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other hand, stronger effect of *CYP1A1* ^{462}*Val* found in previous pooled analysis among nonsmokers (6) was not observed in Asian populations investigated in our study.

Le Marchand et al. (19) hypothesized that genetic susceptibility to polycyclic aromatic hydrocarbons (based on high-risk genotypes for *CYP1A1* and *GSTM1*) predominantly causes squamous cell carcinoma. In the multiethnic study conducted by Le Marchand et al. (19), *CYP1A1* ^{6235}*C* allele was associated with a 3.1-fold risk of squamous cell carcinoma when combined with a *GSTM1* deletion. Decreasing trend of squamous cell carcinoma, relative to the increase in adenocarcinoma, associated with filter-tipped cigarettes in developed country indirectly supports this hypothesis (33). The increased risk of squamous cell carcinoma in relation with the *GSTM1*-null genotype observed in our study is consistent with the results of previous studies, including those of a meta-analysis (10, 19, 34, 35). The effect of the *CYP1A1* ^{6235}*C* allele, especially when combined with a *GSTM1*-null genotype, also tended to be associated with a higher risk of squamous cell carcinoma among Asians (5); in our study, *CYP1A1 TC* or *CC* genotype was associated with significant elevation of squamous cell carcinoma risk compared with *TT* genotype (OR, 1.6) and Ile-C haplotype was significantly associated with squamous cell carcinoma risk (OR, 2.1).

BPDE is known to induce G:C to T:A transversion mutations in the hotspot codons of the *p53* tumor suppressor gene (36), which is found more frequent in squamous cell carcinoma than in adenocarcinoma (37). Cigarette smoke is also known to be causally related to BPDE-DNA adducts (38, 39), and BPDE-DNA adduct

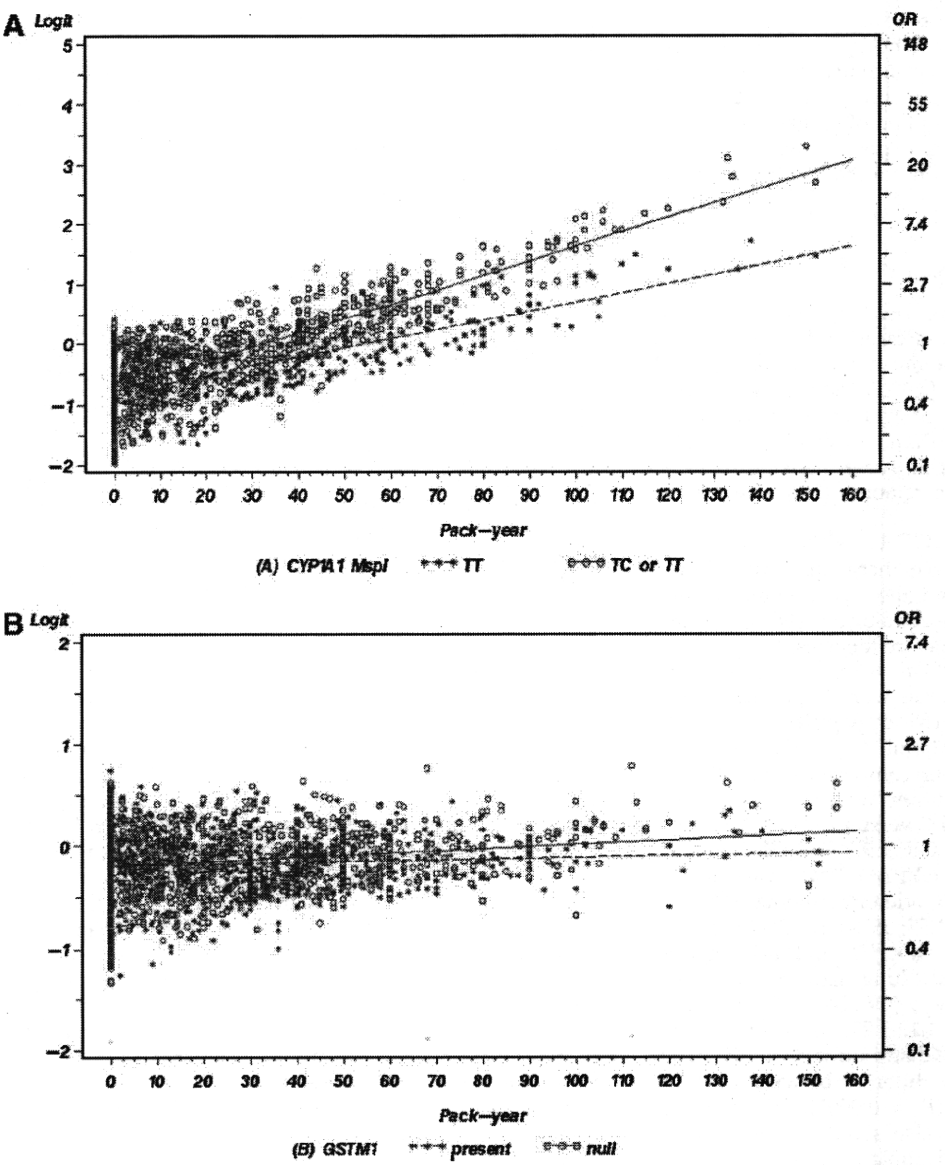

Figure 1. The smoking effect on lung cancer stratified by the *CYP1A1* $T^{6235}C$ *(MspI)* (**A**) and *GSTM1* null/present (**B**). When the interaction was evaluated with smoking, increasing trend of lung cancer risk as pack-year increased was stronger among those with the *CYP1A1* 6235 TC/CC genotype compared with those with TT genotype ($P_{interaction}$ = 0.001). Although the association between smoking and lung cancer was stronger among those with the *GSTM1*-null genotype compared with the present type, it was only marginally significant with the assumption of common intercept ($P_{interaction}$ = 0.08).

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level is elevated in the lung parenchyma of smokers with *GSTM1*-null genotype (40). Moreover, the combined genotypes of *CYP1A1* ^{462}*Val* and *GSTM1* null have been associated with increased adduct level in lung tissues of squamous cell carcinoma patients (41). Thus, it is speculated that our finding of an association between *GSTM1* and *CYP1A1* polymorphisms with the risk of squamous cell carcinoma is related to polycyclic aromatic hydrocarbon exposure derived from smoking because polycyclic aromatic hydrocarbons are primarily metabolized by *CYP1A1* and *GSTM1*. The greater effects observed among smokers also support this smoking-related etiology of squamous cell carcinoma in Asian population.

Our study is the largest pooled analysis conducted for Asian populations to evaluate the role of polymorphisms in carcinogen-metabolizing genes (i.e., *CYP1A1*, *GSTM1*, and *GSTT1*) in lung cancer development. We simultaneously evaluated the potential effect of four polymorphisms on lung cancer and the modification of those effects by smoking exposure reporting significant interaction between *CYP1A1* ^{6235}*C* allele and smoking. Subtype-specific results in Asian population are also noteworthy.

However, our study has several limitations to be considered. First, not all published Asian studies were included in this study. However, there was no evidence of significant publication bias for this pooled analysis. In terms of heterogeneity, only marginally significant heterogeneity was found for *CYP1A1* ^{462}*Val/Val* compared with *Ile/Ile* (P = 0.08). We note that when the adjusted values were considered, the heterogeneity did not remain. Other limitation of our study may be the relatively small sample size in subgroup analyses. We found that the *GSTT1*-null genotype was marginally associated only with small cell lung cancer risk, whereas no association with lung cancer was observed for either Asians or Caucasians in the previous pooled analysis for *GSTT1*-null genotype (13). Although relatively higher variant allele frequencies, compared with other ethnic groups (13, 32), may compensate for the relative small sample size in terms of statistical power, we cannot exclude chance for the explanation of the significant association between the *GSTT1*-null genotype and small cell lung cancer risk, considering that only 71 cases were available. Sizable exclusion of subjects for missing data on smoking and pathologic subtypes also limits the conclusion from our results for interactive effects between the polymorphisms and smoking, and subtype-specific analysis. Thus, our findings need to be replicated in a larger study. Future study should also include the measurement of dietary factors, such as isothiocyanates, which are involved in the detoxification of tobacco-related carcinogens (42) and may have protective effects on lung cancer especially among smokers or those with *GST*-null genotypes, as observed in a Chinese population (24, 43).

In summary, the results of our study suggest that genetic polymorphism in *CYP1A1* and *GSTM1* plays a role in lung cancer susceptibility in Asian populations and that the effects are strongest for squamous cell carcinoma. Although our results are generally consistent with previous studies and are supported by epidemiologic and experimental observations, additional large studies are needed to help to elucidate the role of genetic polymorphisms in xenobiotic-metabolizing genes in lung cancer development. The interaction between environmental exposure other than smoking (e.g., indoor coal combustion) and these polymorphisms still remains to be evaluated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Downregulation of *EphA1* in colorectal carcinomas correlates with invasion and metastasis

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The Eph gene family has important roles in the developmental processes and may also be involved in the initiation, progression, and metastasis of certain types of cancers. In the present study, quantitative real-time reverse-transcriptase PCR was performed to detect the expression of *EphA1* transcript in 5 colon cancer cell lines and 75 colorectal carcinomas. Immunohistochemical staining was used to check the expression of *EphA1* protein in 20 colorectal adenomas and in 111 colorectal carcinomas specimens. *EphA1* protein expression was not completely consistent with transcript expression. *EphA1* protein was expressed in all adenomas and reduced in 54% colorectal cancers. Reduced expression of *EphA1* protein occurred more often in male patients ($P=0.028$) and in patients with poor differentiation ($P=0.027$), greater depth of wall invasion ($P=0.003$), lymph node metastasis ($P=0.034$), and advanced tumor stage ($P=0.003$). Patients with reduced *EphA1* expression had a poor overall survival ($P=0.059$). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively). Our data indicate that the *EphA1* may play different roles during the different stages of colorectal carcinoma progression.

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Keywords: *EphA1*; colorectal carcinoma; invasion; metastasis; survival

Colorectal carcinoma is one of the most common malignant tumors. There are estimated 112 340 new cases of colon cancer and 41 420 new cases of rectum cancer in USA, and estimated 52 180 cases of colorectal cancer patients died in 2007. The incidence and mortality rate of colorectal cancer rank the third in all types of cancers both of male and female patients.[1] The search for new molecular targets of early diagnosis, rational therapy, and prognosis is the current research hot spot.

