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Letter

The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands

Hideyuki Takeshima,[1] Satoshi Yamashita,[1] Taichi Shimazu,[2] Tohru Niwa,[1] and Toshikazu Ushijima[1,3]

[1]*Carcinogenesis Division, National Cancer Center Research Institute, 104-0045 Tokyo, Japan;* [2]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, 104-0045 Tokyo, Japan*

Instructive mechanisms are present for induction of DNA methylation, as shown by methylation of specific CpG islands (CGIs) by specific inducers and in specific cancers. However, instructive factors involved are poorly understood, except for involvement of low transcription and trimethylation of histone H3 lysine 27 (H3K27me3). Here, we used methylated DNA immunoprecipitation (MeDIP) combined with a CGI oligonucleotide microarray analysis, and identified 5510 and 521 genes with promoter CGIs resistant and susceptible, respectively, to DNA methylation in prostate cancer cell lines. Expression analysis revealed that the susceptible genes had low transcription in a normal prostatic epithelial cell line. Chromatin immunoprecipitation with microarray hybridization (CHiP-chip) analysis of RNA polymerase II (Pol II) and histone modifications showed that, even among the genes with low transcription, the presence of Pol II was associated with marked resistance to DNA methylation (OR = 0.22; 95% CI = 0.12–0.38), and H3K27me3 was associated with increased susceptibility (OR = 11.20; 95% CI = 7.14–17.55). The same was true in normal human mammary epithelial cells for 5430 and 733 genes resistant and susceptible, respectively, to DNA methylation in breast cancer cell lines. These results showed that the presence of Pol II, active or stalled, and H3K27me3 can predict the epigenetic fate of promoter CGIs independently of transcription levels.

[Supplemental material is available online at http://www.genome.org. The microarray data from this study have been submitted to Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE15154.]

Epigenetic alterations, along with genetic alterations, are known to play critical roles in human carcinogenesis and other acquired diseases (Laird and Jaenisch 1996; Robertson 2005; Jones and Baylin 2007). Especially, DNA methylation of promoter CpG islands (CGIs) has been known to be involved in silencing of tumor-suppressor and other genes (Ushijima 2005; Eckhardt et al. 2006; Jones and Baylin 2007). In addition, a critical role of methylation of the nucleosome-free region (NFR) just upstream of a transcription start site (TSS) was recently demonstrated in nucleosome occupation and thus in gene silencing (Li et al. 2007; Lin et al. 2007).

Epigenetic alterations, different from genetic alterations, have unique natures, such as gene specificity (Costello et al. 2000; Esteller et al. 2001; Keshet et al. 2006; Nakajima et al. 2009; Oka et al. 2009), high levels of accumulation in normal-appearing tissues (Kondo et al. 2000; Maekita et al. 2006; Ushijima 2007), and deep involvement of inflammation in their induction (Issa et al. 2001; Ushijima and Okochi-Takada 2005; Maekita et al. 2006). Especially, the presence of gene specificity, originally suggested by the presence of tumor type-specific DNA methylation patterns (Costello et al. 2000; Esteller et al. 2001), is now confirmed by methylation of specific genes in non-cancerous tissues exposed to specific carcinogenic factors (Nakajima et al. 2009; Oka et al. 2009). Selection biases for genes with growth advantage can be avoided by analysis of non-cancerous, therefore polyclonal, tissues (Mihara et al. 2006). The gene specificity of DNA methylation induction depending on cell types and carcinogenic factors shows that there are instructive mechanisms for DNA methylation induction, in contrast to the random nature of mutation induction.

As mechanisms for instructive induction, limited information is available so far, including low transcription levels and some histone modifications. Exogenous and endogenous genes are likely to become methylated only when they have low transcription levels (Song et al. 2002; De Smet et al. 2004). Most genes methylated in cancer tissues had no or low transcription in their normal counterpart cells (Ushijima 2005; Keshet et al. 2006). Transcription factors, such as SP1/SP3 and MLL, protected CpG sites from becoming methylated, independent of and dependent on transcription levels, respectively (Boumber et al. 2008; Erfurth et al. 2008). In addition, trimethylation of histone H3 lysine 27 (H3K27me3), a target of Polycomb repressive complex (PRC) 2 (Hansen et al. 2008), was enriched in normal cells and embryonic stem (ES) cells at genes that can be methylated in cancers (Ohm et al. 2007; Schlesinger et al. 2007; Widschwendter et al. 2007; Hahn et al. 2008; Rodriguez et al. 2008). Nevertheless, at a genome level, many genes have low transcription levels and H3K27me3 but are still resistant to DNA methylation induction, indicating that some critical factors are likely to be still missing.

In this study, we hypothesized that RNA polymerase II (Pol II) binding around TSSs can function as a protective factor for DNA methylation induction. Accumulation of Pol II at genes with low transcription levels (stalled Pol II) was recently found in as high as 12% of protein-coding genes in *Drosophila melanogaster* (Muse et al. 2007; Zeitlinger et al. 2007) and in humans (Guenther et al. 2007). We demonstrate in a genome-wide manner that Pol II binding, active or stalled, and histone modifications in normal cells predict genes resistant and susceptible to DNA methylation in cancers.

Results

Identification of genes with promoter CGIs resistant and susceptible to DNA methylation

To identify genes with promoter CGIs resistant and susceptible to induction of DNA methylation in human prostate cancers, four

[3]**Corresponding author.**
E-mail tushijim@ncc.go.jp; fax 81-3-5565-1753.
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prostate cancer cell lines (PC3, LNCaP, 22Rv1, and Du145), along with a normal prostatic epithelial cell line (RWPE1), were analyzed using methylated DNA immunoprecipitation (MeDIP) combined with a human CGI oligonucleotide microarray that covered 27,800 CGIs (MeDIP-CGI microarray analysis).

First, appropriate cutoff values of our original output values "DNA methylation values" (Me values) were determined using 145 samples (29 CGIs in five cell lines) (Supplemental Table S1). As cutoff values with high specificity and little compromise of sensitivity, cutoff values of 0.6 and 0.4 were selected for methylated and unmethylated CGIs, respectively (Supplemental Fig. S1). The specificity and sensitivity for methylated (unmethylated) CGIs with these values were 0.95 (0.96) and 0.85 (0.82), respectively. DNA methylation status of a CGI or putative NFR was judged as unmethylated (UM), moderately methylated (MM), and highly methylated (HM) when the average of Me values of the probes within the region was 0–0.4, 0.4–0.6, and 0.6–1.0, respectively. The validity of our methods was also supported by the fact that promoter CGIs were more likely to be unmethylated (68%–82%) than those in gene bodies (54%–63%), which conformed with previous observations (Supplemental Table S2; Ushijima et al. 2003; Eckhardt et al. 2006; Rakyan et al. 2008).

The susceptibility of genes was determined by methylation analysis of 8930 NFRs (Li et al. 2007). Genes with NFRs unmethylated (Me value, 0–0.4) in the normal cell line and all the four cancer cell lines were defined as DNA methylation-resistant genes. On the other hand, those unmethylated in the normal cell line but highly methylated (Me value, 0.6–1.0) in at least one of the four cancer cell lines were defined as DNA methylation-susceptible genes (Fig. 1A). Susceptible genes were further divided into S1, S2, S3, and S4 subclasses according to the DNA methylation frequency in cancer cell lines (highly methylated in one, two, three, and four, respectively, of the four cancer cell lines). In addition, genes unmethylated in the normal cell line but moderately methylated (Me value, 0.4–0.6) in at least one of the four cancer cell lines were defined as genes with intermediate susceptibility (intermediate genes). In prostate cancers, 5510, 1330, and 521 genes with promoter CGIs were classified as resistant, intermediate, and susceptible genes, respectively (Fig. 1B). DNA methylation levels of NFRs were largely consistent with those of further upstream regions up to −800 bp, and downstream regions up to +800 bp (Fig. 1C).

To avoid any tissue bias and statistical errors, we also analyzed three human breast cancer cell lines (MCF7, ZR-75-1, and MDA-MB-468), along with normal human mammary epithelial cells (HMEC). As in the prostate, the promoter CGIs were more likely to be unmethylated (68%–90%) than the CGIs located in gene bodies (52%–70%) (Supplemental Table S2). Using the same definition as in the prostate cancers, 5430, 1913, and 733 genes with promoter CGIs were classified as resistant, intermediate, and susceptible genes, respectively (Fig. 1B). As in prostate cancers, DNA methylation levels were also largely consistent among the NFRs, further upstream regions, and downstream regions in human breast cancers (Supplemental Fig. S2). Between breast and prostate cancers, only 261 genes, 36% of the susceptible genes in breast cancers and 50% of those in prostate cancers, were commonly susceptible, showing the presence of tissue specificity.

To explore possible selection bias for the resistant and susceptible genes due to gene functions, functional annotation analysis of resistant and susceptible genes was performed. In the prostate, 203 and 154 processes out of 16,621 biological processes were enriched among the resistant and susceptible genes, respectively. Among the resistant genes, processes involved in basic cellular processes such as metabolic process, RNA processing, and RNA splicing were enriched. In contrast, among the susceptible genes, biological processes involved in the developmental processes of

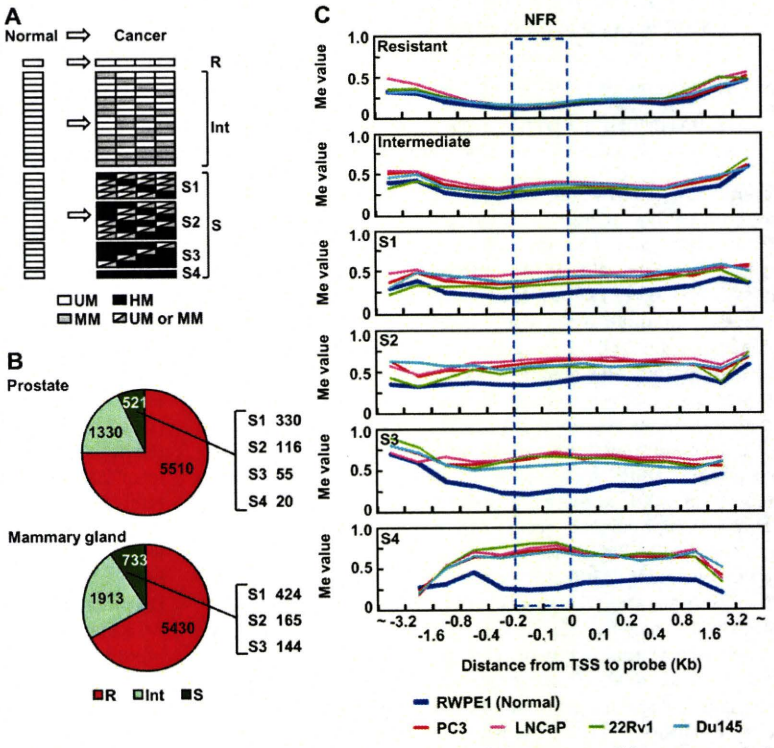

Figure 1. Identification of methylation-resistant and methylation-susceptible genes and their methylation profiles in various genomic regions against TSSs. (*A*) Definition of genes resistant and susceptible to induction of DNA methylation. Genes unmethylated (UM) (white) in the normal cell line (cells) and all cancer cell lines were defined as resistant genes (R). Genes unmethylated in the normal cell line (cells) but highly methylated (HM) (black) in at least one of the four cancer cell lines were defined as susceptible genes (S). Susceptible genes were further divided into four subclasses according to DNA methylation frequency in the cancer cell lines (S1–S4). Genes unmethylated in the normal cell line (cells) but moderately methylated (MM) (gray) in the cancer cell lines were defined as genes with intermediate susceptibility (intermediate genes: Int). (*B*) The fractions of resistant (red), intermediate (light green), and susceptible (green) genes in the prostate and the mammary gland. (*Right* side of the pie graph) Numbers of susceptible genes in each subclass (S1–S4). (*C*) DNA methylation levels at various positions against the TSSs in the normal prostatic cell line and four cancer cell lines. Average Me values of CGIs continuous from their NFRs are shown. (Blue dotted rectangle) The NFRs. Methylation levels of the NFRs were similar to those of upstream regions up to −800 bp and downstream regions up to +800 bp.

specific cells or tissues, such as nervous system development, and embryonic development, were enriched (Table 1). Similar enrichment of genes involved in specific biological processes was also observed in the mammary glands.

