with endoscopic RHG are at even higher risk.

In subjects with nonatrophic stomach, the establishment of *H. pylori* infection increases serum levels of PG, particularly PGII, together with *H. pylori* antibody, and the extent of the increase in these serum markers reportedly shows positive correlations with the activity of *H. pylori*-associated inflammation.[35,36] The basic mechanisms underlying the elevation of serum PG levels are not fully known. While *H. pylori* antibody level reflects the complex interaction between bacterial infection and immunological host response, PG levels are considered to reflect local mucosal damage. These two serum tests thus appear to indicate two different aspects of *H. pylori* infection. As described above, *H. pylori*-infected subjects with nonatrophic stomach were classified into the three groups of Bα, Bβ and Bγ, and the activity of *H. pylori*-associated gastritis appeared to increase in the order of Group Bα, Bβ and Bγ, as revealed by serum levels of PGII and *H. pylori*-antibody. Cancer development occurred in the same order, and Group Bγ showed the highest cancer risk among these three groups. Furthermore, stratification of Group Bγ based on the *H. pylori*-antibody level led to the identification of subjects with more active inflammation and higher cancer risk, especially for diffuse-type cancer, showing good accordance with the reported chronic active inflammation-based carcinogenesis for this type of cancer.[5,15]

Meanwhile, highly active inflammation in the corpus of the nonatrophic stomach reportedly leads to the formation of enlarged gastric folds.[22,23] Although enlarged folds can be caused by various pathological processes, including inflammatory, or tumorous infiltration, or by hyperplasia of the foveolae or glands,[37,38] *H. pylori* infection is considered to represent the leading cause of gastric enlarged folds, that is, gastritis known variously as RHG or enlarged-fold gastritis based on endoscopic or barium X-ray findings, respectively.[22–31] Previous studies analyzing the relationship