Eph receptors, the largest subfamily of the receptor tyrosine kinases, are divided into two subfamilies, EphA and EphB, based on the sequence homology of their extracellular domains and their affinity to bind corresponding ligands, EphrinA and EphrinB (Eph Nomenclature Committee, 1997).[2] The Eph family of receptor tyrosine kinases has important roles in diverse biological processes including nervous system development,[3–5] angiogenesis,[6] and vascular system development.[7] A number of Eph receptors and their ephrins ligands are implicated in carcinogenesis.[8–16] *EphA1*, the first member of Eph receptors tyrosine kinase, was isolated from erythropoietin-producing hepatocellular carcinoma cell lines and is located on chromosome 7q34.[17] It is widely expressed in normal tissues including lung, small intestinal, kidney, bladder, thymus, and colon.[18] The expression level of *EphA1* in human cancers is variable. Overexpression of *EphA1* was observed in certain types of tumors including ovarian carcinoma,[19] and head and neck squamous carcinoma.[20] Reduced expression of *EphA1* was detected in prostate cancer cell lines,[21] breast carcinoma cell lines,[12] and basal-cell carcinomas and squamous-cell carcinoma specimens of the skin.[22] There was a marginal study of *EphA1*

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expression in colorectal carcinoma specimens, particularly, with respect to clinicopathologic parameters. In this study, we performed quantitative real-time reverse-transcriptase PCR (RT-PCR) and immunohistochemistry to detect the expression of *EphA1* mRNA and protein in a set of colorectal carcinomas and adenomas. And the association of *EphA1* expression levels with clinicopathologic parameters of colorectal carcinomas was analyzed. To our knowledge, this is the first description of the role of *EphA1* in colorectal progression and prognosis.

Materials and methods

Five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 were used in the present study. The cells were routinely cultured in Dulbecco's modified Eagle's medium (NISSUI Pharmaceutical Co., Tokyo, Japan), supplemented with 1 mmol/l L-glutamine, 10% fetal bovine serum (FBS; Life Technologies Inc.), and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

All the tissue samples in our study were collected from 111 patients with colorectal carcinoma and 20 patients with colorectal adenomas, as part of a study approved by the Research Ethics Board of the Nanjing Jinling Hospital, Clinical School of Medical College of Nanjing University. These patients had undergone surgery in Nanjing Jinling Hospital between 2004 and 2006 without any preoperative therapy. Among the 111 cases of colorectal carcinomas, 75 fresh tissue samples including cancer tissues and matched normal mucosas were immediately frozen in liquid nitrogen after the resection and then stored at −80°C for the preparation of the total RNA. Formalin-fixed and paraffin-embedded tumor tissues were sectioned at 4 μm thickness and stained with hematoxylin and eosin for the pathological identification. The patients included 66 men and 45 women. Ages ranged from 23 to 84 (median age: 59 years). The distribution of the tumors by sites of origin was as follows: the cecum and ascending colon, 26 tumors; the transverse colon, 4 tumors; the sigmoid colon, 14 tumors; and the rectum, 67 tumors. The clinicopathologic variables of the 111 patients of colorectal carcinoma were shown in Table 1. The tumor stage was classified according to the TNM classification of World Health Organization of 2007.

Quantitative Real-Time RT-PCR

The total RNA was extracted using the RNA extraction reagent TRIzol (Invitrogen, CA, USA) according to the manufacturer's protocol. Single-strand cDNA was synthesized using 2 μg total RNA with an oligo(dT) primer. Quantitative real-time RT-PCR was performed to detect the *EphA1* transcript expression in colorectal carcinoma on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). The experiments were run in triplicate. The sense primer, antisense primer, and TaqMan probe for detection of *EphA1* were designed according to the *EphA1* mRNA sequence (GenBank accession number: NM_005232). The sense primer is 5′-ATCTTTGGGCTGCTGCTTGG-3′ and the antisense primer is 5′-GCTTGTCCTCTCGATCCACA TC-3′. The PCR products are 127 bp long. The TaqMan probe is 5′-(FAM) CGGTCACGCTGC CTCTGCTGCC (Eclipse)-3′. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control (GenBank accession number: NM_002046). The sense primer is 5′-CCAGGTGGTCTCCTCTGACTT-3′ and the antisense primer is 5′-GTTGCTGTAGCCAAATTCGTT GT-3′. The PCR products are 130 bp long. The probe is 5′-(FAM) AACAGCGACACCCACTCCTCCACC (Eclipse)-3′. The values of *EphA1* mRNA expression were normalized using the *GAPDH* expression. The primers and probes for *EphA1* and *GAPDH* were synthesized by TaKaRa Biotechnology Inc. (Dalian, China).

The reaction mixture consisted of 3.0 μl 10 × buffer; 3.0 μl 2.0 μmol/l deoxy-ribonucleoside triphosphates (dNTPs; Invitrogen); 3.0 μl 3.0 μmol/l sense primer; 3.0 μl 3.0 μmol/l antisense primer; 1.0 μl 3.0 μmol/l fluorescence probe; 0.20 μl 5 U/μl Takara ExTaq Hotstart Taq (TaKaRa Biotechnology), 0.6 μM 5-carboxy-X-rhodamine reference dye (Invitrogen), 2.0 μl cDNA template, and distilled water for a total volume of 30 μl. The PCR cycling conditions were used as follows: 2 min at 95°C, followed by 40 amplification cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 1 min.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 2 μm thickness. All the sections were deparaffinized using xylene, dehydrated by gradient ethanol, and then rehydrated with deionized water. Heat-mediated antigen retrieval was run by autoclave treatment (120°C for 2 min in 1 mmol/l EDTA, pH 8.0) and then followed by cooling at room temperature. Incubation with a polyclonal antibody raised against the COOH terminus of the human *EphA1* receptor (dilution 1:100, ABGENT, San Diego, USA) was performed overnight at 4°C. After washing with phosphate-buffered saline (pH 7.4), the sections were then incubated with secondary antibody (Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3′-diaminobenzidine. Nuclei were counterstained with hematoxylin.

Table 1 Correlation between expression of EphA1 transcript and clinicopathologic parameters in 75 colorectal carcinomas

	Case number	*N/T>2*	*N/T0.5-2*	*N/T<0.5*	P-*value*
Overall	75	28	17	30	—
Sex					
Male	44	17	10	17	0.952
Female	31	11	7	13	
Age (years)					
≤55	36	13	8	15	0.96
>55	39	15	9	15	
Location					
Rectum+sigmoid colon	57	23	13	21	0.556
Colon	18	5	4	9	
Tumor size (cm)					
≤5	49	18	11	20	0.98
>5	26	10	6	10	
Depth of wall invasion					
Mucosa+submucosa	4	3	1	0	0.278
Muscularis propria	19	7	2	10	
Subserosa+serosa	52	18	14	20	
Tumor differentiation					
Well+moderate	48	15	13	20	0.36
Poor+mucinous	27	13	4	10	
Lymph node metastasis					
Negative	46	18	9	19	0.72
Positive	29	10	8	11	
Clinical stage (TNM)					
I	20	9	2	9	0.617
II	25	8	7	10	
III+IV	30	11	8	11	
Stage (Dukes)					
A+B	45	17	9	19	0.78
C	30	11	8	11	

N/T, normal–tumor; TNM, tumor, node, metastasis.

The immunostaining results were evaluated independently by three pathologists. The different results were unified by consensus. The score of *EphA1* expression was made semiquantitatively by assessing the percentage of stained cells and the staining intensity in both tumor tissue and normal mucosa. The percentage of positive cells was rated as follows: 0 score for 0–5%, 1 score for 6–25%, 2 scores for 26–50%, and 3 scores for more than 50%. The staining intensity was rated as follows: 0 score for no staining, 1 score for weak staining, 2 scores for moderate staining, and 3 scores for strong staining. The scores from the percentage and intensity were added to an overall score. The expression of the *EphA1* protein in colorectal carcinomas was categorized into downregulation, upregulation, and no difference by comparing the overall score in tumor tissue *vs* matched normal mucosa.

Methylation-Specific PCR

Genomic DNA was modified by sodium bisulfite, as described by Clark *et al*.[23] Primers were designed to discriminate between methylated and unmethylated alleles after sodium bisulfite treatment. Primer sequences were chosen for the regions containing frequent CpG near the 3′-end of the primers to provide maximum discrimination between methylated and unmethylated DNA. Aliquots (2 μl) were amplified in a 30-μl reaction mixture consisting of 1 × buffer (10 mM Tris-HCl, 2.0 mM $MgCl_2$, 50 mM KCl, pH 8.3), 1 U Takara ExTaq Hotstartaq, 260 μM dNTPs, and 0.3 μM of the primer sets. The PCR reaction involved 2 min at 95°C, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and finally 10 min at 72°C. The methylation-specific primers were 5′-ATTCGGGTTATTGTTTTAGGTT TC-3′ (forward) and 5′-GAAAATCGATACCTTC CTTAACG-3′ (reverse). The PCR products were 129-bp long. Unmethylation-specific primers were 5′-ATTTGGGTTATTGTTTTAGGTTTTG-3′ (forward) and 5′-ACAAAAATCAATACCTTCCTTAACAC-3′ (reverse). Primer sets for detection of methylated and unmethylated DNA were located at the same sites of genomic sequence (forward primer was located at −35 to −12 from translation start site; reverse primer was located at 71–93; Figure 4). The

PCR products were 131-bp long. The PCR products were separated on 8% nondenaturing polyacrylamide gel, followed by ethidium-bromide staining.

Statistical Analysis

The χ^2-test was adopted to determine differences among intergroup variables by use of SPSS 15.0. software (SPSS, Chicago, IL, USA). Kaplan–Meier survival analysis was used to examine the relationship between categorical groups and survival for univariate analysis. A *P*-value <0.05 was considered statistically significant.

Results

Expression of the *EphA1* Transcript in Colon Cancer Cell Lines and in Colorectal Carcinomas

RT-PCR was performed to detect the expression of *EphA1* transcript in colon cancer cell lines DLD1, HT29, HCT116, SW480, and SW620. *EphA1* mRNA was detected in all the colon cancer cell lines (Figure 1). Quantitative real-time RT-PCR was used to detect the expression of *EphA1* transcript in 75 fresh specimens of colorectal carcinomas. The ratio of normal–tumor (N/T) was based on the relative expression level of *EphA1* transcript in paired normal mucosa and the tumor tissues of the same patient. The results were classified into three groups according to the ratio of the two: downregulation (N/T > 2), upregulation (N/T < 0.5), and no difference (N/T 0.5–2). Down and upregulation of *EphA1* transcript were observed in 37% (28 of 75) and 40% (30 of 75) of the specimens, respectively (Table 1). The association of expression of *EphA1* transcript with the sex, age, tumor site, size, depth of wall invasion, differentiation, clinical stage, lymph node metastasis, and Dukes stage were analyzed. No any significant difference between the *EphA1* transcript expression and these clinicopathologic parameters was found. The data were summarized in Table 1.

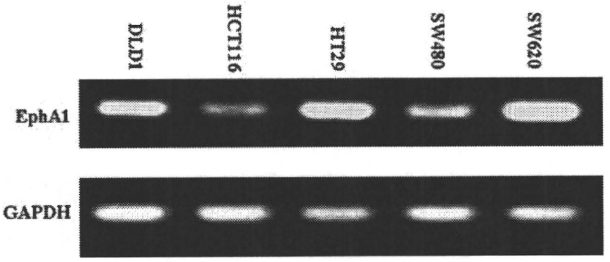

Figure 1 Expression of *EphA1* in colon cancer cell lines DLD1, HCT116, HT29, SW480, SW620. Housekeeping gene *GAPDH* was used as an internal control.