Low transcription levels of DNA methylation-susceptible genes in normal cell lines

For a limited number of genes, the susceptibility of genes with low transcription to DNA methylation has been reported in cell lines (Song et al. 2002; De Smet et al. 2004) and in human tissue (Ushijima and Okochi-Takada 2005; Nakajima et al. 2009). To analyze this susceptibility in a genome-wide manner, we performed expression analysis in the normal prostatic cell line using a GeneChip oligonucleotide microarray. Owing to the difference of array platforms between the CGI oligonucleotide microarray and the GeneChip oligonucleotide expression microarray, we were able to measure transcription levels of the 7574 genes out of 8930 genes with promoter CGIs in the normal prostatic cell line. The accuracy of the transcription levels obtained by the GeneChip oligonucleotide microarray was validated by observing a strong correlation between the microarray data and mRNA levels obtained by quantitative RT-PCR (correlation coefficient = 0.95 and 0.97 in RWPE1 and HMEC, respectively) (Supplemental Fig. S3). When the transcription levels were analyzed according to the DNA methylation status in the normal prostatic cell line itself, as expected, highly methylated genes had remarkably low transcription levels (Supplemental Fig. S4).

Genes highly methylated in prostate cancer cell lines had low transcription levels in the normal prostatic cell line (Fig. 2A). When transcription levels of resistant, intermediate, and susceptible genes were compared, susceptible genes had lower transcription levels than resistant genes. Even among the susceptible genes, genes with frequent DNA methylation had lower transcription levels than those with infrequent DNA methylation (Fig. 2B). When fractions of genes with high, moderate, and low transcription levels were analyzed in the 7574 total, 4567 resistant, and 479 susceptible genes, the susceptible genes had a significantly larger fraction of genes with low transcription (63%) than the total genes (38%; $P < 0.001$, χ^2 test) (Fig. 2C). Even among the susceptible genes, genes with more frequent DNA methylation had the larger fraction of genes with low transcription (Supplemental Fig. S5). These results showed that aberrant DNA methylation is preferentially induced in genes with low transcription, as previously reported (Song et al. 2002; De Smet et al. 2004; Ushijima 2005; Keshet et al. 2006; Nakajima et al. 2009), in a genome-wide manner.

In the mammary glands, the susceptible genes also had a significantly larger fraction of genes with low transcription (74%) than the total genes (37%; $P < 0.001$, χ^2 test) (Supplemental Figs. S5, S6).

Levels of histone modifications and Pol II binding were associated with DNA methylation susceptibility

Although most genes susceptible to DNA methylation in cancers had low transcription in the normal cell line (cells), the converse was not true: 1237 of 2852 (prostate) and 1048 of 2750 (breast) genes with low transcription in the normal cell line (cells) were still resistant to DNA methylation in cancers (Fig. 2C; Supplemental Fig. S6). This indicated that factors besides low transcription are also involved in DNA methylation susceptibility. To address this issue, we analyzed both active (acetylation of histone H3 [H3Ac] and trimethylation of histone H3 lysine 4 [H3K4me3]) and inactive (trimethylation of histone H3 lysine 9 [H3K9me3] and H3K27me3) histone modifications and Pol II binding at and adjacent to the NFRs in a genome-wide manner. Since the length of sheared DNA used for chromatin immunoprecipitation (ChIP) analysis ranged mainly from 200 to 1000 bp, analysis of probes within the NFRs automatically reflected histone modifications adjacent to the NFRs even if nucleosomes were absent in the NFRs.

Table 1. Functional annotation analysis of genes with different DNA methylation susceptibility

	Prostate		Mammary gland	
Category	Term	*P*-value	Term	*P*-value
Resistant	Primary metabolic process	3.72E-22	Cellular metabolic process	3.16E-20
	Macromolecule metabolic process	6.27E-22	Metabolic process	1.74E-19
	Cellular metabolic process	1.08E-21	Primary metabolic process	4.50E-19
	Metabolic process	5.98E-21	Macromolecule metabolic process	2.33E-17
	Biopolymer metabolic process	2.92E-15	RNA processing	9.75E-17
	RNA processing	1.34E-14	RNA splicing	6.54E-14
	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	1.49E-14	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	1.30E-13
	mRNA metabolic process	9.65E-14	Macromolecule localization	1.51E-13
	RNA splicing	3.94E-13	mRNA metabolic process	1.29E-12
	Protein transport	7.19E-13	Biopolymer metabolic process	1.29E-12
Susceptible	Multicellular organismal process	2.41E-16	Multicellular organismal process	8.20E-30
	Multicellular organismal development	3.33E-12	Multicellular organismal development	4.77E-23
	System development	4.77E-11	System development	2.57E-18
	Anatomical structure development	1.10E-09	Anatomical structure development	6.61E-17
	System process	3.09E-08	Developmental process	5.18E-16
	Nervous system development	9.59E-08	Nervous system development	3.96E-13
	Developmental process	4.48E-07	Cell-cell signaling	2.27E-12
	Organ development	7.93E-07	Organ development	2.85E-12
	Cell-cell signaling	3.03E-06	Embryonic development	6.96E-11
	Biological adhesion	9.69E-06	System process	1.01E-10

Enrichment of specific biological processes in Gene Ontology criteria among resistant and susceptible genes was analyzed by DAVID bioinformatics resources. The top 10 significantly enriched biological processes in each gene category are listed. The significance (*P*-value) of enrichment is shown.

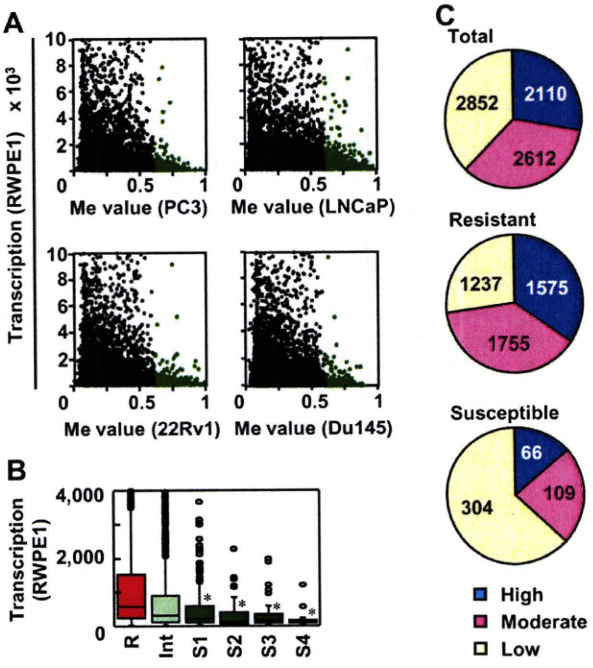

Figure 2. Low transcription levels of DNA methylation-susceptible genes in the normal prostatic cell line (RWPE1). (*A*) The association between DNA methylation levels (Me value of the NFRs) in each of the four prostate cancer cell lines (PC3, LNCaP, 22Rv1, and Du145) and transcription levels in RWPE1. (Green dots) Genes highly methylated in a cancer cell line. Genes highly methylated in a cancer cell line had low transcription levels in the normal cell line. (*B*) Transcription levels of resistant (R), intermediate (Int), and susceptible (S1–S4) genes in RWPE1. The boxes represent the 75th and 25th percentiles, and the line in the box represents the 50th percentile (the median). Whiskers represent the maximum data within (75th percentile + 1.5 × [75th percentile − 25th percentile]) and the minimum data within (25th percentile − 1.5 × [75th percentile − 25th percentile]). (Dots) The data not included between the whiskers. Transcription levels of Int, S1, S2, S3, and S4 were compared to that of R by the Mann-Whitney *U*-test ($*P < 1 \times 10^{-5}$). Susceptible genes had significantly lower expression levels than resistant genes. (*C*) The fraction of genes with high (blue) (signal intensity > 1000), moderate (pink) (250–1000), and low (yellow) (<250) transcription. Susceptible genes had a significantly larger fraction of genes with low transcription than the total genes.

The data obtained by the ChIP with microarray hybridization (ChIP-chip) analysis were validated by analyzing correlations between the signal ratio (immunoprecipitated DNA [IP]/whole cell extract [WCE]) obtained by ChIP-chip and those obtained by quantitative ChIP-PCR (Supplemental Fig. S7).

Using only genes with low transcription, we analyzed the association between the candidate instructive factors in the normal prostatic cell line and susceptibility to DNA methylation in prostate cancer cell lines. It was clear that H3Ac and H3K4me3 were elevated in resistant genes, and H3K27me3 was elevated in susceptible genes (Fig. 3A). In contrast, the H3K9me3 level was not different between resistant and susceptible genes. Notably, Pol II binding was remarkably higher in resistant genes (Fig. 3B). When further upstream regions and downstream regions were analyzed, resistant genes had elevated H3Ac and H3K4me3 mainly in their downstream regions, and susceptible genes had elevated H3K27me3 in their downstream regions and further upstream regions (Fig. 3C). Pol II binding was elevated mainly in the NFRs and then in downstream regions of resistant genes (Fig. 3C). In the mammary glands, exactly the same tendency was observed (Supplemental Fig. S8).

Next, within the normal prostatic cell line, the association between histone modifications and transcription levels was analyzed. Conforming to previous reports (Barski et al. 2007; Wang et al. 2008), genes with high and low transcription had elevated active and inactive histone modifications (Supplemental Fig. S9). Notably, among genes with low transcription, those without DNA methylation had elevated H3K27me3, confirming a previous report that H3K27me3 is involved in gene silencing independent of DNA methylation (Kondo et al. 2008). Within the normal mammary epithelial cells, the same tendency was observed.

Strongest association of Pol II binding with resistance to DNA methylation

The combination effect of H3K27me3 and one of the three active factors (H3Ac, H3K4me3, and Pol II binding) on DNA methylation susceptibility was then examined (Fig. 3D). All the three combinations were informative in distinguishing the resistant and susceptible genes, while Pol II binding gave the clearest discrimination. Multivariate logistic regression analysis was then performed to compare precisely the independent effects of H3Ac, H3K4me3, H3K9me3, H3K27me3, and Pol II binding on DNA methylation susceptibility. The genes with low transcription in the normal cell line (cells) were divided into quintiles according to the amounts of H3Ac, H3K4me3, H3K9me3, H3K27me3, and Pol II binding at the NFRs. Compared with the genes in the lowest quintile, multivariate-adjusted odds ratios (ORs) of genes in the other quintiles to become moderately or highly methylated in cancers (Int, and S1–S4 for the prostates; Int, and S1–S3 for the mammary glands) were calculated (Table 2). In the prostates, Pol II binding had the strongest independent association with resistance, and H3K27me3 had a strong and significant association with susceptibility. In the mammary glands, similar associations were observed. If the analysis was performed for the multivariate-adjusted odds ratio of genes to become highly methylated (S1–S4 for the prostates; and S1–S3 for the mammary glands), the association of Pol II binding became even clearer (Supplemental Table S3).

Finally, regardless of their transcription levels, all the genes were classified into genes with "active Pol II" (high/moderate transcription, high Pol II), those with "stalled Pol II" (low transcription, high Pol II), and those with "low Pol II" (low Pol II). The group of genes with low Pol II was further subdivided into those with and without H3K27me3. In the normal prostatic cell line, 47%, 13%, and 40% of genes had active, stalled, and low Pol II, respectively (Fig. 4A). Both genes with active Pol II and genes with stalled Pol II consisted mostly of resistant genes (Fig. 4B). In contrast, genes with low Pol II contained larger fractions of susceptible genes, and the presence of H3K27me3 remarkably increased the fraction. Similar results were obtained also in the mammary glands (Supplemental Fig. S10).

