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 enzyme 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. This study was approved by the Institutional Review Board of Hamamatsu University School of Medicine.

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 (BER) pathway (4,5). NTH1 protein, also known as NTHL1 protein, has both DNA glycosylase and 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-hydroxy-2-deoxythymine, 5,6-dihydroxy-2-deoxyuridine, 5-hydouracil, 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.

Materials and methods

Samples

Gastric cancer cell lines MKN28, T2M1, 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% CO2 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 diffus type in 28 cases. Normal gastric mucosa tissue from 80 gastric cancer patients 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'-GATGGCCACCATGGCTAGT-3' and 5'-CCACAGTCTCCC-TAGGGAG-3' for the NTH1 transcript; 5'-GTTCTGTAACGGCCATACGG-3' and 5'-TCCCCGTGTGGGACATACGG-3' for the PBGD transcript and 5'-GCTTCAGACCATGGGGGAG-3' and 5'-TGTAGTTGGACATG-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 (pH 7.5), 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

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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 5% skim 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 hors eradish 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′-GCCGATTTCACGTGTAGTGCCTGCAAGGTCT-3′ and 5′-GCCGATCTCCAGACCTGGGCGCGCGCA-3′. The FLAG-NTH1 cDNA fragment was amplified by using the NTH1 expression vector and the following primer set: 5′-GCCGATTCACATGAGAACTCGAGCACGAG-3′ and 5′-GCCGATCTCCAGACCTGGGCGCGCGCA-3′. Various sized DNA fragments (1.2, 1.0, 0.8, 0.6, and 0.4 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′-CGCTTTCACTTGGCAA-TATCTCTACTCG3′ for the 1.2 kb fragment; 5′-GAAGATCTCCACCTACCACC-TCTCCACAGAAC-3′ for the 1.0 kb fragment; 5′-GAAGATCTCCATCAAGCCTATCGGAT-3′ for the 0.8 kb fragment; 5′-GAAGATCTCCACCGACCTGAGAA-3′ for the 0.6 kb fragment; 5′-GAAGA-TCTCGTGCGACAGGAGGCTCTG-3′ for the 0.4 kb fragment and 5′-GAAGATCTCTCGCGTGGGAGCTCTG-3′ for the 0.1 kb fragment. All of these sense primers were used in combination with the same antisense primer: 5′-CGCAAGATCTCTTCGCGCCCAGATG-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 of the promoter as a template, and the following primer set: 5′-GAAGAAGCATTCCCGCTGCGTTGCG-3′ and 5′-TCCCGGGCCATCGAGGACCC-3′. The luciferase reporter plasmid for the 1.2 kb fragment; 5′-GAAGAAGCATTCCCGCTGCGTTGCG-3′ and 5′-TCCCGGGCCATCGAGGACCC-3′. All of these sense primers were used in combination with the same antisense primer: 5′-CGCAAGATCTCTTCGCGCCCAGATG-3′. Each fragment was then cloned into the BglII site of the pGL4.10 vector (Promega, Madison, WI).

Establishment of stable transfectants

AGS cells were transfected with a pcDNA3.1 parental plasmid vector or a pcDNA3.1-NTH1 plasmid vector by using Lipofectamine 2000 reagent (Invitrogen). G418-resistant clones were isolated by culturing in a medium containing 400 μg/ml of G418 (Invitrogen). G418-resistant clones were isolated by culturing in a medium containing 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 5′-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 fluoromicroscope 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′-GAGAATCTCACTACAGCCAGACGC-3′ and 5′-GAGCCTCGAGATCCCTTATG-3′ for region 1 and 5′-GAGAATCTCACTACAGCCAGACGC-3′ and 5′-GAGCCTCGAGATCCCTTATG-3′ for region 2. The PCR products of region 2 were digested with Aflav 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 electrophoresis, 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 age, gender (±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.
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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 performed 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 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
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.

**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...
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 \textit{NTH1} gene and found

![Fig. 3.](image)

\textbf{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 \mu m and 25 \mu m, respectively. The immunohistochemical findings in the 50 gastric cancer cases are denoted at the row of NTH1 localization in (A).
two novel promoter polymorphisms, c.-163C\(\rightarrow\)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 DNA, the low NTH1 activity might be involved in the pathogenesis of a subset of gastric cancers.

**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\(\rightarrow\)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\(\rightarrow\)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 each 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, \(\*P < 0.05\).
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 localized 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 nucleic DNA, especially in organs exposed to severe oxidative stress.

### Supplementary material

Supplementary Table SI can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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### References


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