Expression of *EphA1* Protein in Colorectal Carcinomas and Adenomas

The *EphA1* protein was detected in most of the normal mucosa cells (Figure 2a①). The adenoma cells expressed *EphA1* protein diffusely (Figure 2a②). A heterogeneous *EphA1*-staining pattern between cells was observed in carcinoma tissue sections. The up and downregulations of the *EphA1* protein were observed in 31% (34 of 111) and 54% (60 of 111) cases of colorectal carcinomas, respectively (Table 2; Figure 2a③④). The immunostaining of *EphA1* was observed as particles in cytoplasm or distributed homogenously in cytoplasm (Figure 2b⑤⑥).

The Significance of Reduced Expression of the *EphA1* Protein in Colorectal Carcinoma

The expression of *EphA1* protein was significantly related to sex, depth of wall invasion, differentiation, lymphatic metastasis, and clinical stage. The reduced expression of *EphA1* was more often occurred in poorly differentiated colorectal carcinomas and mucinous adenocarcinomas than in well- and moderately differentiated cases ($P=0.027$). The patients with reduced *EphA1* protein had deeper serosa and subserosa invasiveness than those without *EphA1* downregulation ($P=0.003$). Colorectal carcinomas with reduced *EphA1* expression had more advanced tumor stage ($P=0.003$) and lymph node metastasis ($P=0.034$). Reduced expression of the *EphA1* protein was more often detected in male than in female patients ($P=0.028$). There was no significant association with other clinicopathologic variables (Table 2).

Reduced *EphA1* Protein Expression is Associated with Poor Survival in Patients with Colorectal Carcinoma

We examined the association of *EphA1* protein expression with clinical outcome. The Kaplan–Meier survival analysis showed patients with reduced *EphA1* expression had shorter survival than those with high *EphA1* expression (log-rank test, $P=0.059$; Figure 3①). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively; Figure 3②③).

Detection of Methylated *EphA1* DNA in Colon Cancer Cell Lines

Five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 were checked for methylation status at promoter-associated region of *EphA1* by methylation-specific PCR. Methylated DNA of *EphA1* was detected in all five tested colon cancer cell lines (Figure 4).

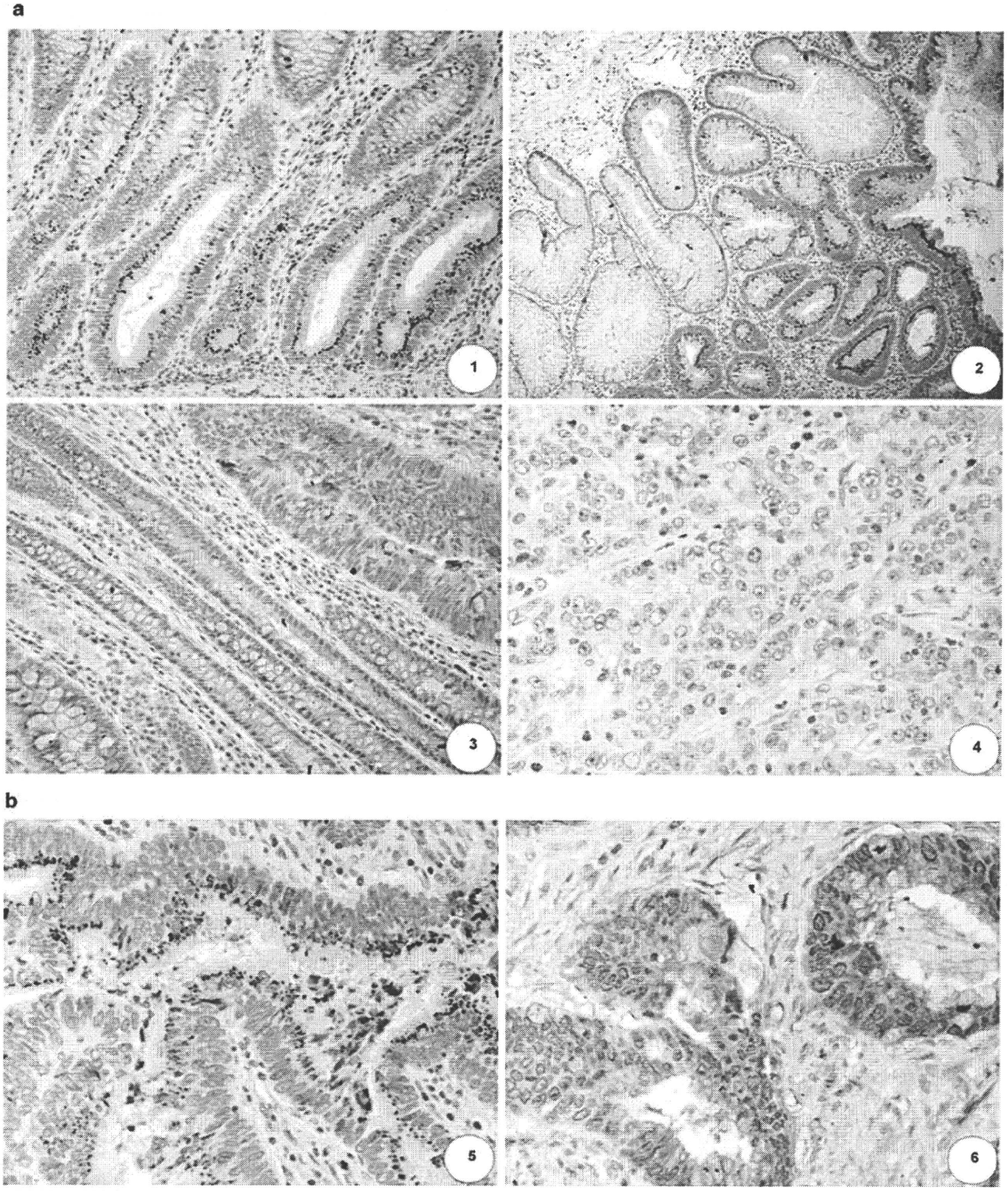

Figure 2 **(a)** Immunohistochemical staining of *EphA1* in colorectal carcinomas. ① The expression of *EphA1* in normal mucosa (EnVision, original magnification × 200). ② Upregulation of *EphA1* in adenoma cells (right lower) compared with normal mucosas (left upper; EnVision, original magnification × 100). ③ Downregulation of *EphA1* in carcinoma cells (right upper) compared with normal mucosas (left lower; EnVision, original magnification × 200). ④ Lost expression of *EphA1* in poorly differentiated carcinoma cells (EnVision, original magnification × 400). **(b)** ⑤ Strong immunoreactivity was detected in moderately differentiated carcinoma cells. The subcellular localization of *EphA1* protein revealed the accentuation of golgiosome (EnVision, original magnification × 400). ⑥ *EphA1* protein was expressed homogeneously in cytoplasm (EnVision, original magnification × 400).

Table 2 Correlation between EphA1 protein expression and clinicopathologic parameters in 111 colorectal carcinomas

	Case number	*Downregulation*	*No difference*	*Upregulation*	P-value
Overall	111	60	17	34	—
Sex					
Male	66	37	14	15	0.028
Female	45	23	3	19	
Age (years)					
≤55	49	31	8	10	0.109
>55	62	29	9	24	
Location					
Rectum+sigmoid colon	81	40	12	29	0.144
Colon	30	20	5	5	
Tumor size (cm)					
≤5	80	40	14	26	0.352
>5	31	20	3	8	
Depth of wall invasion					
Mucosa+submucosa	10	3	3	4	0.003
Muscularis propria	24	7	3	14	
Subserosa+serosa	77	50	11	16	
Tumor differentiation					
Well+moderate	80	37	15	28	0.027
Poor+mucinous	31	23	2	6	
Lymphatic metastases					
Negative	66	29	12	25	0.034
Positive	45	31	5	9	
Clinical stage (TNM)					
I	28	7	5	16	0.003
II	37	22	7	8	
III+IV	46	31	5	10	
Stage (Dukes)					
A+B	65	29	12	24	0.06
C	46	31	5	10	

N/T, normal–tumor; TNM, tumor, node, metastasis.

Discussion

The Eph gene family is the largest subfamily of receptor tyrosine kinase including at least 16 receptors and 9 ligands. The role of Eph family in developing processes has been well documented. The interaction of Eph receptors and their ligands controls the cells repulsion and movement during tissue patterning in embryonic development. However, the roles of Eph and Ephrin proteins in tumorigenesis are not clearly established. The Eph family was initially known as a putative oncogene based on their overexpression in certain types of human cancers. The *EphA2* receptor is overexpressed in colorectal,[24] gastric,[25] ovarian,[11] and esophageal squamous-cell carcinoma.[26] The *EphA4* receptor is overexpressed in pancreatic ductal adenocarcinoma,[27] and *Ephrin B1* is overexpressed in ovarian carcinoma.[28] More recently, increasing data have shown that some members of Eph receptors and Ephrin ligands have roles of tumor suppressor. A representative example is that certain EphB subfamily proteins, including *EphB2*, *EphB4*, suppress colorectal cancer progression through Wnt signal pathway.[9,10,29]

The *EphA1* receptor is widely expressed in human normal tissues. However, its expression levels in different types of human tumors are greatly diverse, and its role in tumorigenesis is still very vague. In the present study, we described the expression of the *EphA1* transcript and protein in colorectal carcinomas and analyzed the association of expression of *EphA1* with clinicopathologic parameters.