Discussion

In this study, we showed that Pol II binding in the NFRs in normal cell lines (cells) was closely associated with resistance to DNA methylation in cancer cell lines (cells) for the first time. The association between Pol II binding and resistance to DNA methylation was independent of transcriptional levels. It was also independent from the promoting effect of H3K27me3, and the combination of Pol II binding and H3K27me3 could explain a large part of the

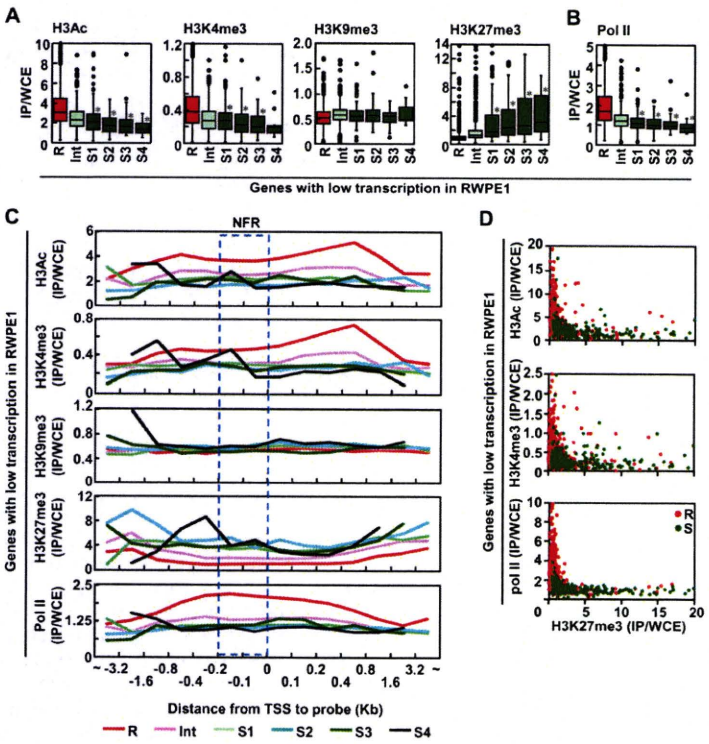

Figure 3. The association between the levels of candidate instructive factors in RWPE1 and DNA methylation susceptibility, among genes with low transcription in RWPE1. (*A*) Histone modification levels of genes with different susceptibilities to DNA methylation. For the box plot and statistical methods, refer to the legend to Figure 2B. Active histone modifications were elevated in resistant genes, and H3K27me3 was elevated in susceptible genes. (*B*) The association between Pol II binding and DNA methylation susceptibility. Pol II binding was associated with resistance even among genes with low transcription. (*C*) Levels of histone modifications and Pol II binding at various positions against the TSSs in RWPE1. Average levels of histone modifications and Pol II binding of CGIs continuous from their NFRs are shown. (Blue dotted rectangle) The NFRs. (*D*) The combination effect of one of the three active factors (H3Ac, H3K4me3, and Pol II binding) (*y*-axis) and H3K27me3 (*x*-axis) on resistance and susceptibility of genes with low transcription. (Red dots) DNA methylation-resistant genes; (green dots) DNA methylation-susceptible genes; they were separated by any of the three combinations.

instructive mechanisms for induction of DNA methylation. These data provided fundamental information on how the epigenetic fate of promoter CGIs is determined. The association between Pol II binding and resistance to DNA methylation can be potentially useful in the prediction of genes that will become silenced in cancer and other diseases.

Our multivariate analysis involving Pol II binding and histone modifications showed that the association between active histone modifications and resistance to DNA methylation was mostly overridden by that of Pol II binding, while the association between H3K27me3 and susceptibility to DNA methylation remained. It was reported that active histone modifications are involved in anchoring of the basal transcription factor TFIID (Vermeulen et al. 2007), which forms a transcription complex with Pol II. H3K4me3 is recognized by the PHD domain of TFIID, and acetylation of histone H3 lysine 9 and lysine 14 potentiates this interaction. It was therefore suggested that Pol II binding more directly works as a protection mechanism than active histone modifications, and that H3K27me3 has an independent mode of action.

Pol II forms a huge transcription complex of ~3 MDa with general transcription factors and other proteins (Boeger et al. 2005), and such a huge complex around promoter CGIs is expected to compete with DNA methyltransferases and their associated proteins. On the other hand, H3K27me3 is recognized by PRC2/3 (Hansen et al. 2008), which contains EZH2. Since EZH2 interacts with DNMT3A and DNMT3B (Vire et al. 2006), H3K27me3 is expected to signal binding of DNMT3A and DNMT3B. Taken together, Pol II binding and H3K27me3 are likely to function by preventing and promoting, respectively, recruitment of DNA methylation complexes.

Functional annotation analysis revealed that most of the susceptible genes were involved in the developmental processes of specific cells or tissues. Genes in this category were considered unnecessary for normal cells that have already differentiated. This raised alternative possibilities: The lack of current need for a gene is one of the instructive factors, or an unnecessary gene has a low level of Pol II, which is associated with methylation susceptibility. To distinguish these two possibilities, we examined overrepresentation of susceptible genes among genes with low Pol II levels after classification of genes by their function (Supplemental Table S4). As a result, in any categories of genes, susceptible genes were overrepresented among the genes with low Pol II levels, showing that the presence of Pol II was an independent factor for resistance to DNA methylation from functions of genes.

Specific genome structures are also known to be involved in the specificity of genes methylated, in addition to the instructive factors analyzed here. The presence of a repetitive sequence has been reported to be capable of functioning as a source of aberrant DNA methylation (Yates et al. 1999). In addition to methylation induction of individual genes, a cluster of genes can be methylated simultaneously in a cancer (Frigola et al. 2006). In this study, 64% and 50% of the susceptible genes in breast and prostate cancers, respectively, were unique to individual tumors. The susceptibility specific to a tissue is more likely to be due to Pol II binding and H3K27me3 rather, while susceptibility common to different tissues can be due to specific genome structures.

Genes moderately methylated were considered to be methylated in a fraction of cancer cells and thus to have been methylated after clonal expansion started. Genes highly methylated were considered to be present in all the cancer cells, and thus to have been methylated before clonal expansion. Therefore, DNA methylation susceptibility in normal cell line (cells) might be more precisely measured using genes highly methylated (Supplemental Table S3) than using genes highly and moderately methylated (Table 2).

As materials, we used normal and cancer cell lines to perform efficient and precise ChIP experiments. It is known that cancer cell

Table 2. The association between the levels of candidate instructive factors and susceptibility to DNA methylation (Int and S)

	Lowest quintile	2nd quintile	3rd quintile	4th quintile	Highest quintile
Prostate					
H3Ac	1	0.78 (0.54–1.12)	0.86 (0.56–1.31)	0.86 (0.54–1.37)	0.91 (0.53–1.57)
H3K4me3	1	0.99 (0.70–1.41)	1.09 (0.74–1.61)	0.92 (0.61–1.38)	0.52 (0.34–0.82)
Pol II	1	0.83 (0.58–1.18)	0.78 (0.52–1.17)	0.40 (0.25–0.62)	0.22 (0.12–0.38)
H3K9me3	1	1.47 (1.00–2.15)	1.26 (0.85–1.86)	1.22 (0.82–1.80)	1.20 (0.81–1.78)
H3K27me3	1	1.41 (0.92–2.17)	2.88 (1.89–4.40)	5.95 (3.87–9.13)	11.20 (7.14–17.55)
Mammary gland					
H3Ac	1	0.95 (0.68–1.35)	0.63 (0.43–0.91)	0.44 (0.30–0.66)	0.42 (0.26–0.67)
H3K4me3	1	0.96 (0.68–1.34)	1.02 (0.71–1.47)	0.59 (0.40–0.87)	0.49 (0.31–0.75)
Pol II	1	1.22 (0.88–1.71)	1.29 (0.90–1.86)	1.14 (0.77–1.68)	0.67 (0.43–1.04)
H3K9me3	1	1.03 (0.76–1.41)	1.07 (0.78–1.47)	1.43 (1.03–1.99)	0.89 (0.64–1.25)
H3K27me3	1	1.61 (1.20–2.18)	2.44 (1.78–3.34)	3.96 (2.86–5.48)	6.44 (4.56–9.10)

Multivariate-adjusted odds ratio (OR) (95% confidence interval; 95% CI) to become methylated (Int, and S1–S4 for the prostates; and Int, and S1–S3 for the mammary glands) is shown for each group. The multivariate-adjusted OR (95% CI) was derived from analyses in which all other listed variables were included into the mode.

lines generally show a larger number of methylated genes than primary tumor cells when a single cancer cell line and a primary tumor sample are compared. However, when a large number of primary tumor samples are analyzed, most DNA methylation found in cancer cell lines is also observed in at least one of the primary tumor samples (Sato et al. 2003; Lodygin et al. 2005; Yamashita et al. 2006). Therefore, it is considered that DNA methylation susceptibility identified in cancer cell lines reflects that in the primary cancer cells as a whole.

In summary, Pol II binding and H3K27me3 in normal cell lines (cells) could predict the epigenetic fate of genes with promoter CGIs in cancer cell lines independently of transcription activity and are major components of instructive mechanisms of DNA methylation induction.

Methods

Cell culture

PC3, LNCaP, 22Rv1, Du145, MCF7, ZR-75-1, and MDA-MB468 (American Type Culture Collection) were maintained in RPMI1640. RWPE1 (American Type Culture Collection) was maintained in keratinocyte-SFM containing 5 ng/mL rEGF, 50 μg/mL bovine pituitary extract (Invitrogen). HMEC (Clonetics) was maintained in mammary epithelial cell serum-free growth medium containing 1% growth supplement (CELL Applications).

ChIP assay

About 1×10^7 cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and washed with ice cold 1× PBS (–) twice. Cells were re-suspended in lysis buffer (50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 1% [w/v] SDS), incubated for 10 min on ice, and then sonicated to shear DNA to an average length ranging from 200 to 1000 bp with a Bioruptor UCD-250 (Cosmo Bio). After DNA shearing, the lysate was centrifuged at 13,000 rpm for 10 min, and supernatant was recovered. The volume of supernatant containing 30 μg of sheared DNA was adjusted to 100 μL with lysis buffer, and then was diluted with 900 μL of dilution buffer (50 mM Tris-HCl at pH 8.0, 167 mM NaCl, 1.1% [w/v] Triton X-100, 0.11% [w/v] sodium deoxycholate [DOC]). Twenty microliters of sheared chromatin was recovered and was used as input DNA.

Diluted lysate was incubated with 2 μg of antibody against H3K4me3 (07-473; Millipore), H3K9me3 (07-442; Millipore), H3K27me3 (07-449; Millipore), H3Ac (06-599; Millipore), or Pol II (ab5095; Abcam), which was reported to be capable of detecting stalled Pol II (Muse et al. 2007) overnight at 4°C with rotation, and then immuno-complexes were collected with 25 μL of Dynabeads Protein A (Invitrogen Dynal AS). Collected beads were washed with 1× RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% [w/v] Triton X-100, 0.1% [w/v] SDS, 0.1% [w/v] DOC) containing 150 mM NaCl twice, 1× RIPA buffer containing 500 mM NaCl twice, LiCl wash buffer (10 mM Tris-HCl at pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% [w/v] NP-40, 0.5% [w/v] DOC), and 1× TE containing 50 mM NaCl. Beads were re-suspended with 1× TE, and the cross-links were reversed in the presence of 200 mM NaCl overnight at 65°C. DNA was recovered with RNase A and proteinase K treatment, followed by phenol extraction and ethanol precipitation, and dissolved in 100 μL of 1× TE. One microliter of DNA was used for quantitative ChIP-PCR to confirm the specificity of our

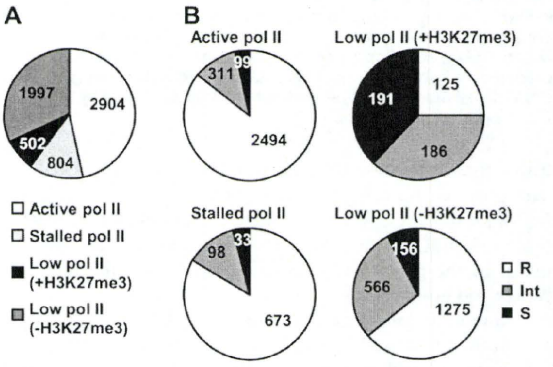

Figure 4. The association between Pol II binding and DNA methylation resistance in the total 6207 genes, regardless of transcription levels. (*A*) Classification of genes by Pol II status and H3K27me3 in the normal prostatic cell line. We were able to analyze transcription levels for 4567 of 5510 resistant, 1161 of 1330 intermediate, and 479 of 521 susceptible genes (total 6207 of 7361 genes) due to a difference in microarray platforms. Genes with high Pol II levels and high/moderate transcription levels were considered as those with "active Pol II." Genes with high Pol II levels but low transcription levels were considered as those with "stalled Pol II." Genes with low Pol II were further subdivided into those with and without H3K27me3. The numbers of genes with active, stalled, and low Pol II are shown. (*B*) The fractions of resistant, intermediate, and susceptible genes according to the Pol II and H3K27me3 statuses. Genes with either active or stalled Pol II had a larger fraction of resistant genes, and genes with low Pol II had a larger fraction of susceptible and intermediate genes.

ChIP technique (Supplemental Fig. S11) or to validate microarray results (Supplemental Fig. S7). Quantitative ChIP-PCR was performed using SYBR Green I (BioWhittaker Molecular Applications) and an iCycler Thermal Cycler (Bio-Rad Laboratories) as described previously (Nakajima et al. 2009). The primers used in quantitative ChIP-PCR are listed in Supplemental Table S5 (Kirmizis et al. 2004).

MeDIP

Five micrograms of genomic DNA was sheared by sonication using a VP-5s homogenizer (TAITEC) to a length of ~300 bp (Supplemental Fig. S12). Generally, there are nine to 53 CpG sites in 300-bp regions of promoter CGI (Nakajima et al. 2009), and this number of CpG sites is sufficient for efficient immunoprecipitation by MeDIP (Keshet et al. 2006). After heat denaturation for 10 min at 95°C, DNA was incubated with 5 μg of antibody against 5-methyl cytidine (Diagnode) in 1× IP buffer (10 mM Na-phosphate at pH 7.0, 140 mM NaCl, 0.05% [w/v] Triton X-100) overnight at 4°C with rotation. Immuno-complexes were collected with 70 μL of Dynabeads Protein A, washed with 1× IP buffer four times, and were recovered by Proteinase K treatment, followed by phenol extraction and ethanol precipitation. DNA was dissolved in 26 μL of 1× TE.

CGI oligonucleotide microarray analysis

Genome-wide analysis of DNA methylation, histone modifications, and Pol II binding was carried out using a human CGI oligonucleotide microarray (Agilent technologies) that contained 237,220 probes in or within 95 bp of CGI covering 27,800 CGIs, with an average probe spacing of 100 bp.

For MeDIP-CGI microarray analysis, immunoprecipitated DNAs from 4.33 μg of sonicated DNA and 0.96 μg of input DNA, without any amplification, were labeled with Cy5 and Cy3, respectively, using an Agilent Genomic DNA Labeling kit PLUS (Agilent technologies). Labeled DNA was hybridized to the microarray for 40 h at 67°C with constant rotation (20 rpm), and then scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). The scanned data were processed using Feature Extraction Ver.9.1 (Agilent Technologies), and the IP (Cy5) and WCE (Cy3) signal values were obtained. These two values were normalized using background subtraction, and signal log ratio [$\log_2$(IP/WCE)] and *P*[Xbar] were obtained using Agilent G4477AA ChIP Analytics 1.3 software (Agilent Technologies). Xbar is a signal value for a probe that takes account of signals for neighboring probes (within 1 kb), and *P*[Xbar] is a probability of how the Xbar value is deviated from a normal distribution of Xbar values of the entire genome of a sample.

For ChIP-chip analysis, 500 ng of immunoprecipitated and input DNA, without any amplification, was labeled with Cy5 and Cy3, respectively, and then hybridized with the microarray. A scan of the microarray and the data processing were performed as described above. The levels of each histone modification or Pol II binding were assessed by the signal ratio (IP/WCE). Genes were classified into those with high and low levels of each histone modification or Pol II binding when they had signal intensities higher and lower, respectively, than the average signal intensity of total probes. The microarray data (MeDIP-CGI microarray and ChIP-chip analyses) were submitted to the GEO database under accession no. GSE15154.

Calculation of Me value

The Me value of each probe was calculated as Me value = [signal log ratio × (1 − *P*[Xbar]) − 1.3]/2.6 + 0.5. The Me value was developed to give a value between 0 and 1 that linearly correlates with the amount of methylated DNA molecules at a specific locus and is not influenced by the genome-overall methylation levels. The Me value of a single probe is known to correlate well with an average DNA methylation level of CpG sites within 200 bp from the probe (Yamashita et al. 2009).

Definition of genomic regions

The position of each probe against a TSS was determined using UCSC hg18 (NCBI Build 36.1, March 2006). A CGI was defined as an assembly of probes with intervals <500 bp. CGIs were classified into four categories, promoter CGIs (within 10 kb upstream of the TSS), divergent CGIs (within 10 kb upstream of the TSSs of two genes that are transcribed in opposite directions), gene body CGIs, and downstream CGIs (within 10 kb downstream from genes). A CGI spanning both a promoter region and gene body was split into a promoter CGI and a gene body CGI. A putative NFR was defined as a region between a TSS, determined by UCSC hg18 (NCBI Build 36.1, March 2006), and its 200 bp upstream. Since TSSs are inherently variable for some genes (Suzuki et al. 2001), and the size of NFRs are different according to studies (Yuan et al. 2005; Gal-Yam et al. 2006), the locations are approximate, but expected to be correct as a whole. According to these definitions, 34,697 assemblies of probes were defined as CGIs, and 9624 assemblies were defined as NFRs. Genes with multiple NFRs because of their multiple TSSs were analyzed as different genes. DNA methylation status and histone modifications/Pol II binding in each CGI (or NFR) were assessed by an average Me value and signal ratio, respectively, of the probes located within each CGI (or NFR). A single CGI (or NFR) contains 6.8 (2.0) probes on average.

Gene expression analysis by oligonucleotide microarray

Expression microarray analysis was performed by a GeneChip Human Genome U133 Plus 2.0 expression microarray (Affymetrix) that contained 54,000 probe sets from 39,000 genes. From 8 μg of total RNA, the first-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) and a T7-(dT) 24 primer (Amersham Bioscience). Double-stranded cDNA was then synthesized, and biotin-labeled cRNA was synthesized using a BioArray HighYield RNA transcript labeling kit (Enzo). Twenty micrograms of labeled cRNA was fragmented and hybridized to the GeneChip oligonucleotide microarray. The microarray was stained and scanned according to the protocol from Affymetrix. The scanned data were processed using GeneChip operating software (ver. 1.4). The signal intensity of each probe was normalized so that the average signal intensity of all the probes on a microarray would be 500. Average signal intensity of all the probes for a gene was used as its transcription level. Genes were classified into those with high (>1000), moderate (250–1000), and low (<250) transcription according to their signal intensities.

Multivariate analysis and other statistical tests

To evaluate the independent contribution of each predictor variable (H3Ac, H3K4me3, Pol II binding, H3K9me3, or H3K27me3 level) in relation to the other four predictor variables on DNA methylation susceptibility (an outcome variable), multivariate logistic regression analysis was performed. Susceptible genes were defined as (1) those moderately and highly methylated in cancer cell lines (Int, and S1–S4 for the prostates; Int, and S1–S3 for the mammary glands), or (2) those highly methylated in cancer cell lines (S1–S4 for the prostates; and S1–S3 for the mammary glands). The predictor variables were classified into quintiles according to

H3Ac, H3K4me3, Pol II binding, H3K9me3, or H3K27me3 levels of the NFRs to create dummy variables. This was done because a log linear relationship was unclear between the raw value (signal ratio of each gene) and DNA methylation susceptibility. Multivariate-adjusted ORs and 95% confidence intervals (CIs) of genes in each quintile for DNA methylation susceptibility were calculated, including all predictor variables simultaneously in the model using SAS software, ver. 9.1 (SAS Institute Inc, *SAS/STAT 9.1 User's Guide*, SAS Institute Inc., Cary, NC). Using the lowest quintile as a reference, we calculated multivariate-adjusted ORs of genes in each quintile, which reflect DNA methylation susceptibility relative to the reference while controlling for the simultaneous effect of all the other predictor variables included in the model.

The fractions of genes with low transcription were compared between different groups of genes by the χ^2-test. The transcription, histone modification, and Pol II binding levels were compared between two groups of genes by the Mann-Whitney's *U*-test.

Functional annotation analysis

Functional annotation analysis was performed by DAVID bioinformatics resources (Dennis et al. 2003; Huang et al. 2009). The enrichment of genes in a biological process (a Gene Ontology criterion) was analyzed by comparing a fraction of genes with an ontology among the resistant (or susceptible) genes with that among all the genes.

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Eradication of *Helicobacter pylori* prevents cancer development in subjects with mild gastric atrophy identified by serum pepsinogen levels

Kimihiko Yanaoka[1], Masashi Oka[1], Hiroshi Ohata[2], Noriko Yoshimura[3], Hisanobu Deguchi[1], Chizu Mukoubayashi[1], Shotaro Enomoto[1], Izumi Inoue[1], Mikitaka Iguchi[1], Takao Maekita[1], Kazuki Ueda[1], Hirotoshi Utsunomiya[1], Hideyuki Tamai[1], Mitsuhiro Fujishiro[4], Masataka Iwane[2], Tatsuya Takeshita[5], Osamu Mohara[2] and Masao Ichinose[1]*

[1]*Department of Gastroenterology, School of Medicine, Wakayama Medical University, Wakayama-city, Wakayama, Japan*
[2]*Wakayama Wellness Foundation, Wakayama-city, Wakayama, Japan*
[3]*Department of Joint Disease Research, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[4]*Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[5]*Department of Public Health, School of Medicine, Wakayama Medical University, Wakayama-city, Wakayama, Japan*

A longitudinal cohort study was conducted in *Helicobactor pylori*-infected middle-aged Japanese males to evaluate the preventive effects of *H. pylori* eradication on the development of gastric cancer according to the extent of chronic atrophic gastritis (CAG). The extent of CAG was monitored by baseline serum pepsinogen (PG) levels. We followed 3,656 subjects with persistent *H. pylori* infection and 473 subjects with successful *H. pylori* eradication for cancer development for a mean (SD) of 9.3 (0.7) years. Groups with and without extensive CAG were categorized based on PG test-positive criteria to detect extensive CAG of PG I ≤ 70 ng/ml and PG I/II ratio ≤ 3.0. During the study period, 5 and 55 gastric cancers developed in *H. pylori*-eradicated and the noneradicated subjects, respectively, indicating no significant reduction in cancer incidence after *H. pylori* eradication. Among the noneradicated subjects, 1,329 were PG test-positive and 2,327 were PG test-negative. Gastric cancer was confirmed in 30 and 25 subjects, respectively. Among subjects whose infection was eradicated, 155 were PG test-positive and 318 were PG test-negative. Of these subjects, gastric cancer was confirmed in 3 and 2 subjects, respectively. Significant reduction in cancer incidence after eradication was observed only in PG test-negative subjects ($p < 0.05$; log-rank test). The results of this study strongly indicate that cancer development after eradication depends on the presence of extensive CAG before eradication and that *H. pylori* eradication is beneficial to most PG test-negative subjects with mild CAG as defined by the aforementioned criteria.

Key words: pepsinogen; *Helicobacter pylori*; eradication; cancer prevention; gastric cancer

Helicobacter pylori is recognized as a major pathogenic factor for persistent inflammation in the human stomach.[1,2] Chronic active gastritis induced by *H. pylori* is involved in the development of peptic ulceration[3] and is also considered a critical component that triggers the first stage of a series of precancerous processes, that is, the atrophy-metaplasia-dysplasia-cancer sequence.[4] In 1994, the International Agency for Research on Cancer (IARC) classified *H. pylori* infection as a definite (class I) carcinogen.[5] Nowadays, it is widely accepted that *H. pylori* is a major risk factor for developing gastric cancer together with its precursor lesions based on extensive evidence derived from clinico-epidemiologic studies[6–15] and experiments with *H. pylori*-infected animals.[16–22] Therefore, it appears quite reasonable to conclude that gastric cancer is theoretically preventable by the eradication of *H. pylori*.