The *EphA1* transcript was detected in all five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 by RT-PCR (Figure 1). The expression levels of *EphA1* mRNA in five colon cancer cell lines were different. There is a CG-rich region around *EphA1* translation start site (Figure 4a), the methylation, and unmethylation-specific primer sets were designed by using web software MethPrimer (http://www.urogene.org//methprimer/). Methylated and unmethylated DNA

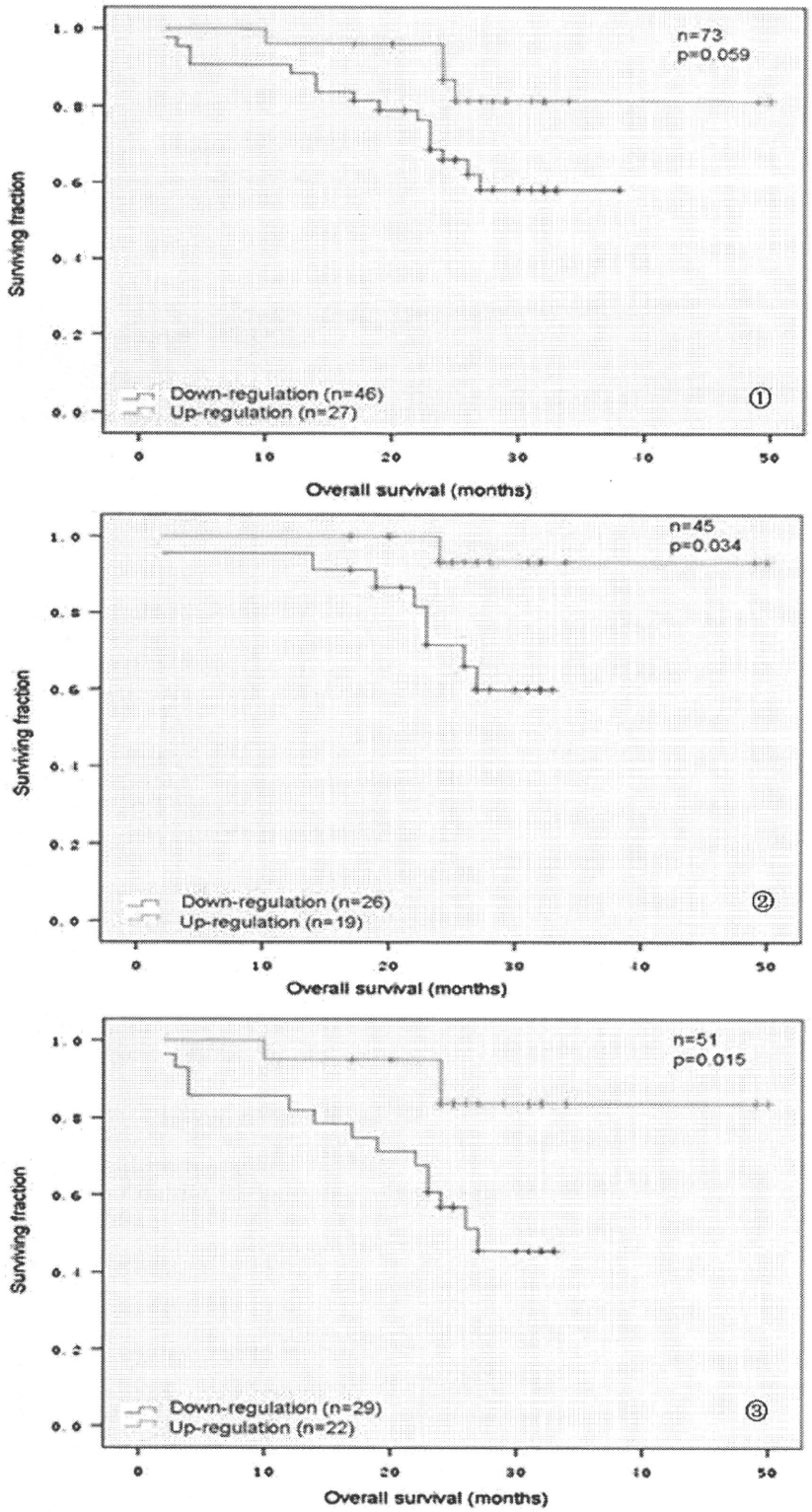

Figure 3 ① Kaplan–Meier plots of overall survival showed patients with *EphA1* downregulation had a shorter survival than those with *EphA1* upregulation ($P = 0.059$). ②③ Kaplan–Meier plots of overall survival showed reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P = 0.034$ and 0.015, respectively).

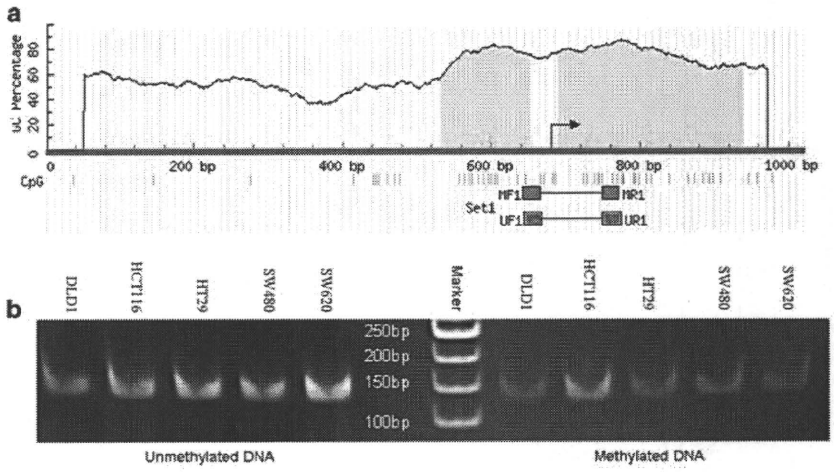

Figure 4 (**a**) Schematic show of the promoter-associated CpG island in *EphA1* and the location of PCR primer sets for specific detection of methylation and unmethylation *EphA1* DNA. The arrow showed the translation start site of *EphA1*. (**b**) Methylated and unmethylated DNAs of *EphA1* were detected in colon cancer cell lines.

of *EphA1* was detected in all tested five colon cancer cell lines (Figure 4b). To explore the methylation status of the promoter-associated CpG island of *EphA1* in tissue DNA of colorectal carcinomas and the association of the methylation with clinicopathologic parameters will be our next project.

The expression of *EphA1* transcript was downregulated in 37% and upregulated in 40% tested samples. No significant relation between the *EphA1* transcript expression and clinicopathologic parameters was found (Table 1). The *EphA1* protein was detected in most of the normal mucosa cells and diffusely expressed in adenoma cells (Figure 2). However, in the colorectal carcinomas, the *EphA1* expression was showed heterogeneity in carcinoma cells both of intra- and inter-samples. Our data suggest that *EphA1* protein was partly lost in the transition from adenomas to adenocarcinomas. Reduced expression of *EphA1* protein occurred more often in male patients ($P=0.028$) and in patients with poor differentiation ($P=0.027$), greater depth of wall invasion ($P=0.003$), lymph node metastasis ($P=0.034$), and advanced tumor stage ($P=0.003$). These data show that *EphA1* may be involved in the progression of colorectal carcinomas. Although *EphA1* has a closer homologous sequence and more similar structure to *EphA2* than any other Eph receptor,[30] our results show that there are great different features between them in terms of protein expression and its relation to clinicopathologic parameters in colorectal carcinomas. *EphA2* expression is present at the cytomembrane of the normal colorectal epithelium. However, *EphA2* immunoreactivity in colorectal carcinoma cells was diffusely distributed throughout the cytoplasm, with little staining of the cytomembrane.[24] In this study, different subcellular localization of EphA1 was found. In normal mucosa gland and adenoma cells, the immunostaining of EphA1 was showed dense brown particles in the Golgi's body. This staining pattern was only showed in parts of well-differentiated carcinoma cells. In poorly differentiated carcinoma and parts of well-differentiated carcinoma cells, the EphA1 was diffusely stained in cytoplasm. The staining pattern of EphA1 in skin ulcers was also altered, in which EphA1 was expressed in keratinocytes adjacent to the rim of the ulcer with an intense cytoplasmic staining, but with a membranous staining in those distant from the rim of the same ulcer.[22] The mechanism for this altered EphA1 immunostaining pattern is unknown. In addition, overexpression of *EphA2* is associated with metastasis and stage of the cancer.[24] However, in this study, reduced expression of *EphA1* occurred more often in patients with advanced tumor stage and lymph node metastasis. Our data suggest that *EphA1* and *EphA2* play different roles in the progression of colorectal carcinomas.

The expression of *EphA1* protein was not completely consistent with the transcript expression in 75 samples, in which only 35% (26 of 75) showed consistency. This pattern was also reported on *EphB4* expression in breast carcinomas,[31] *EphA2* expression in bladder carcinomas,[32] and *EphA7* expression in hepatocellular carcinomas.[18] The post-transcription regulation mechanisms interpreted *EphB4* and *EphA2* differential expression between protein and transcript. Upregulation of *EphA7* mRNA in hepatocellular carcinomas may be attributable to higher vascularization in the investigated tumor, resulting in intercellulalr cross-contaminations. In the present study, *EphA1* staining was not observed in stromal cells, vascular endothelial cells, or lymphocytes; we postulate that post-transcription, post-translation regulation mechanisms, or quick degradation of unstable *EphA1* protein are the reasons of inconsistent expression of *EphA1* mRNA and protein.

Follow-up information was available in 73 patients with a follow-up duration of 2–50 months (median time: 25 months), including 46 cases with reduced *EphA1* expression and 27 cases with *EphA1* upregulation. The overall survival rate in patients with *EphA1* downregulation was shorter than that in patients with *EphA1* upregulation (log-rank test, $P=0.059$). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively). The protective roles of *EphA1* protein in aged colorectal patients and patients with rectal cancers and sigmoid colon cancers are more obvious.

In summary, reduced expression of the *EphA1* protein in colorectal carcinomas is related to invasiveness, differentiation, metastasis, stage, and prognosis. Our data implicate that *EphA1* receptor may play different roles in the different stage of progression of colorectal carcinomas.

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Altered expression of the human base excision repair gene *NTH1* in gastric cancer

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A base excision repair enzyme, NTH1, has activity that is capable of removing oxidized pyrimidines, such as thymine glycol (Tg), from DNA. To clarify whether the *NTH1* gene is involved in gastric carcinogenesis, we first examined the NTH1 expression level in eight gastric cancer cell lines, and the results showed that NTH1 expression was downregulated in all of them, including cell line AGS. Next, a comparison of excisional repair activity against Tg by empty vector-transfected AGS clones and FLAG-NTH1-expressing AGS clones showed that a low NTH1 expression level led to low capacity to repair the damaged base in the gastric epithelial cells. Reduced messenger RNA expression of NTH1 was also detected in 36% (18/50) of primary gastric cancers. Moreover, immunohistochemical analysis revealed that NTH1 was predominantly localized in the cytoplasm in 24% (12/50) of the primary gastric cancers in contrast to the nuclear localization in non-cancerous tissue, suggesting impaired excisional repair ability for nuclear DNA. No associations between clinicopathological factors and NTH1 expression level or localization pattern were detected in the gastric cancers. Next, we found two novel genetic polymorphisms, i.e. c.-163C>G and c.-241_-221del, in the *NTH1* promoter region, and a luciferase assay showed that both were associated with reduced promoter activity. However, there were no associations between the polymorphisms and risk of gastric cancer in a gastric cancer case–control study. These findings suggested that downregulation of NTH1 expression and abnormal localization of NTH1 may be involved in the pathogenesis of a subset of gastric cancers.

Introduction

Reactive oxygen species generated in living cells during normal cellular metabolism and in response to exogenous inducers cause the formation of oxidatively damaged bases in DNA (1–3). These oxidized base lesions are primarily removed by the base excision repair (BER) pathway (4,5). NTH1 protein, also known as NTHL1 protein, is one of the human BER enzymes and it has both DNA glycosylase activity and apurinic/apyrimidinic (AP) lyase activity, both of which function in the initiation of BER (6,7). NTH1 recognizes and removes a wide range of oxidized pyrimidine derivatives, including thymine glycol (Tg), 5-hydroxycytosine, 5-hydroxy-6-hydrothymine, 5,6-dihydroxycytosine, 5-hydrouracil, 5-formyluracil and formamidopyrimidines (8–10).

A variety of factors, including sodium chloride intake, *Helicobacter pylori* infection and smoking, induce inflammation of gastric tissue, and the gastric tissue is exposed to oxidative stress as a result (11–13). Low BER activity is speculated to lead to the accumulation of huge amounts of oxidative DNA damage in gastric epithelium and to be a causative factor of gastric cancer as a result of mutations in cancer-related genes. Moreover, abnormal expression of BER genes in gastric cancer and BER polymorphisms associated with low BER activity and/or risk of gastric cancer have actually been reported (14–18). In addition to these reports, an inherited abnormality of the BER gene *MYH* is well known to be responsible for multiple colorectal adenomas and carcinomas (19). Based on all above, we hypothesized that low DNA repair ability due to an NTH1 abnormality is involved in gastric carcinogenesis. Since there have been no reports of abnormal NTH1 expression in gastric cancer or of *NTH1* polymorphisms associated with low NTH1 promoter activity, low NTH1 activity or gastric cancer risk, in this study, we investigated whether NTH1 expression is abnormally regulated in gastric cancer, then searched for *NTH1* promoter polymorphisms associated with low NTH1 promoter activity and investigated the relationship between the polymorphisms that were identified and gastric cancer risk.

Abbreviations: AP, apurinic/apyrimidinic; 5-aza-dC, 5-aza-deoxycytidine; BER, base excision repair; cDNA, complementary DNA; GAPD, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; PCR, polymerase chain reaction; PBGD, porphobilinogen deaminase; QRT-PCR, quantitative real-time polymerase chain reaction; SSCP, single-strand conformation polymorphism; Tg, thymine glycol.

Materials and methods

Samples

Gastric cancer cell lines MKN28, TMK1, MKN74, KATOIII, AGS, MKN1, MKN45 and HSC39 were obtained from the Human Science Research Resource Bank (Osaka, Japan) or the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum under 5% CO_2 atmosphere at 37°C. Gastric cancer tissue and corresponding normal gastric mucosa tissue from a total of 50 sporadic cases of gastric cancer were obtained from Hamamatsu University Hospital, Shizuoka, Japan and used for analysis of NTH1 expression. The mean age of the patients was 65.1 years (standard deviation: 12.2), and the sample included 40 men and 10 women. The tumors were classified histologically as the intestinal type in 22 cases and the diffuse type in 28 cases. Normal gastric mucosa tissue from 80 gastric cancer patients was obtained from Hamamatsu University Hospital and used to search for genetic polymorphisms in the *NTH1* gene promoter region. This study was approved by the Institutional Review Board of Hamamatsu University School of Medicine.

Quantitative real-time polymerase chain reaction

Expression of the NTH1 messenger RNA (mRNA) transcript was measured by quantitative real-time polymerase chain reaction (QRT-PCR) with a LightCycler instrument (Roche, Palo Alto, CA). Total RNA was extracted with an RNeasy Plus Mini Kit (QIAGEN, Valencia, CA), and 2–5 μg of total RNA was converted to complementary DNA (cDNA) with a SuperScript First-Strand System for reverse transcription–PCR (Invitrogen, Carlsbad, CA). PCR amplification of the NTH1 transcript and the transcript of the control housekeeping gene porphobilinogen deaminase (*PBGD*) or glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) was performed with the cDNA and a QuantiTect SYBR Green PCR kit (QIAGEN). The following PCR primers were used: 5′-GATGGCACACCTGGCTATG-3′ and 5′-CCACAGCTCCCTAGGCAG-3′ for the NTH1 transcript; 5′-GTCTGGTAACGGCAATGCGG-3′ and 5′-TCCCCTGTGGTGGACATAGC-3′ for the PBGD transcript and 5′-GCTCAGACACCATGGGGAAG-3′ and 5′-TGTAGTTGAGGTCAATGAAGGGG-3′ for the GAPD transcript. The relative amounts of NTH1 transcript were normalized to those of the PBGD or GAPD transcript. T/N ratios were calculated by dividing the normalized transcript amounts in the cancerous tissue by the amounts in the non-cancerous tissue.

Western blot analysis

Cells were harvested in lysis buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM ethylenediaminetetraacetic acid, 100 mM sodium fluoride, 1 mM sodium orthovanadate and

protease inhibitor cocktail (Sigma, St Louis, MO), and the whole-cell extracts were mixed with an equal volume of 2× sodium dodecyl sulfate sample buffer and boiled. An 8 or a 40 μg sample of extracts was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was electrophoretically transferred to a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The membrane was blocked with non-fat milk and incubated with an anti-NTH1 monoclonal antibody (R&D system, Minneapolis, MN), an anti-FLAG M2 monoclonal antibody (Sigma) or an anti-β-tubulin monoclonal antibody (Sigma). After washing with Tris-buffered saline containing 0.05% Tween-20, the membrane was incubated with an anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare Bio-Sciences Corp.). The membrane was then washed with Tris-buffered saline containing 0.05% Tween-20, and immunoreactivity was visualized with an electrochemiluminescence system (GE Healthcare Bio-Sciences Corp.).

5-Aza-deoxycytidine and trichostatin A treatment

A stock solution of the DNA methyltransferase inhibitor 5-aza-deoxycytidine (5-aza-dC; Sigma) and a stock solution of the histone deacetylase inhibitor trichostatin A (WAKO, Tokyo, Japan) were prepared in acetic acid and methanol, respectively. Cells were treated with 2 μM 5-aza-dC for 48 h or 330 nM trichostatin A for 24 h. The medium containing 5-aza-dC was changed every 24 h.

Plasmid construction

NTH1 expression vectors containing and not containing a FLAG were constructed by inserting the NTH1 cDNA with the FLAG sequence at the N-terminus and the NTH1 cDNA alone, respectively, into the BamHI site of a pcDNA3.1(-) plasmid vector (Invitrogen). The NTH1 cDNA fragment was amplified by using cDNA derived from non-cancerous tissue and the following primer set: 5′-CCGGAATTCCACCATGTGTAGTCCGCAGGAGTC-3′ and 5′-CGCGGATCCTCAGAGACCCTGGGCGGCCGGGCA-3′. The FLAG-NTH1 cDNA fragment was amplified by using the NTH1 expression vector and the following primer set: 5′-CGCGGATCCACCATGGACTACAAGGACGACGATGACAAGTGTAGTCCGCAGGAGTC-3′ and 5′-CGCGGATCCTCAGAGACCCTGGGCGGCCGGGCA-3′.

Various sized DNA fragments (1.2, 1.0, 0.8, 0.6, 0.4 and 0.1 kb) of the upstream region of the *NTH1* gene were amplified by PCR of genomic DNA derived from a blood sample collected from a healthy individual. The sense primers used for each region were: 5′-GGAGATCTTCCACCTGGACATATCCTTACCTG-3′ for the 1.2 kb fragment; 5′-GAAGATCTTCAACCCGTCTCCCACAGAAC-3′ for the 1.0 kb fragment; 5′-GAAGATCTTCCTTCAAGCCTGAATCTTTGC-3′ for the 0.8 kb fragment; 5′-GAAGATCTTCCAGGCACCTAGGAAAGCCC-3′ for the 0.6 kb fragment; 5′-GAAGATCTTCCTGCAGCAGAGACCCGTTC-3′ for the 0.4 kb fragment and 5′-GAAGATCTTCCGCCTTGGGAGCTTGCTG-3′ for the 0.1 kb fragment. All of these sense primers were used in combination with the same antisense primer: 5′-CGAAGATCTTCCCGGCGGCCCATGC-3′. Each fragment was then cloned into the BglII site of the pGL4.10[*luc2*] vector (Promega, Madison, WI).

A luciferase reporter plasmid for the -163G polymorphism type was generated by site-directed mutagenesis with a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), pGL4.10 plasmid vector containing 0.4 kb fragment of *NTH1* promoter as a template, and the following primer set: 5′-GAAAGGCGCACCCCGCTGGCCCCGGGA-3′ and 5′-TCCCGGGGCCAGCGGGGTGCGCCTTTC-3′. A luciferase reporter plasmid for the -241_-221del type was constructed by inserting the DNA fragment amplified from the genomic DNA derived from the non-cancerous gastric mucosa of an individual with the deletion type into the BglII site of the pGL4.10 vector. The PCR was carried out by using the primer set for the 0.4 kb fragment of the *NTH1* promoter.

All of the plasmid vectors were confirmed by DNA sequencing.

Establishment of stable transfectants

AGS cells were transfected with a pcDNA3.1 parental plasmid vector or a pcDNA3.1-NTH1 plasmid vector by using a Lipofectamine 2000 reagent (Invitrogen). G418-resistant clones were isolated by culturing in a medium containing 400 μg/ml of G418 (Gibco BRL, Grand Island, NY).

DNA cleavage activity assay

Cells were harvested and incubated in lysis buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM ethylenediaminetetraacetic acid and protease inhibitor cocktail (Sigma). After centrifugation at 4°C for 45 min at 85 000g, the supernatant was recovered and used for DNA cleavage activity assay. Next, 30mer oligonucleotides containing and not containing a single 5R,6S-Tg (5′-CTGGTGGCCTGAC[Tg or T]CATTCCCCAACTAGTG-3′) were chemically synthesized and purified by polyacrylamide gel electrophoresis (Japan Bio Services, Saitama, Japan). The oligonucleotides were ^{32}P-labeled at the 5′ terminus with a MEGALABEL kit (Takara, Osaka, Japan) and a [γ-^{32}P]adenosine triphosphate (PerkinElmer, Tokyo, Japan) and annealed to a complementary strand containing an adenine opposite the Tg or T. The reaction was performed at 37°C for 30 min with 20 μl of a mixture containing 3 μg of cell extracts, 20 mM sodium phosphate (pH 7.5), 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol and 2.5 nM labeled oligonucleotide. After the reaction, the mixture was treated with 0.1 M NaOH at 95°C for 4 min. Denaturing formamide dye was added to the mixture, and after heating at 95°C for 3 min, the mixture was separated by 20% polyacrylamide gel electrophoresis. A marker oligonucleotide was also ^{32}P-labeled at the 5′ terminus and used as a size marker for the cleaved products. The radioactivity of intact and cleaved oligonucleotides was quantified by using a FLA-3000 fluoroimage analyzer (Fuji Film, Tokyo, Japan) and ImageGauge software (Fuji Film).

Immunohistochemical analysis

Paraffin-embedded tissue sections were deparaffinized, rehydrated and boiled at 95°C for 30 min in citric acid buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation for 5 min in a 3% hydrogen peroxide solution. Next, the slides were incubated with an anti-NTH1 polyclonal antibody (Novus Biologicals, Littleton, CO) at a dilution of 1:800 for 30 min at room temperature and then with dextran polymer conjugated with goat anti-rabbit immunoglobulin G and horseradish peroxidase (ChemMate Envision Kit, DAKO, Kyoto, Japan) for 30 min at room temperature. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. The analysis was performed with a DAKO autostainer (DAKO) (20).