Evidence from the murine model of *H. pylori* infection provides strong support for the reversibility of *H. pylori*-induced mucosal changes in the stomach with early bacterial eradication, and that eradication therapy, even in longstanding *H. pylori* infection, would prevent stomach carcinogenesis.[23–25] To date, several randomized and nonrandomized human intervention trials have been conducted to prove the effectiveness of *H. pylori* eradication in the control of gastric cancer.[15,26–31] However, unlike the case with peptic ulcer disease in which *H. pylori* eradication can significantly modify the natural history by preventing recurrence,[32–35] it remains unclear whether the eradication of *H. pylori* is an effective strategy for reducing the incidence of and mortality from gastric cancer.

Several previous studies revealed that the more advanced the stage of *H. pylori*-related chronic atrophic gastritis (CAG)—that is, the extent of gastric atrophy together with intestinal metaplasia—the greater the cancer risk.[36–40] Thus, the evaluation of CAG is especially important in the analysis of cancer prevention by *H. pylori* eradication. However, previous studies have not assessed the extent of coexisting CAG[15,31] or have assessed it only with endoscopic findings[29,30] and/or histopathology on endoscopic biopsy.[26–28] Both of these methods of diagnosis of gastric atrophy depend on subjective judgment without a gold standard; the reported intra- and inter-observer agreement among these methods are not satisfactory.[41,42] A randomized controlled trial would be an alternative approach. However, this type of trial would not be ethically feasible with today's knowledge about *H. pylori* infection and the development of cancer. Furthermore, the only 2 randomized controlled trials of *H. pylori* eradication in a high-risk area of China had inconsistent results, whereas 1 indicated a possibility of a beneficial effect of eradication in an early phase of *H. pylori* infection,[26] the other showed that *H. pylori* eradication did not lead to a significant reduction in cancer regardless of the severity of coexisting CAG.[28] These 2 studies stratified the extent of CAG based on the histopathology of endoscopic biopsy samples. Since CAG, including intestinal metaplasia, is a multifocal process, a certain degree of random error in the evaluation of CAG will also occur in the analysis of endoscopic biopsy samples. Meanwhile, a considerable number of studies, including ones by us, indicated that serum pepsinogen (PG) levels give an objective and reliable measure for the extent of CAG.[43–45]

Our objective in this study was to elucidate the preventive effects of *H. pylori* eradication according to the extent of *H. pylori*-related CAG, using PG levels to ascertain the degree of CAG. We performed long-term follow-up (mean follow-up period ± SD, 9.3 ± 0.7 y) on the development of gastric cancer in a cohort of *H. pylori*-infected subjects, with and without eradication, in whom serum PG levels were measured.

Abbreviations: CAG, chronic atrophic gastritis; ELISA, enzyme-linked immunosorbent assay; *H. pylori*, *Helicobacter pylori*; IgG, immunoglobulin G; PG, pepsinogen; RIA, radioimmunoassay; SD, standard deviation.

A part of this study was presented at the AGA 2007 conference in Washington, D.C., and was selected as a poster of distinction.

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***Correspondence to:** Department of Gastroenterology, School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama-shi, Wakayama 641-0012, Japan. Fax: +81-734-45-3616.
E-mail: ichinose@wakayama-med.ac.jp

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Subjects and methods

Study subjects

The study was conducted in Wakayama City, Wakayama Prefecture, located in the southwestern part of the main island of Japan. The gastric cancer mortality rate in Wakayama Prefecture is among the highest in Japan. It ranked fourth highest in 2005, with a mortality rate of 53.0/100,000 person-years, whereas for all of Japan the mortality rate for gastric cancer was 39.9/100,000 person-years. Between April 1994 and the end of March 1995, 8,420 factory workers (8,236 males, 184 females) aged 40–60 years underwent an annual multiphasic health screening program. This type of screening program is a common activity in various workplaces throughout Japan. It is done to detect incident diseases in their early stages. Thus, subjects were symptom-free and those who had symptoms requiring prompt medical care were excluded. Therefore, the subjects could be considered to represent the healthy Japanese population.

The study subjects underwent a series of screening tests and procedures: an interview to ascertain the general state of health, physical examination, chest radiography, electrocardiogram, laboratory blood tests, urinalysis, fecal occult blood test, and barium X-ray with digital radiography (DR). Subjects who had 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 medication that might affect gastrointestinal function, such as proton pump inhibitors, adrenocortical steroids, or non-steroidal anti-inflammatory drugs, were excluded from the study. Furthermore, the subjects with *H. pylori* infection were selected for the study using serum-specific antibody titers as described in the following section. Thus, 5,776 subjects with *H. pylori* infection (5,645 males, 131 females) were eligible for this study. Some of these subjects had been investigated in a previous cohort study.[46]

Diagnosis of extensive CAG and H. pylori *infection*

The 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 PG levels and anti-*H. pylori* IgG titers. Serum PG (PG I and II) levels were measured using a modification (RIA-beads Kit, Dainabott, Tokyo, Japan) of our previously reported RIA.[47] Subjects with extensive CAG were diagnosed based on the PG test-positive criteria of PG I $\leq$ 70 ng/ml and PG I/II ratio $\leq$ 3.0.[48,49] These criteria offer a sensitivity of 70.5% and a specificity of 97% in the diagnosis of extensive CAG, using pathological diagnosis as the gold standard.[48] Serum anti-*H. pylori* IgG titers were measured using ELISA (MBL Inc., Nagoya, Japan).[50] Subjects with antibody titers >50 U/ml were classified as *H. pylori*-infected and those with antibody titers <30 U/ml were regarded as infection negative. Subjects with a titer level that was $\geq$30 U/ml and $\leq$50 U/ml were considered indeterminate. The infection-negative and indeterminate subjects were excluded from the study. The sensitivity and specificity of the ELISA test used in this study were 93.5 and 92.5%, respectively.[50]

Treatment of H. pylori *and follow-up*

From April 1994 to March 1997, 5,776 *H. pylori*-infected subjects were informed of their infection and were advised to visit the clinic at their workplace. At the clinic they were told about the possible role of *H. pylori* infection in gastroduodenal disorders, including peptic ulcer disease and gastric cancer, and also about the possible side effects of *H. pylori* eradication therapy. As a result, 852 *H. pylori*-infected subjects (728 males, 124 females) underwent *H. pylori* eradication with dual therapy consisting of the proton pump inhibitor omeprazole 20 mg twice a day and amoxicillin 750 or 500 mg twice a day for 2 weeks, or with triple therapy consisting of the proton pump inhibitor omeprazole 20 mg twice a day, amoxicillin 750 mg twice a day, and clarithromycin 200 mg twice a day for 1 week. *H. pylori* status was assessed by serum *H. pylori* antibody level at the annual health check-up. All subjects were followed until the end of the study period of 10 years, which was the end of March 2004. Subjects underwent the aforementioned health screening program annually and were also screened to identify incident gastric cancer as described in the following section. The incident day of gastric cancer was defined as the day of the health check-up when the cancer was detected. Duration of the observation period was calculated for each subject from the time of the baseline survey to the diagnosis of gastric cancer. The ethics committee of the workplace approved the protocol, and informed consent was obtained from all participating subjects.

Screening for gastric cancer

Cancer screening was performed by double-contrast barium X-ray with digital radiography (DR) and by serum PG as filter tests. For upper gastrointestinal barium X-ray, a remote controlled X-ray fluoroscope (TU-230XB, Hitachi Medical Corporation, Tokyo, Japan) and real-time DR (DR-2000H, Hitachi Medical Corporation, Tokyo, Japan) were used. The double-contrast upper gastrointestinal X-ray series used 150 ml of high-concentration barium at 200%, and 11 films were taken for each subject as described previously.[46] Subjects were also screened using the serum PG filter test. Those with positive barium X-ray findings and/or PG test-positive result were further examined by upper gastrointestinal endoscopy (XQ-200, Olympus, Tokyo, Japan).

Resected specimens of gastric cancer obtained by endoscopy or surgery were assessed histopathologically and classified according to Lauren's classification into intestinal or diffuse type.[51] Location of the cancer in the stomach was classified as cardia or noncardia based on clinical and histopathological records.

Statistical analysis

Data were analyzed using SPSS 11.0 (SPSS, Chicago, IL) and STATA (STATA Corp., College Station, TX). Differences were tested for significance using the *t* test for comparisons between 2 groups, analysis of variance (ANOVA) for comparisons among multiple groups, and Scheffe's LSD test for comparisons of pairs of groups. The chi-square test and Fisher's exact test were used to compare categorical variables. Long-term effects of *H. pylori* eradication on gastric cancer development were analyzed by the Kaplan-Meier method, and statistical differences between curves were tested by the log-rank test.

Results

Among the 5,776 *H. pylori*-positive subjects, 852 received eradication therapy. Of these subjects, 743 (87.2%) (643 males, 100 females) became *H. pylori*-negative after eradication. The remaining 4,924 subjects (4,917 males, 7 females) did not receive eradication therapy (Fig. 1). Since there was an imbalance in the number of female subjects included in the groups with and without eradication, all results of the female subjects were excluded from the analysis. Among the 743 successfully eradicated subjects, 169 either dropped out or became *H. pylori*-positive during the follow-up period. Among the 4,924 subjects with persistent *H. pylori infection*, 1,253 dropped out, underwent eradication therapy after March 1997, or experienced spontaneous resolution of their infection. These subjects were also excluded from the analysis. In addition, we excluded the 1 case of gastric cancer in the *H. pylori*-eradicated subjects and the 8 cases in the noneradicated subjects that had developed in the first year of the study since the cancers might have existed at the start of the study. Thus, to ensure completeness of the diagnosis of *H. pylori* infection or gastric cancer development during the study period, only data from the survivors in March 2004-that is, the 473 subjects with successful eradication and 3,656 subjects with persistent infection-were analyzed.

As shown in Table I, there were no significant differences in baseline characteristics between the subjects with and without

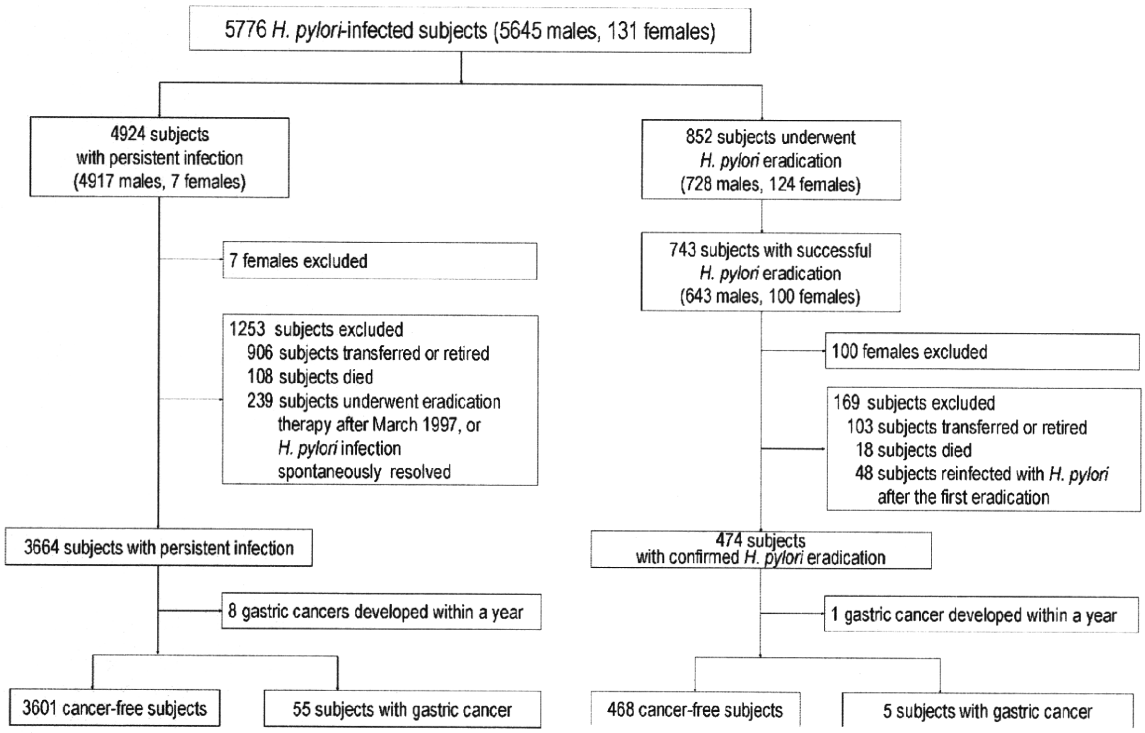

FIGURE 1 – Schematic flow of the study subjects.

eradication in terms of age, current smoking, and current drinking habits. Of the 3,656 subjects with persistent *H. pylori* infection, 36.4% (n = 1,329) had extensive CAG based on serum PG levels using the aforementioned PG test-positive criteria of PG I $\leq$ 70 ng/ml and PG I/II ratio $\leq$ 3.0.[48,49] This figure is not significantly different from that in the 473 *H. pylori*-eradicated subjects, that is, 32.8% (n = 155). Furthermore, the mean levels of serum PG I and PG II and the PG I/II ratio did not differ significantly between the *H. pylori*-eradicated and noneradicated subjects, indicating that the extent of coexisting gastric atrophy was similar in the 2 groups (Table I).