Luciferase reporter assay

AGS cells were cotransfected with the *firefly* luciferase reporter vector pGL4.10 containing a fragment of the *NTH1* promoter and the *Renilla* luciferase reporter vector pGL4.74 [*hRluc*/TK] (Promega) by using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested 24 h after transfection. Luciferase activity was quantified by a Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated according to the manufacturer's instructions.

PCR–single-strand conformation polymorphism analysis and DNA sequencing

DNA was extracted with a DNeasy Tissue Kit (QIAGEN). The *NTH1* promoter sequence was divided into two regions (region 1 and 2), and each region was amplified by PCR with HotStarTaq DNA polymerase (QIAGEN). The following primers were used: 5′-GAGAACTCAACTACAGCCCAGC-3′ and 5′-GAGCTCCGAGCATCCCTTAG-3′ for region 1 and 5′-GCAGAGACCCGTTCGCAC-3′ and 5′-CGGACTCCTGCGGACTAC-3′ for region 2. The PCR products of region 2 were digested with AvaI before PCR–single-strand conformation polymorphism (SSCP) to adjust its size to <200 bp. The PCR products were diluted with two volumes of loading solution and applied to 8% polyacrylamide gels containing and not containing 5% glycerol. The products were electrophoresed at room temperature and 4°C and detected by silver staining. PCR products exhibiting the abnormally shifted band in the PCR–SSCP analysis were directly sequenced in both directions with a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and an ABI 3100 Genetic Analyzer (Applied Biosystems) (21).

Genotyping

The region containing the c.-163C>G and c.-241_-221del polymorphisms was amplified by PCR with a HotStarTaq DNA polymerase (QIAGEN), and the primer set used to amplify the promoter region 2 in PCR–SSCP analysis was used in this analysis. Half of the PCR product was used for genotyping of the c.-241_-221del polymorphism by simple agarose gel elecrtrophoresis, and the other half was used for genotyping of the c.-163C>G polymorphism by PCR–restriction fragment length polymorphism analysis with restriction enzyme MvaI.

Case–control study

A multicenter, hospital-based case–control study of gastric cancer was conducted at four hospitals in Nagano Prefecture, Japan, from October 1998 to March 2002. Newly diagnosed eligible patients ranging in age from 20 to 74 years were identified during the survey at the hospitals. Two controls were matched to each case by gender, age (±3 years) and area of residence. The controls were selected from persons who came to the same four hospitals for routine health examinations during the same period and were confirmed not to have cancer. As a result, we collected a total of 153 cases and 303 controls (22), and we analyzed the data for the 148 cases and 292 controls that met the criteria for inclusion in this study. The genotyping method used for the *NTH1* promoter polymorphisms is described in the section above. This study was approved by the Institutional Review Boards of National Cancer Center.

Statistical analysis

Odds ratios, 95% confidence intervals and *P* values for trends in the case–control study were obtained by conditional logistic regression analysis to assess the association between the genotype of each polymorphism and risk of gastric cancer. Both crude and adjusted odds ratios were calculated. Odds ratios for gastric cancer were adjusted for family history, *H.pylori* infection, smoking status, alcohol intake, vegetable consumption, salt intake, body mass index and Japan Agriculture membership. The statistical analyses to evaluate associations between genotype and risk of gastric cancer were performed with the SAS program (SAS Institute, Cary, NC).

Results

Downregulation of NTH1 expression in gastric cancer cell lines

To determine the status of NTH1 expression in the gastric cancer, we first preformed a QRT-PCR analysis for NTH1 mRNA transcripts in eight gastric cancer cell lines: MKN28, TMK1, MKN74, KATOIII, AGS, MKN1, MKN45 and HSC39. The mRNA expression levels were significantly lower in all (8/8, 100%) of the cell lines than in the non-cancerous gastric mucose of unrelated individuals (Figure 1A). Next, we measured the level of NTH1 protein expression in the cell lines by western blot analysis, and the results showed that their expression in all of the cell lines was below the level of detection by the method used (Figure 1B), indicating that NTH1 expression was downregulated in the gastric cancer cell lines.

To explore the mechanism of the downregulation of NTH1 expression, we treated six gastric cancer cell lines (AGS, TMK1, MKN1, MKN28, MKN45 and MKN74) with the cytosine methylation inhibitor 5-aza-dC or the histone deacetylase inhibitor trichostatin A and measured the level of NTH1 expression by QRT-PCR. However, NTH1 expression was not restored in any of the cell lines by treatment with either inhibitor (Figure 1C), suggesting that the epigenetic regulatory mechanism demonstrated by treatments with them (24) is not involved in the downregulation of NTH1 expression in gastric cancer cell lines.

Low level of Tg DNA glycosylase activity in AGS cells

To determine whether downregulation of NTH1 expression leads to a low capacity for repair of oxidatively damaged DNA in gastric cancer cells, we tried comparing the Tg DNA glycosylase activity of AGS cells stably expressing FLAG-NTH1 and of mock control cells. First, we established AGS-derived clones that overexpressed and did not overexpress NTH1 by means of transfection and subsequent G418-selection, and western blot analysis confirmed a marked difference in level of NTH1 protein expression between the empty vector-transfected clones and FLAG-NTH1-expressing clones (Figure 2A). The Tg DNA glycosylase activity of the cells was then tested by determining its capacity to cleave a double-stranded oligonucleotide containing a Tg:A base pair. The cleaved products were analyzed on a denaturing polyacrylamide gel, and the mobility of the products was compared with that of a marker oligonucleotide. No clear cleavage products were detected when oligonucleotide containing an unmodified T:A base pair was reacted with the cellular protein, but cleaved products with the same mobility as the marker oligonucleotide were detected when reacted with oligonucleotide containing a Tg:A base pair. More importantly, the activity of empty vector-transfected clones was significantly lower than in the FLAG-NTH1-expressing clones (Figure 2B and C), suggesting that downregulation of NTH1 expression leads to low capacity to repair oxidatively damaged DNA in gastric cancer cells.

Downregulation of NTH1 expression in primary gastric cancers

Next, we investigated whether NTH1 expression is also downregulated in primary gastric cancer. NTH1 mRNA expression in 50 primary gastric cancers and corresponding non-cancerous tissues was measured by QRT-PCR, and the ratio of the level of NTH1 mRNA expression in the tumor to the level in the corresponding non-cancerous tissue (T/N ratio) was calculated in each case. Reduced NTH1 expression (T/N ratio <0.5) was observed in 18 of the 50 (36%) primary

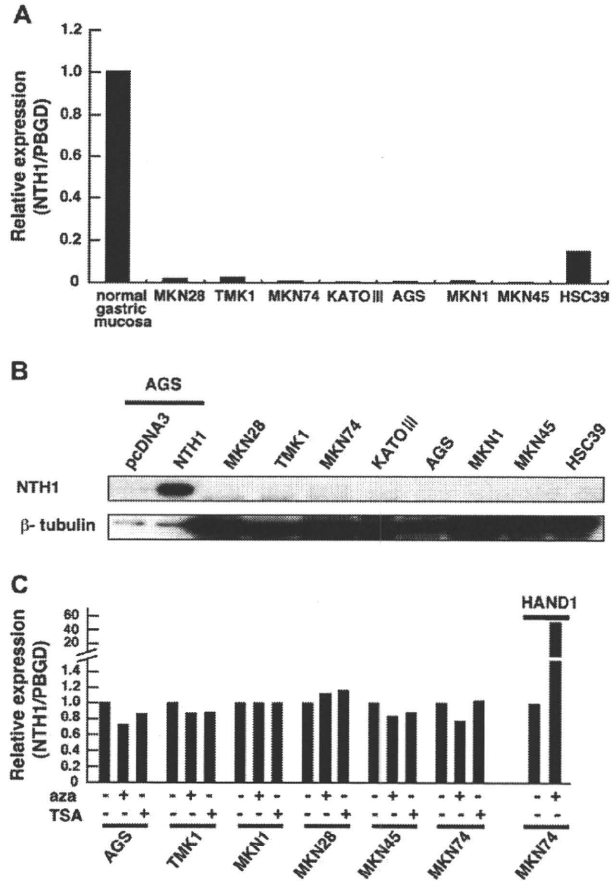

Fig. 1. Downregulation of NTH1 expression in gastric cancer cell lines. (**A**) Measurement of the level of expression of NTH1 transcripts in gastric cancer cell lines by QRT-PCR with a LightCycler instrument. The amounts of NTH1 transcripts normalized to the amount of transcripts of a housekeeping gene, *PBGD*, in eight gastric cancer cell lines are shown in the graph. The average expression level of four normal gastric mucosa samples was measured as a control and set equal to 1.0. (**B**) Measurement of the expression level of NTH1 protein in gastric cancer cell lines by western blot analysis. The levels of NTH1 protein expression in eight gastric cancer cell lines were measured by western blot analysis with anti-NTH1 monoclonal antibody. AGS cells transiently transfected with NTH1 expression vector or empty pcDNA3.1 vector were also used, and 8 μg (corresponding to one-fifth the weight applied in other lanes) of the total cell extract of these AGS cells was applied for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Expression of β-tubulin protein was analyzed as an internal control. (**C**) Examination of the effect of 5-aza-dC and trichostatin A (TSA) on the NTH1 mRNA expression level in six gastric cancer cell lines showing a low level of NTH1 expression. Cells were treated or not treated with 2 μM 5-aza-dC for 48 h or 330 nM TSA for 24 h, and NTH1 mRNA expression was measured by QRT-PCR. The amount of NTH1 transcripts normalized to the amount of PBGD transcripts is shown in the graph. The expression level in the cells not treated with 5-aza-dC or TSA was set equal to 1.0 in each cell line. Expression of HAND1 was also measured as a positive control with reference to a previous paper (23).

gastric cancers (Figure 3A), suggesting that NTH1 mRNA expression is downregulated in a subset of primary gastric cancers.