During the study period, 55 subjects developed gastric cancer among the 3,656 subjects with persistent *H. pylori* infection, and 5 subjects developed cancer among the 473 subjects cured of the infection. The Kaplan-Meier analysis revealed that cancer development in the subjects with persistent infection occurred steadily throughout the study period (Fig. 2) In the *H. pylori*-eradicated subjects, on the other hand, cancer development was null until 4 years after the start of the follow-up, and the first cancer was detected 5 years after eradication. Thereafter, cancer development was observed to increase steadily to the level of the noneradicated subjects by the end of the study period. The cancer incidence rates in the *H. pylori*-eradicated and noneradicated subjects were 117/100,000 person-years and 157/100,000 person-years, respectively. There was not a significant reduction in cancer development according to *H. pylori* eradication in the cohort as a whole (p = 0.55; log-rank test).

Macroscopically, 2 cancers (3.3%) that developed in the noneradicated subjects were located in the cardia, and the other 58 cancers (96.7%) were located elsewhere in the stomach. The mean size of the cancers was smaller in the *H. pylori*-eradicated subjects than in the noneradicated subjects, although the difference was not statistically significant. Among the cancers that developed in the noneradicated subjects, 9.1% (5/55) were detected at an advanced stage and were invading the muscularis propria, 16.4% (9/55) were submucosal, and 74.5% (41/55) were mucosal. In contrast, the cancers in the *H. pylori*-eradicated subjects were all mucosal.

As for the 2 histopathological types, the intestinal-type was the dominant phenotype regardless of *H. pylori* eradication, and there was no significant difference in the incidence rate of this type of cancer between the *H. pylori*-eradicated and noneradicated subjects (94/100,000 person-years vs. 103/100,000 person-years). In contrast, the proportion of diffuse-type cancer was significantly reduced by eradication; the proportions were 20% (1/5) in the *H. pylori*-eradicated subjects and 34.5% (19/55) in the noneradicated subjects ($p < 0.05$). The incidence rate of this type of cancer was significantly lower in the *H. pylori*-eradicated subjects (23/100,000 person-years) compared with the noneradicated subjects (54/100,000 person-years) ($p < 0.05$) (Table I).

Next, we analyzed the effects of *H. pylori* eradication on cancer development according to the extent of coexisting gastric atrophy as evaluated by serum PG levels. The study subjects were classified into PG test-positive and PG test-negative groups based on the aforementioned criteria. In both the *H. pylori*-eradicated and noneradicated subjects, the PG test-positive group had more smokers and more alcohol drinkers than the negative group, but the difference was significant only in the noneradicated subjects ($p < 0.05$). The serum PG levels (PG I, PG II) and the PG I/II ratio, did not differ significantly between the *H. pylori*-eradicated and noneradicated subjects, irrespective of the positivity of the PG test, except that the mean PG I level of the PG-negative group was significantly higher in the *H. pylori*-eradicated subjects than in the noneradicated subjects (Table I).

Figure 3 shows the cumulative incidence of cancer in the *H. pylori*-eradicated and noneradicated subjects stratified by PG test criteria (PG I $\leq$ 70 ng/ml, PG I/II ratio $\leq$ 3.0). In the PG test-positive group with extensive CAG, gastric cancer was confirmed

TABLE I – EFFECT OF *H. PYLORI* ERADICATION ON GASTRIC CANCER DEVELOPMENT ACCORDING TO PG TEST RESULTS, BASED ON POSITIVE CRITERIA OF PG I LEVEL ≤ 70 mg/l AND PG I/II RATIO ≤ 3.0

	Group with *H. pylori* infection			Group with *H. pylori* eradication		
	Total	PG test-negative	PG test-positive	Total	PG test-negative	PG test-positive
Subjects	3,656	2,327	1,329	473	318	155
Person-years	35101.5	22,436	12665.5	4,263	2,918	1,345
Age, y [mean(SD)]	49.8 (4.6)	49.5 (4.7)	50.4 (4.3)	49.6 (5.5)	49.3 (5.6)	50.9 (5.5)
Current smoking. *n* (%)	2,087 (57.1)	1,242 (53.4)	845 (63.6)**	262 (55.4)	169 (53.1)	93 (60.0)
Alcohol use, *n* (%)	2,432 (66.5)	1,479 (63.6)	953 (71.7)**	310 (65.5)	204 (64.2)	106 (68.4)
Follow-up, y [mean(SD)]	9.6 (1.0)	9.6 (0.9)	9.5 (1.1)	9.1 (1.4)	9.2 (1.3)	8.7 (1.6)
PG I, ng/ml [mean(SD)]	62.0 (32.5)	75.8 (31.1)	37.9 (17.5)	74.2 (34.6)	92.5 (57.0)**	36.8 (18.2)
PG II, ng/ml [mean(SD)]	20.0 (11.0)	20.3 (12.4)	19.5 (7.8)	23.1 (14.6)	24.9 (16.5)	19.5 (8.4)
PG I/II [mean(SD)]	3.4 (1.6)	4.3 (1.4)	2.0 (0.7)	3.4 (1.5)	4.1 (1.3)	1.9 (0.7)
Total gastric cancer						
Age, y [mean(SD)]	50.8 (3.9)	50.6 (4.3)	50.9 (3.6)	53.4 (5.5)	50.5 (3.5)	55.3 (6.4)
Size of the cancer, mm [mean(SD)]	12.8 (8.3)	14.4 (10.4)	11.4 (5.3)	12.0 (5.7)	12.5 (3.5)	11.7 (7.6)
Depth of cancer invasion (m:sm:mp)[1]	41:9:5	15:7:3	26:2:2	5:0:0	2:0:0	3:0:0
Follow-up, y [mean(SD)]	5.9 (2.5)	4.3 (2.4)	5.6 (2.5)	6.0 (1.0)	5.0 (0)	6.7 (0.6)
Cases/incidence rate[2]	55/157	25/111	30/237	5/117	2/69**	3/223
Intestinal gastric cancer						
Cases/incidence rate[2]	36/103	14/62	22/174	4/94	1/34	3/223
Diffuse gastric cancer						
Cases/incidence rate[2]	19/54	11/49	8/63	1/23*	1/34	0

*Significantly different from the total subjects in group with *H. pylori* infection ($p < 0.05$).
**Significantly different from PG test-negative group with *H. pylori* infection ($p < 0.05$).
[1]Abbreviations used are as follows: m, mucosa; sm, submucosa; mp, muscularis propria.–[2]Per 100,000 person-years.

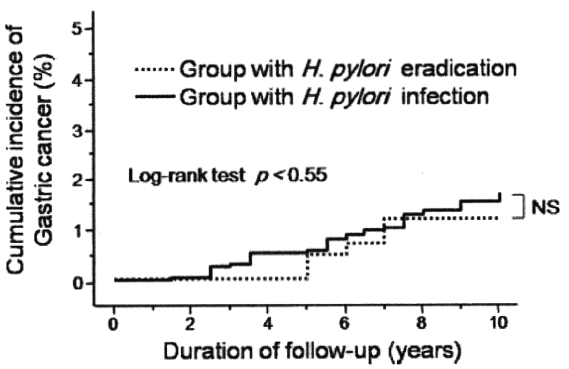

FIGURE 2 – Kaplan-Meier analysis of gastric cancer development in subjects with persistent *H. pylori* infection and in those whose infection was eradicated. The cancer incidence rates of the former and the latter were 157/100,000 person-years and 117/100,000 person-years, respectively. There was no significant difference in cancer development between the 2 groups ($p = 0.55$; log-rank test).

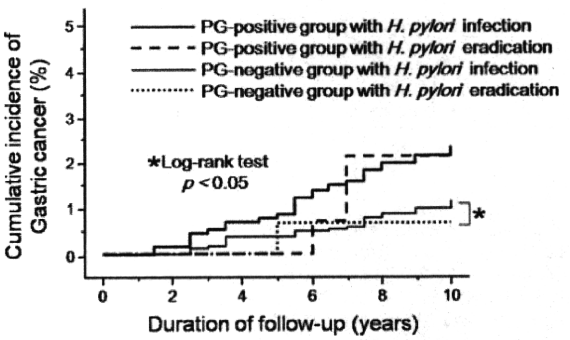

FIGURE 3 – Kaplan-Meier analysis of gastric cancer development in the PG test-positive group with extensive CAG and PG test-negative group with mild CAG according to *H. pylori* infection status. The positive criteria for PG test to detect extensive CAG were PG I level ≤ 70 μg/l and PG I/II ratio ≤ 3.0. Among the subjects with persistent *H. pylori* infection, the cancer incidence rates for the PG test-positive and PG test-negative groups were 237/100,000 person-years and 111/100,000 person-years, respectively. Meanwhile, comparing the *H. pylori*-eradicated subjects, the cancer incidence rates of the PG test-positive and PG test-negative groups were 223/100,000 person-years and 69/100,000 person-years, respectively. Eradication of *H. pylori* significantly reduced cancer development in the PG test-negative group with mild CAG ($p < 0.05$; log-rank test), but not in the PG test-positive group with extensive CAG.

in 3 and 30 subjects among the *H. pylori*-eradicated and noneradicated subjects, respectively, thus, putting the cancer incidence rate at 223/100,000 person-years and 237/100,000 person-years, respectively. Likewise, in the PG test-negative group with mild CAG, cancer was confirmed in 2 and 25 subjects among the *H. pylori*-eradicated and noneradicated subjects, respectively, and the cancer incidence rates were 69/100,000 person-years and 111/100,000 person-years, respectively. Significant reduction in cancer development according to eradication was observed only in the PG test-negative group ($p = 0.04$; log-rank test).

Eradication tended to decrease the development of both intestinal-type and diffuse-type cancer in the PG test-negative group, both showing a p value of less than 0.1. The effect of eradication on the incidence rate of intestinal-type cancer was of marginal significance ($p = 0.06$). In the PG test-positive group, eradication led to an increase in the incidence rate of intestinal-type cancer, although it was not a statistically significant difference. In contrast, the development of diffuse-type cancer in the PG test-positive group was null after eradication (Table I).