Abnormal cellular localization of NTH1 protein in primary gastric cancers

Since abnormal cellular localization of NTH1 protein has recently been reported in colorectal cancer (25), we examined the subcellular localization of NTH1 protein in 50 primary gastric cancers by immunohistochemical analysis with the same anti-NTH1 antibody as used

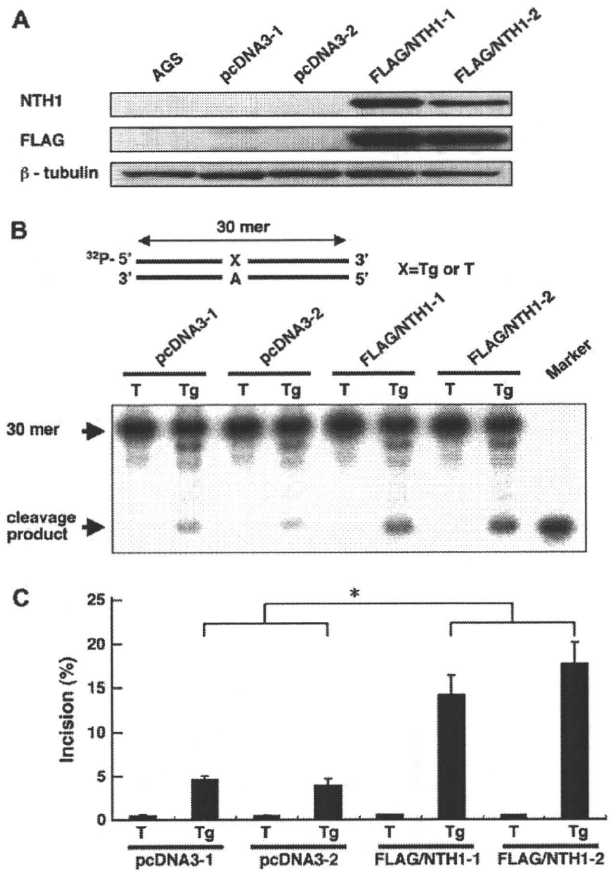

Fig. 2. Low level of Tg DNA glycosylase activity in AGS cells. (**A**) Western blotting of FLAG-NTH1 protein in parental AGS cells, empty vector-transfected AGS clones and FLAG-NTH1 stably expressing AGS clones with anti-NTH1 or anti-FLAG antibodies. Expression of β-tubulin protein was also analyzed as an internal control. (**B** and **C**) Measurement of Tg DNA glycosylase activity in AGS cells. Cleavage activity against double-strand DNA containing base pair Tg:A was measured in empty vector-transfected AGS clones and in stably FLAG-NTH1-expressing AGS clones. Cell extracts and a ^{32}P-labeled double-stranded oligonucleotide containing or not containing a single 5R,6S-Tg:A mispair were incubated and subjected to 20% polyacrylamide gel electrophoresis. A ^{32}P-labeled marker oligonucleotide was used as a size marker for the cleaved products. Representative results are shown in the panel in (B). The intact 30mer oligonucleotides and cleavage products are indicated by the arrows. The amount of cleaved products as percentages of the total were calculated and shown in a bar graph of (C). Values are means ± SDs of three independent experiments. *P* values were calculated by the two-tailed Student's *t*-test, $^{*}P < 0.01$. Activity in the empty vector-transfected clones was significantly lower than in the FLAG-NTH1-expressing clones.

in the colorectal cancer study. The results showed that NTH1 was predominantly localized in the cell nucleus in non-cancerous gastric tissues derived from unrelated 13 individuals (Figure 3B, left panel), consistent with the previous results (10,26), whereas there were two patterns of NTH1 immunostaining in gastric cancer cells. In one pattern, NTH1 was predominantly localized in the nucleus (Figure 3B, middle panel), whereas in the other pattern, it was predominantly localized in the cytoplasm (Figure 3B, right panel), and 12 of the 50 (24%) primary gastric cancers exhibited the cytoplasmic localization pattern. Since NTH1 has a role in repairing oxidatively damaged bases in nuclear DNA, excisional repair by NTH1 protein may be impaired in the nucleus of cells having the cytoplasmic localization pattern of NTH1.

Examination of the clinicopathological characteristics of gastric cancers with altered NTH1 expression

We searched for clinicopathological features of the cases of gastric cancer with downregulation of NTH1 expression or abnormal localization of NTH1, but no associations were found between any of the clinicopathological factors (sex, onset age, tumor stage, tumor histology and p53 immunohistochemistry) and the NTH1 expression levels or cellular localization patterns of NTH1 (supplementary Table SI is available at *Carcinogenesis* online). A combination of the results of QRT-PCR and immunohistochemical analyses demonstrated that a total of 54% of the primary gastric cancers exhibited downregulation of NTH1 expression and/or a cytoplasmic localization pattern of NTH1, but no associations were detected between the clinicopathological factors and altered NTH1 expression either (supplementary Table SI is available at *Carcinogenesis* online).

Novel NTH1 promoter polymorphisms associated with reduced promoter activity

To study the characteristics of NTH1 expression in greater detail, we investigated the promoter activity in the *NTH1* upstream region. Luciferase reporter plasmids containing various sized DNA fragments (1.2, 1.0, 0.8, 0.6, 0.4 and 0.1 kb) of the upstream region of the *NTH1* gene were prepared, and a luciferase reporter assay was performed in AGS cells. The results showed that a luciferase reporter construct with the 0.4 kb-length fragment yielded the greatest enhancement of promoter activity (Figure 4A). We then searched for genetic polymorphisms of the region containing *NTH1* promoter activity by PCR–SSCP analysis using non-cancerous tissue derived from 80 gastric cancer patients. Two novel polymorphisms, c.-163C>G and c.-241_-221del, were found in this region and had an allele frequency of 0.6 and 1.9%, respectively (Figure 4B). We also found the c.-241_-221del polymorphism but not c.-163C>G, in the cases of gastric cancer (Figure 3A). We then compared the promoter activity of the 0.4 kb-length *NTH1* promoter regions containing these polymorphisms or the wild-type by a luciferase reporter assay in AGS cells. Promoter activity of the sequence containing the c.-163C>G or c.-241_-221del polymorphisms was significantly lower than that of the wild-type sequence ($P < 0.05$) (Figure 4C), suggesting that these two novel *NTH1* promoter polymorphisms are associated with reduced promoter activity.

Evaluation of the relation between NTH1 promoter polymorphisms and gastric cancer risk in a case–control study

Since the c.-163C>G and c.-241_-221del polymorphisms in the *NTH1* promoter region are found in gastric cancer patients and are associated with reduced promoter activity, we tested for a presumed association between the *NTH1* promoter polymorphisms and gastric cancer risk in a gastric cancer case–control study. Genotyping of the two polymorphisms was successfully performed in 148 gastric cancer cases and 292 age- and gender-matched healthy controls. Their minor allele frequency was relatively low in both groups (c.-163C>G, 2.4% in the cases and 2.6% in the controls; c.-241_-221del, 1.4% in the cases and 2.4% in the controls). One individual who was homozygous for the minor allele was found, but no individuals had both types of minor allele. No clear association between the genetic polymorphisms and gastric cancer risk was found in this study (Table I).

Discussion

In this study, downregulation of expression of the BER gene *NTH1* based on both the mRNA and protein levels was shown in eight gastric cancer cell lines. Comparison of DNA glycosylase activity toward the oxidatively damaged base Tg in empty vector-transfected clones and FLAG-NTH1-expressing clones of the AGS gastric cancer cell line revealed that a low NTH1 expression level led to low capacity to repair the damaged base in gastric cancer cells. Reduced expression of NTH1 transcripts was also detected in 36% (18/50) of the primary gastric cancers. Immunohistochemical analysis revealed that NTH1 was predominantly localized in the nucleus in non-cancerous tissue, which

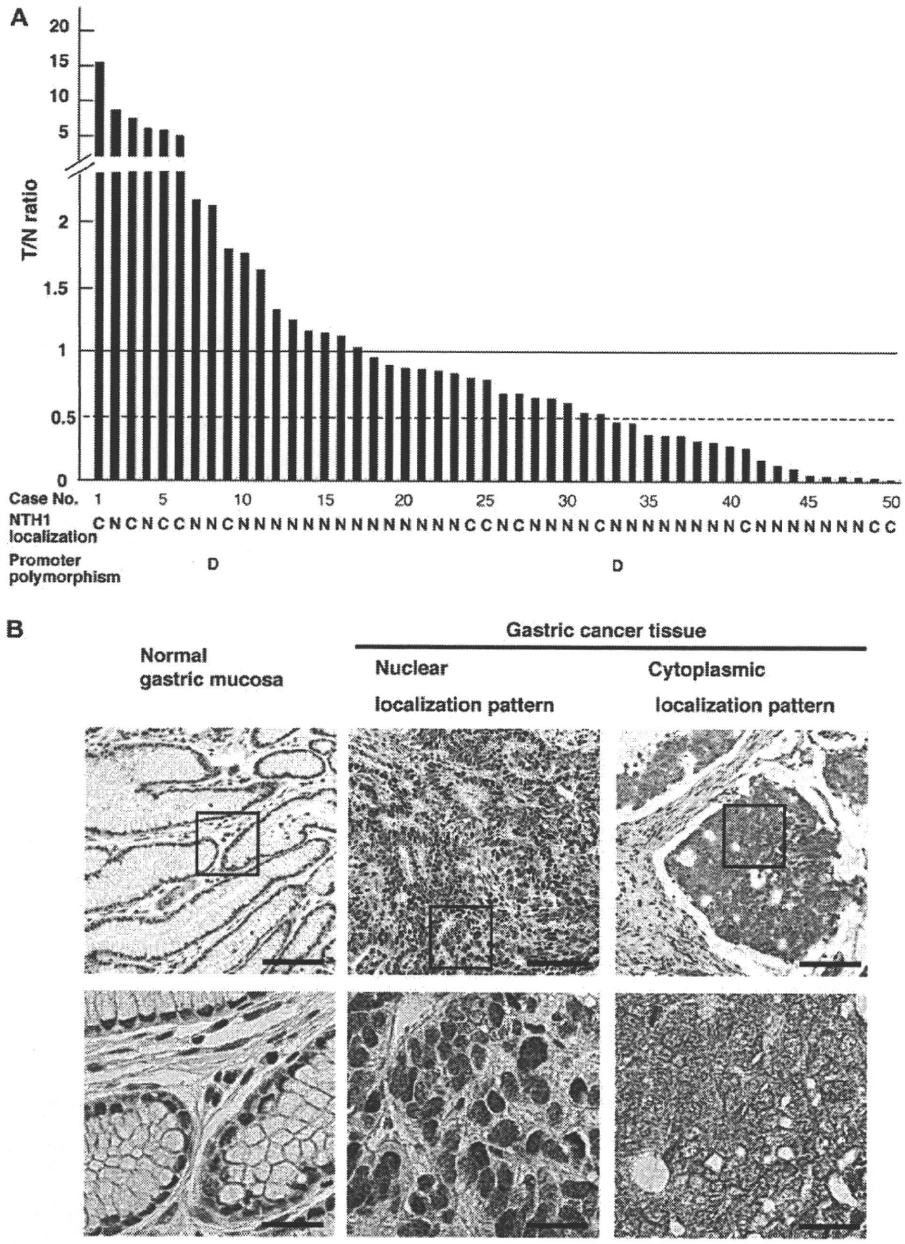

Fig. 3. Low level of NTH1 expression and abnormal cellular localization of NTH1 in primary gastric cancers. (**A**) Comparison of NTH1 mRNA levels in the cancerous tissue of 50 primary gastric cancers and corresponding non-cancerous gastric tissue by QRT-PCR analysis. After the relative amounts of NTH1 transcripts were normalized to those of the GAPD transcript, T/N values were calculated by dividing the normalized transcript amounts in gastric cancer tissue by the amounts in the corresponding non-cancerous tissue. The 'N' and 'C' under each case number indicate the nuclear localization pattern of NTH1 protein and the cytoplasmic localization pattern of NTH1 protein, respectively, which were identified by the immunohistochemical analysis in (B). The results of genotyping NTH1 promoter polymorphisms are also shown under the NTH1 localization pattern row. The 'D' indicates the heterozygote for the c.-241_-221del polymorphism and wild-type. The c.-163C>G polymorphism was not found in any of the cases. Cases no. 18, 25, 28, 35, 42, 45 and 49 were not genotyped for these polymorphisms because of the lack of a genomic DNA sample. (**B**) Determination of the NTH1 localization pattern in primary gastric cancer tissues by immunohistochemical analysis with anti-NTH1 polyclonal antibody. Nuclear localization of NTH1 protein in normal gastric mucosa is shown in the left panels. Gastric cancers showing NTH1 expression predominantly in the nucleus and in the cytoplasm are shown in the middle panels and in the right panels, respectively. The lower panels are a higher magnification of the area enclosed by the square in upper panels. The scale bars in the upper panels and lower panels represent 100 μm and 25 μm, respectively. The immunohistochemical findings in the 50 gastric cancer cases are denoted at the row of NTH1 localization in (A).

would be suitable for its ability to repair nuclear DNA. In 24% (12/50) of the primary gastric cancers, however, NTH1 was predominantly localized in the cytoplasm, suggesting impaired ability for excisional repair of nuclear DNA. A combination of the results of QRT-PCR and immunohistochemical analyses demonstrated that a total of 54% of the primary gastric cancers exhibited downregulation of NTH1 expression and/or a cytoplasmic localization pattern of NTH1; however, no associations were detected between the clinicopathological factors and the altered NTH1 expression in the gastric cancers. We then evaluated the promoter activity of the upstream region of the *NTH1* gene and found

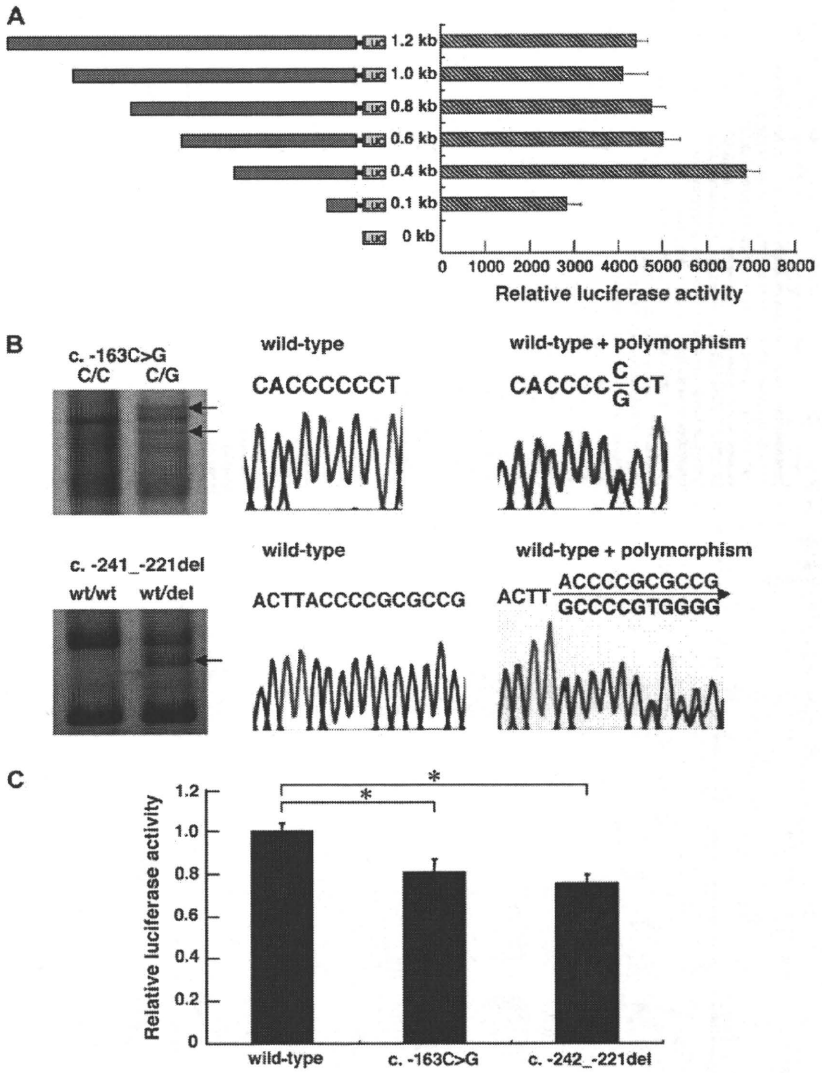

Fig. 4. Measurement of *NTH1* promoter activity and identification of *NTH1* promoter polymorphisms associated with reductions in promoter activity. (**A**) Measurement of luciferase reporter activity in the upstream region of the *NTH1* gene. Luciferase reporter activity was measured 24 h after transfection with reporter plasmids, and the *firefly* luciferase activity was normalized to the *Renilla* luciferase activity. The luciferase activity values are means ± SDs of three independent experiments. The luciferase activity of the cells transfected with each luciferase reporter plasmid is shown relative to the activity when transfected with the empty vector, which has been set equal to 1. (**B**) Identification of the c.-163C>G and c.-241_-221del polymorphisms in the *NTH1* promoter region. The left panels show the results of the PCR–SSCP analysis. Arrows point to abnormally shifted bands obtained by PCR–SSCP analysis. The middle panels and right panels show the results of direct sequencing analysis of the PCR product covering the polymorphic sites. The middle panels show the wild-type allele, and the right panels show both the wild-type and polymorphism alleles. (**C**) Reduction of promoter activity in the *NTH1* promoter sequence containing the c.-163C>G or c.-241_-221del polymorphism revealed by the luciferase reporter assay. Luciferase reporter activity was measured 24 h after transfection with the reporter plasmids, and the *firefly* luciferase activity was normalized to the *Renilla* luciferase activity. The luciferase activity values are means ± SDs of three independent experiments. The luciferase activity of the cells transfected with reporter plasmid containing each polymorphism is shown relative to that of the cells transfected with wild-type reporter plasmid, which has been set equal to 1. *P* values were calculated by the two-tailed Student's *t*-test, $^{*}P < 0.05$.

two novel promoter polymorphisms, c.-163C>G and c.-241_-221del, that were associated with reduced promoter activity. These results suggested that low NTH1 activity arising from downregulation of NTH1 expression and/or abnormal localization of NTH1 may be involved in the pathogenesis of a subset of gastric cancers.

In this study, low ability of the gastric cancer cells to repair oxidatively damaged DNA was demonstrated by the Tg DNA cleavage assay. Tg is one of the main oxidative stress markers (27,28) and one of the substrates of the human NTH1 protein (6–8). However, since the Tg:A mispair is also repaired by another BER enzyme, NEIL1 (15,29), at first we did not know whether the Tg DNA cleavage assay would be suitable for our purpose. Because the results of our study showed that the level of Tg DNA cleavage activity was related to the level of NTH1 protein expression in gastric cancer cells, the Tg DNA cleavage assay was concluded to be an appropriate means of evaluating the effect of low NTH1 expression level on cellular repair activity. The results also suggested that NTH1 is the main BER enzyme for Tg in human gastric cells. The subtle Tg repair ability in empty vector-transfected clones (Figure 2B and C) appears to be attributable to NEIL1 protein or a slight amount of NTH1 protein expressed in the clones. In any event, the results of our study clearly showed that downregulation of NTH1 expression leads to the low cellular repair capacity for Tg in gastric cancer cells. Since NTH1 recognizes and excises many different kinds of oxidatively damaged

Table I. Distribution of the c.-163C>G and c.-241_-221del genetic polymorphisms in the *NTH1* promoter region in Japanese gastric cancer cases and controls

Nucleotide change[a]	Genotype	Case, *n* (%)	Control, *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[b]
c.-163C>G	C/C	141 (95.3)	278 (95.2)	1.00 (reference)	1.00 (reference)
	C/G	7 (4.7)	13 (4.5)	0.55 (0.18–1.70)	0.41 (0.07–2.45)
	G/G	0 (0.0)	1 (0.3)		
c.-241_-221del	wt/wt	144 (97.3)	278 (95.2)	1.00 (reference)	1.00 (reference)
	wt/del	4 (2.7)	14 (4.8)	1.06 (0.41–2.73)	1.52 (0.42–5.57)
	del/del	0 (0.0)	0 (0.0)		

CI, confidence interval; OR, odds ratio.
[a]The 5′ nucleotide of the ATG translation initiation codon is -1.
[b]Adjusted for family history, *H.pylori* infection, smoking status, alcohol intake, vegetable consumption, salt intake, body mass index and Japan Agriculture membership.

bases, it is probably that some of the damaged bases, including mutagenic bases (30–33), are also inefficiently repaired in gastric cancers with a low NTH1 expression level. Such low repair ability of gastric cells may be responsible for gastric carcinogenesis, possibly via induction of mutations in cancer-related genes.

Radak *et al.* (34) used a double-stranded oligonucleotide containing an AP site as a substrate in glycosylase activity assays and found lower NTH1 glycosylase activity in lung cancer than in non-cancerous tissue; however, several other DNA glycosylases besides NTH1 are involved in the repair of AP sites (4,5), and it has not been determined whether NTH1 is a major glycosylase in relation to the AP site. Thus, based on our study, the Tg DNA cleavage assay may be more accurate for making comparisons between the NTH1 activity of cancerous tissue and non-cancerous tissue.

Associations between promoter polymorphisms and cancer risk have been reported for several DNA repair genes, including *XRCC1*, *MLH1* and *MSH2* (35–37). In this study, two novel *NTH1* promoter polymorphisms, c.-163C>G and c.-241_-221del, were identified by screening samples derived from 80 gastric cancer patients and were shown to be associated with a mild reduction of *NTH1* promoter activity. Reduced repair capacity based on reduced *NTH1* promoter activity in the cells with these polymorphisms may lead to an increase in their mutation rate, and the effect would be substantial in organs exposed to severe oxidative stress, such as the stomach. Since the prevalence of these promoter polymorphisms was low, a comparative analysis of the *NTH1* polymorphisms in a larger number of healthy individuals and patients with a variety of cancers is warranted to determine the involvement of the *NTH1* gene in human carcinogenesis.

The immunohistochemical analysis in this study showed that 24% of the gastric cancer tissues examined exhibited predominant NTH1 expression in the cytoplasm. In a previous study, cytoplasmic localization of NTH1 was detected in 35% of primary colorectal cancers (25). These results suggested that NTH1 is abnormally localized predominantly in the cytoplasm in subsets of certain types of human cancers. Interestingly, cytoplasmic localization of another BER enzyme, apurinic/apyrimidinic endonuclease 1 (APE1) protein, has also been reported in cancer (38). It is unclear why NTH1 is predominantly accumulated in the cytoplasm of cancer cells. Although cytoplasmic NTH1 may have played some role in cells, low NTH1 expression in the nucleus would be expected to result in insufficient repair of oxidatively damaged bases in nuclear DNA, especially in organs exposed to severe oxidative stress.

Supplementary material

Supplementary Table SI can be found at http://carcin.oxfordjournals.org/

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