In addition to the PG test-positive criteria of PG I ≤ 70 ng/ml and PG I/II ratio ≤ 3.0, which are the most widely used criteria to detect subjects with extensive CAG in Japan,[48,49] stricter criteria of PG I ≤ 50 ng/ml and PG I/II ratio ≤ 3.0, and PG I ≤ 30 ng/ml and PG I/II ratio ≤ 2.0 are used to detect subjects with more extensive atrophy.[52] In this study, the accuracy of cancer detection among the *H. pylori*-eradicated subjects using the criteria PG I ≤ 50 ng/ml and PG I/II ratio ≤ 3.0 was better and more balanced than those by the other 2 sets of criteria, showing a sensitivity of 60% and specificity of 76.7%. The accuracy of cancer detection by the criteria of PG I ≤ 70 ng/ml and PG I/II ratio ≤ 3.0 had a sensitivity of 60% and specificity of 67.5%. Furthermore, the sensitivity and specificity for the criteria of PG I ≤ 30 ng/ml and PG I/II ratio ≤ 2.0 were 20 and 89.3%, respectively. Therefore, the

TABLE II – EFFECT OF *H. PYLORI* ERADICATION ON GASTRIC CANCER DEVELOPMENT ACCORDING TO PG TEST RESULTS, BASED ON POSITIVE CRITERIA OF PG I LEVEL ≤ 50 mg/l AND PG I/II RATIO ≤ 3.0

	Group with *H. pylori* infection			Group with *H. pylori* eradication		
	Total	PG test-negative	PG test-positive	Total	PG test-negative	PG test-positive
Subjects	3,656	2,690	966	473	361	112
Person-years	35101.5	25943.5	9,158	4,263	3319	944
Age, y [mean(SD)]	49.8 (4.6)	49.5 (4.6)	50.8 (4.3)	49.6 (5.5)	49.1 (5.1)	51.0 (4.6)
Current smoking, *n* (%)	2,087 (57.1)	1,471 (54.7)	616 (63.8)**	262 (55.4)	201 (55.7)	61 (54.5)
Alcohol use, *n* (%)	2,432 (66.5)	1,743 (64.8)	689 (71.3)	310 (65.5)	235 (65.1)	75 (67.0)
Follow-up, y [mean(SD)]	9.6 (1.0)	9.6 (0.9)	9.5 (1.2)	9.1 (1.4)	9.2 (1.3)	8.7 (1.5)
PG I, ng/ml [mean(SD)]	62.0 (32.5)	73.6 (29.6)	29.8 (12.9)	74.2 (34.6)	88.6 (54.6)**	27.8 (12.4)
PG II, ng/ml [mean(SD)]	20.0 (11.0)	21.1 (12.0)	16.9 (6.5)	23.1 (14.6)	25.2 (15.7)	16.5 (6.6)
PG I/II [mean(SD)]	3.4 (1.6)	4.0 (1.5)	1.8 (0.7)	3.4 (1.5)	3.9 (1.3)	1.8 (0.7)
Total gastric cancer						
Age, y [mean (SD)]	50.8 (3.9)	50.3 (4.3)	51.4 (3.4)	53.4 (5.5)	50.5 (3.5)	55.3 (6.4)
Size of the cancer, mm [mean(SD)]	12.8 (8.3)	13.4 (9.2)	11.9 (6.9)	12.0 (5.7)	12.5 (3.5)	11.7 (7.6)
Depth of cancer invasion (m:sm:mp)[1]	41:9:5	19:7:4	22:2:1	5:0:0	2:0:0	3:0:0
Follow-up, y [mean(SD)]	5.9 (2.5)	6.2 (2.5)	5.3 (2.5)	6.0 (1.0)	5.0 (0)	6.7 (0.6)
Cases/incidence rate[2]	55/157	30/116	25/273	5/117	2/60**	3/318
Intestinal gastric cancer						
Cases/incidence rate[2]	36/103	15/58	21/230	4/94	1/30	3/318
Diffuse gastric cancer						
Cases/incidence rate[2]	19/54	15/58	4/43	1/23*	1/30	0

*Significantly different from the total subjects in group with *H. pylori* infection ($p < 0.05$).
**Significantly different from PG test-negative group with *H. pylori* infection ($p < 0.05$).
[1]Abbreviations used are as follows: m, mucosa; sm, submucosa; mp, muscularis propria.–[2]per 100,000 person-years.

effect of eradication on cancer development in the PG test-positive and PG test-negative groups based on the criteria of PG I ≤ 50 ng/ml and PG I/II ratio ≤ 3.0 was also analyzed post hoc. As shown in Table II, the analysis revealed a more pronounced reduction in the cancer incidence rate in the PG test-negative group after eradication regardless of histopathological type of cancer, with an incidence rate in the *H. pylori*-eradicated and the noneradicated subjects of 60/100,000 person-years and 116/100,000 person-years, respectively ($p = 0.04$; log-rank test). At the same time, there was no significant reduction, but rather an increase, in the cancer incidence rate in the PG test-positive group after eradication, with an incidence rate of 318/100,000 person-years and 273/100,000 person-years in the *H. pylori*-eradicated and noneradicated subjects, respectively (Table II). In contrast, the results of the analysis using the other criteria of PG I ≤ 30 ng/ml and PG I/II ratio ≤ 2.0 revealed that the effect of eradication was not remarkable in the control of cancer development in the PG test-positive and PG test-negative groups (not shown).

Discussion

Previous studies analyzing the effect of *H. pylori* eradication on the development of gastric cancer have not sufficiently assessed the degree of background CAG, which is a major risk factor for gastric cancer.[36–40,46] In this study, we monitored the extent of coexisting CAG in the study subjects using serum PG levels. As shown by the serum PG levels in the *H. pylori*-eradicated and noneradicated subjects, there was no significant difference in the extent of coexisting CAG between the 2 groups. The results of this longitudinal cohort study demonstrated that the preventive effect of *H. pylori* eradication on cancer development is not evident in an asymptomatic middle-aged population in a high-risk gastric cancer area of Japan. This is in accord with the results of 2 previous randomized controlled studies in another country with a high risk of gastric cancer (China).[26,28] It is highly probable that *H. pylori* eradication targeted to middle-aged subjects in cancer high-risk areas does not lead to reduction in cancer development at the population level.

It is noteworthy that there was a marked delay in cancer development in the *H. pylori*-eradicated subjects, and the cancers that developed were less advanced compared with those in the subjects with persistent infection; all the cases were early mucosal cancer that could be treated by endoscopic resection. This is consistent with the results of animal experiments demonstrating the role of *H. pylori* infection as a promoter in stomach carcinogenesis.[20–22] It also indicates that the initiated cells in the *H. pylori*-infected stomach mucosa, once established as a result of long-lasting inflammation, tend to remain in the deranged structure of the mucosa even after eradication. Without the promoter action, their proliferation is markedly suppressed; however, they grow steadily, leading in time to clinically detectable cancer. Therefore, if the study period were shorter than 5 years, the results would have been quite different, indicating the effectiveness of eradication in the control of cancer in the general population. The difference between our results and some of the previous studies will probably be explained by the relatively short follow-up periods of the other studies, that is, a mean of around 3 years.[29–31]

In this study, we found that *H. pylori* eradication led to a reduction in cancer development in the PG test-negative group with mild CAG, defined by the aforementioned PG test-positive criteria. On the basis of the serum PG levels, the extent of CAG was similar in the *H. pylori*-eradicated and the noneradicated subjects in the PG test-positive group. However, in the PG test-negative group, the mean serum PG I level was significantly higher in the *H. pylori*-eradicated subjects than in the noneradicated subjects. The results of previous studies demonstrated that serum PG levels are elevated in *H. pylori*-related nonatrophic gastritis, reflecting the severity of histological changes due to mucosal inflammation.[53,54] *H. pylori* eradication reverses the serum PG elevation.[55,56] In general, the inflammatory process in the nonatrophic stomach is reported to induce a series of molecular events leading to the development of cancer, especially the diffuse type.[10,57,58] Thus, in the PG test-negative group, the basal level of activity of gastritis and cancer risk are considered to be no less in the *H. pylori*-eradicated subjects than in the noneradicated subjects. *H. pylori* eradication significantly reduced the development of cancer in the PG test-negative group with mild CAG, though it was not sufficient to eradicate gastric cancer in all subjects. In contrast, the effect of eradication was not so remarkable in the PG test-positive group with extensive CAG.

These results are in line with the results of some of the previous randomized and nonrandomized studies in which the extent of CAG was evaluated based on endoscopic findings or histopathology of endoscopic biopsy specimens, demonstrating that eradication is not beneficial in preventing cancer development in a subgroup of subjects with premalignant lesions, that is, CAG,

intestinal metaplasia, and dysplasia.[26,29] The results also appear to indicate that most, if not all, of the PG test-positive subjects are beyond the point of no return in stomach carcinogenesis. Previous observations regarding the long-term effect of *H. pylori* eradication indicate that the regression of extensive atrophy together with intestinal metaplasia occurs slowly as a function of the square of the *H. pylori*-free time, and complete recovery of intestinal metaplasia takes more than 12 years once the infection has been eradicated.[59] On the other hand, eradication in the early stage of *H. pylori*-related gastritis without intestinal metaplasia will lead to recovery or even normalization of the structure and function of the gastric mucosa within a short period of time.[59] It is presumed that the carcinogenic potential of the *H. pylori*-eradicated stomach mucosa does not return to the basal level of uninfected mucosa until complete regression of gastric atrophy and intestinal metaplasia is attained. Thus, as this results confirm, it is highly probable that cancer incidence in the PG test-positive group with extensive CAG does not decrease as markedly within a period of 10 years after eradication compared with the negative group with mild CAG. Further long-term morphological and genetic studies will be required to clarify whether *H. pylori* eradication will eventually lead to the complete regression of *H. pylori*-related gastritis, including intestinal metaplasia, and also to complete eradication of gastric cancer. From the viewpoint of cancer prevention, our results indicate that cancer-susceptible subjects with extensive CAG remain at high risk even after complete eradication, and that they should be the target of regular follow-up for a period of at least 10 years, even after complete eradication. It is possible, however, that incident cancer in the *H. pylori*-eradicated subjects will be slow growing and of lower malignant potential and, as a result, clinically easier to control.

Between the 2 histopathological types of gastric cancer, the intestinal-type is the most common and tends to develop with the sequence of gastritis-atrophy-metaplasia-dysplasia-cancer. The effect of eradication was slight and insignificant on the incidence of this type of cancer in the total cohort. However, in the PG test-negative group with mild CAG, a substantial reduction in the incidence of this type of cancer was observed, although the significance of the reduction was marginal ($p < 0.057$) and was probably due to the small number of subjects. In contrast, diffuse cancer tends to develop directly from nonatrophic gastric mucosa without passing through the sequence. Eradication significantly reduced the incidence of this type of cancer. These results are also compatible with previous observations that a series of events induced by *H. pylori* infection from histopathological changes reflecting acute inflammation to molecular changes of altered DNA methylation of CpG islands in various genes, including E-cadherin, which is considered to be deeply involved in the development of diffuse-type cancer in nonatrophic stomach, disappeared immediately after successful eradication.[59–61]

In conclusion, *H. pylori* eradication is considered to be an effective strategy for reducing the risk of gastric cancer in middle-aged subjects with mild CAG. Serum PG levels give an objective measure for eradication. Most, if not all, of the subjects identified by negative PG test criteria would benefit from *H. pylori* eradication. Although further studies are required to determine the best criteria for indicating eradication, PG I level $\leq$ 50 μg/l and PG I/II ratio $\leq$ 3.0 appear to be the best among the 3 most widely-used sets of criteria for PG tests in Japan. Using the criteria, the number needed to treat (NTT) for 10 years to prevent 1 case of gastric cancer was 179, whereas for the second best set of criteria of PG I level $\leq$ 70 μg/l and PG I/II ratio $\leq$ 3.0 the NTT was 238. In Japan, 60 million people are infected with *H. pylori,* and PG test-negative subjects constitute a major proportion among middle-aged subjects.[46,62,63] Although the annual incidence rate of cancer in PG test-negative, *H. pylori*-infected middle-aged subjects is by no means low, that is, around 0.1%,[46] the cost-effectiveness of such measures is unknown. We have recently reported on a group of subjects with especially high cancer risk among PG test-negative subjects.[52] This group of subjects constitutes about 10% of all PG test-negative subjects and shows a high annual cancer incidence rate of over 0.2%, a comparable level to that in PG test-positive subjects with extensive CAG, whereas the annual cancer incidence rate of other PG test-negative subjects is around 0.05%. The authors believe that this group is an especially good target for eradication. Finally, it appears that serum PG levels will probably be useful in developing strategies for the prevention of gastric cancer with *H. pylori* eradication.

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Image of the Month

En bloc resection of cardia cancer and lipoma with endoscopic submucosal dissection

S. Ono, M. Fujishiro *, O. Goto, S. Kodashima, M. Omata

Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo, Japan

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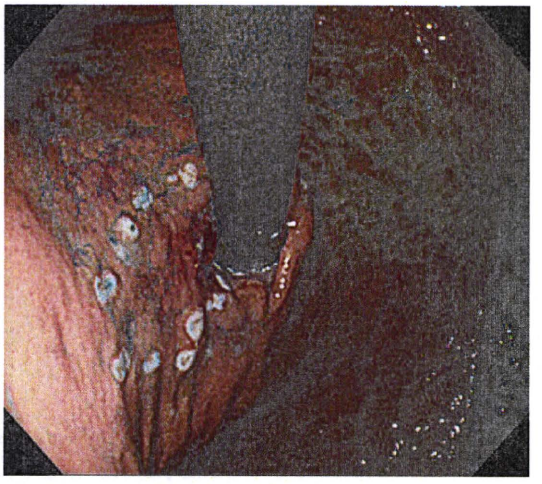
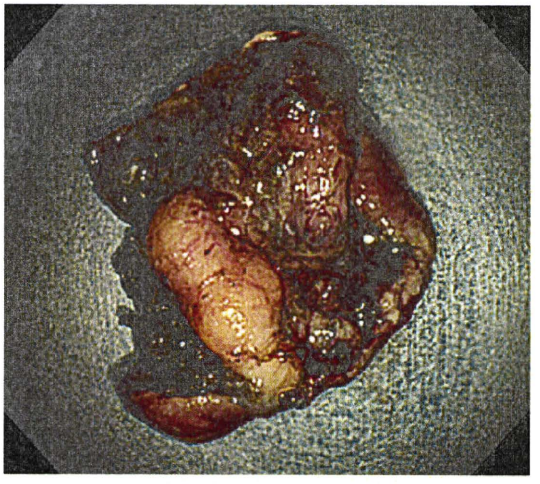


A 64-year-old gentleman was referred to our department for endoscopic submucosal dissection (ESD) of an early gastric cancer [1]. An upper-gastrointestinal endoscopy showed a superficial, shallow depression, 4 cm in diameter, from the lesser curvature to the anterior wall in the cardia. ESD was performed for this intramucosal adenocarcinoma (Fig. 1). Doing the submucosal dissection, we encountered with a yellowish-colored adipose tissue, 2 cm in diameter, beneath the cancerous mucosa unexpectedly. The capsulated adipose tissue was easily differentiated from extraluminal fat, because it was located over the muscularis propria in the submucosal layer and identified as a lipoma. So we carefully cut at the level of the deepest edge of the lipoma, and treated the feeding vessels. Injection agent with indigocarmine added was useful to discriminate the yellowish lipoma from blue-colored submucosa to dissect. Thus, the cancer with the lipoma was successfully resected in an en bloc fashion (Fig. 2). Histological assessment revealed a differentiated type intramucosal adenocarcinoma, 0-IIc, with curative resection and a well-differentiated adipose tumor covered by a fibrous capsule. The most recent endoscopy performed 8 weeks after ESD revealed no recurrence.

The stomach is a rare location for lipomas, and the lipomas represent about 3% of all benign gastric masses [2]. There are few reports of gastric lipoma with early gastric cancer, and no reports of cases treated with ESD procedures [3].



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* Corresponding author. Tel.: +81 3 3815 5411x33019; fax: +81 3 5800 8806.
E-mail address: mtfujish-kkr@umin.ac.jp (M. Fujishiro).

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GASTROENTEROLOGY

Is it possible to predict the procedural time of endoscopic submucosal dissection for early gastric cancer?

Osamu Goto, Mitsuhiro Fujishiro, Shinya Kodashima, Satoshi Ono and Masao Omata

Department of Gastroenterology, The University of Tokyo, Tokyo, Japan

Key words
early gastric cancer, endoscopic submucosal dissection, operation schedule, predictive formula of procedural time, procedural time.

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Correspondence
Dr Mitsuhiro Fujishiro, Department of Gastroenterology, Graduate school of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan. Email: mtfujish-kkr@umin.ac.jp

Abstract

Background and Aim: Endoscopic submucosal dissection (ESD) has been expected to be a possible curative treatment, especially for node-negative early gastric cancer (EGC). We investigated the influential factors on the procedural time of gastric ESD with a Flex knife for the estimation.

Methods: In 222 intestinal-type EGC resected by ESD experts with established techniques, age, sex, location, circumference, gross type, tumor size, tumor depth, ulcerative findings, the period of ESD, the operator, and the experience of the operator were retrospectively analyzed. Predictors with a significant difference, as determined by multivariate analysis, were used to compose a predictive formula of procedural time.

Results: Location, gross type, tumor depth, ulcerative findings, and tumor size were considered influential factors on the procedural time by univariate analysis. Location in the upper-third of the stomach, presence of ulcerative findings, and > 20 mm in size were independent factors, as determined by multivariate analysis. Procedural time (min) was nearly equal to the maximal tumor size (mm) multiplied by 2.5, and an additional 40 min was required if the tumor was located in the upper-third of the stomach or had ulcerative findings (in both situations, an additional 80 min was needed).

Conclusion: The procedural time of ESD with a Flex knife for EGC can be predicted by tumor size, location, and existence of ulcerative findings. The estimation of procedural time may be very useful to determine the operation schedule.

Introduction

Endoscopic submucosal dissection (ESD) is a recently-developed endoluminal surgical technique for intramucosal neoplasms of the gastrointestinal tract, characterized by a circumferential mucosal incision and submucosal dissection beneath the lesion.[1–3] It is expected to be a possible curative method, especially for node-negative early gastric cancer (EGC),[4,5] with the advantage of preserving the whole stomach.

One of the shortcomings of ESD is, however, that it takes longer to resect the lesion, compared to other endoscopic treatments.[5,6] In Japan, ESD is usually performed in the left lateral decubital position under only intravenous administration of some sedatives or analgetics without an anesthesiologist. When the operation is prolonged, this can lead to accompanying complications, for example, postoperative aspiration pneumonia, deep vein thrombosis, or cardiorespiratory instability due to an overdose of anesthetic drugs. If the procedural time can be predicted, it would be very useful for arranging the operation schedule to prevent possible complications. Although some reports refer to the factors that prolong ESD,[7–10] to our knowledge, there has been no investigation about the prediction of the procedural time so far. Therefore, we retrospectively assessed the influential factors on the procedural time of ESD for EGC from our consecutive data and validated the possibility of whether the procedural time of ESD can be predicted before it takes place.

Methods

From August 2003, when the technical methodology of ESD was established in our hospital (The University of Tokyo, Tokyo, Japan) to January 2008, 347 consecutive EGC were resected by ESD. In this study, 222 lesions with a histological diagnosis of intestinal-type EGC were retrospectively investigated. Those excluded included 47 lesions resected by beginners who performed gastric ESD for 30 cases or less; 42 lesions resected in the initial phase of experts, where the total number of resection reached up to 30; eight lesions with a histological diagnosis of diffuse-type EGC, because these lesions were principally resected by gastrectomy in our hospital due to the possibility of the rapid growth of residual cancer cells, or the difficulty in the demarcation of the tumor, or the difficulty to distinguish ulcerative findings caused by biopsy from those by the tumor in some cases; six

Table 1 Univariate analysis of predictors for procedural time

	n	Mean procedural time (min)	*P*-value
Sex (male : female)	183 : 39	77.7 : 69.2	0.3898
Location (U : M : L)	65 : 79 : 78	99.9 : 73.6 : 59.1	< 0.0001*
Circumference (AW : GC: LC: PW)	42 : 29 : 91 : 60	80.2 : 58.3 : 75.1 : 83.8	0.2256
Gross type (0–I/IIa : 0–IIb/IIc : combined)	68 : 136 : 18	82.7 : 68.9 : 107.2	0.0116**
Tumor depth (mucosa : submucosa)	169 : 53	70.7 : 94.0	0.0077
Ulcerative findings (presence : absence)	42 : 180	98.3 : 71.1	0.0041
Period of ESD (early : late)†	136 : 86	78.1 : 73.3	0.5377
Operator (A : B : C : D)	54 : 121 : 36 : 11	86.4 : 72.9 : 77.9 : 57.3	0.3174
Experience of ESD (51 or more : 31–50)	191 : 31	76.9 : 71.8	0.6332
	Mean ± SD	*r*	
Age (years)	68.1 ± 9.3	0.083	0.2203
Tumor size (mm)	21.7 ± 15.2	0.506	< 0.0001

*Significantly different between upper-third (U) and middle-third (M), and between U and lower-third (L) by Fisher's Fisher's protected least significance difference (PLSD). **Significantly different between flat/depressed and combined by Fisher's PLSD. AW, anterior wall; GC, greater curve; LC, lesser curve; PW, posterior wall. †Early, 2003–2005; Late, 2006–2008.

lesions in a remnant stomach after gastrectomy or in a gastric tube after esophagectomy, because the number was small and the specific conditions might affect subsequent analyses; and 22 lesions from patients whose medical records were insufficient for retrospective analyses.

All patients provided written, informed consent before undergoing treatment. All lesions were resected by four very experienced ESD experts, each of whom had performed ESD with a Flex knife for more than 30 cases of EGC or gastric adenoma.[11–13] The technical outcomes and major complication rates of ESD for these cases were as follows: en bloc resection rate, 97.3%; complete resection rate (the rate of en bloc resection with tumor-free lateral and basal margins), 88.3%; delayed bleeding rate, 6.3%; and perforation rate, 2.7%.

ESD was indicated according to the criteria of node-negative EGC by Gotoda *et al.*[14] The ESD techniques have been described elsewhere.[1–3] In brief, a Flex knife (KD-630L; Olympus, Tokyo, Japan)[3,15] was used as the main electrosurgical knife, and other knives, such as an insulation-tipped (IT) knife,[1,2] a hook knife,[16] or a needle knife, were used when required. An endoscope with a water-jet system (GIF-Q260J; Olympus, Japan) was mainly used in the study. A mixture of 10% glycerin mixed with a 5% fructose and 0.9% saline preparation (Glyceol; Chugai Pharmaceutical, Tokyo, Japan) containing 0.005% indigo carmine and 0.0005% epinephrine was used to make a submucosal fluid cushion.[17] Hyaluronic acid was added to the injection solution for resection of a difficult lesion.[18] Hemostatic forceps (HDB2422W; Pentax, Tokyo, Japan) were used for hemostasis.[19]

Procedural time was defined as the duration from circumferential marking around the lesion to the completion of hemostasis on the mucosal defect after resection. To determine the influential factors on procedural time, the following variables were analyzed: age, sex, location (upper-third, middle-third, or lower-third), circumference (anterior wall, posterior wall, lesser curve, or greater curve), gross type (0–I/IIa, 0–IIb/IIc, or combined type), tumor size (maximal diameter of the resected tumor actually measured), tumor depth (mucosal tumor or submucosal invasive tumor), ulcerative findings in the submucosal layer (endoscopical presence or absence), the period of ESD (early [2003–2005], late [2006–2008]), the operator (A, B, C, and D), and the experience of ESD (more than 50 cases or 31–50 cases).

Table 2 Multivariate analysis of predictors for procedural time over 120 min

		Odds ratio (95% confidence interval)	*P*-value
Location	Lower	1	
	Middle	1.006 (0.288–3.513)	0.9920
	Upper	4.649 (1.393–15.513)	0.0124
Gross type	0–I/IIa	1	
	0–IIb/IIc	0.345 (0.109–1.089)	0.0695
	Combined	2.600 (0.691–9.784)	0.1575
Tumor depth	Mucosa	1	
	Submucosa	0.665 (0.229–1.929)	0.4527
Ulcerative findings	Absence	1	
	Presence	4.914 (1.480–16.318)	0.0093
Tumor size	≤ 20 mm	1	
	> 20 mm	8.261 (2.786–24.493)	0.0001

A preliminary univariate analysis was performed using Pearson's correlation coefficient for age and tumor size; Student's *t*-test for sex, tumor depth, ulcerative findings, the period of ESD, and the experience of ESD; and one-way ANOVA for location, circumference, gross type, and the operator. Predictors with a significant difference or correlation, as determined by univariate analysis, were included in the multivariate analysis using a logistic regression model. Predictors with a significant difference, as determined by multivariate analysis, were included in a step forward linear regression model to compose a predictive formula of procedural time. A *P*-value of < 0.05 in each analysis was considered statistically significant.

Results

The univariate analysis of variables for the procedural time is shown in Table 1. Location, gross type, tumor depth, ulcerative findings, and tumor size were considered influential factors on

