

# E-CADHERIN GENE ALTERATIONS IN GASTRIC CANCERS IN DIFFERENT ETHNIC POPULATIONS

**Introduction:** A retrospective study of nucleotide sequence alterations in exons 7–9 of the E-cadherin gene and expression of E-cadherin and  $\beta$ -catenin in gastric tumors from African American, Asian, Caucasian and Hispanic patients was carried out to determine differences potentially related to race/ethnicity in these groups.

**Methods:** Paraffin-embedded tissue sections archived at the Memorial Sloan Kettering Cancer Center were used for immunohistochemical staining of sections for membranous E-cadherin protein and nuclear localization of  $\beta$ -catenin. DNA from tumor tissue extracted from the paraffin sections was used as template for amplification of the E-cadherin gene exonic regions.

**Results:** Sequence analyses of the high-frequency mutation region along E-cadherin exons 7–9 revealed a number sequence alterations in the patient group as a whole, mostly within exon 8. The alterations were mainly single nucleotide insertions, but a palindromic duplication of exon 8 in a Caucasian patient and several extragenic insertions in a Hispanic and an African American patient were also found. Nuclear localization of  $\beta$ -catenin was correlated with inactivating sequence alterations in three patients.

**Conclusions:** Exon 8 was found to display the most extensive alterations in all the groups studied. As a group, the most extensive sequence alterations were found in tumors from Caucasian patients. A finding of potential significance as a biomarker related to ethnicity was a C insertion at nt 76,598 found independently in two African American patients. (*Ethn Dis.* 2008;18[Suppl 2]:S2-70–S2-74)

**Key Words:** E Cadherin Alterations, Gastric Tumors, Ethnic Biomarkers

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## INTRODUCTION

Cancer of the stomach remains a significant worldwide health problem that is associated with wide ethnic and racial variance that remains unexplained. It is the third most commonly diagnosed malignancy worldwide and is the second most common cause of cancer-related death.<sup>1</sup> There is marked ethnic variation; minority US populations have an age-standardized risk for gastric cancer almost twice that of the US White population.<sup>2</sup> Gastric cancer clearly falls within the area of health disparities that disproportionately affects minority communities. According to statistics provided by the National Cancer Institute (SEER data; <http://seer.cancer.gov/>) for the years 1998–2002, Caucasians as a whole are only 57% as likely as African Americans to be diagnosed with stomach cancer. When compared with Hispanics, Pacific Islanders, and Indians (Native American and Alaskan), the relative incidences of gastric cancer in Caucasians are 57%, 46.5%, and 62%, respectively. Death rates from gastric cancers show similar trends in racial variance. Both Pacific Islander/Asians and Blacks are  $\approx 2.2$  times as likely as Whites to die from the disease. Mortality for Hispanics is 75% greater than the mortality for Caucasians. Interestingly, although Asian/Indian populations are 62% more likely than Caucasians to be diagnosed with stomach cancer, they are only  $\approx 35\%$  more likely to die from it. Together, these data highlight the possibility of a fundamental biologic disparity in gastric cancer diagnosed in different ethnic populations.

Not only is gastric cancer a racially diverse disease with a significantly increased incidence in minority populations that is almost twofold higher than in White populations, there is also a marked

racial health disparity as well; Black patients have a more aggressive phenotype and worse prognosis, and Asians have the best prognosis. Dietary and socioeconomic factors among these groups are unlikely to explain such variation in incidence and mortality. Mutations in the genes encoding Wnt components are associated with various cancers, including those of the gastrointestinal tract, and in particular, gastric cancer. Diffuse gastric cancer is associated with loss of E-cadherin function in  $\approx 50\%$  of cases.<sup>3</sup> Germline mutations in E-cadherin (CDH1) associated with loss of E-cadherin function are associated with the familial form of diffuse gastric cancer, hereditary diffuse gastric cancer.<sup>4–8</sup> Because E-cadherins are components of adheren junctions, this observation is consistent with the loose cell-cell attachment characteristic of the histology of diffuse-type gastric tumors.

Also, because the E-cadherin/ $\beta$ -catenin complex normally sequesters a fraction of the total complement of  $\beta$ -catenin in the intracellular membrane compartment, loss of membrane-bound E-cadherin is expected to result in an increase in the cytoplasmic and nuclear pools, and a number of studies have found an increase in nuclear localization of  $\beta$ -catenin in diffuse gastric cancer that shows loss of membrane-bound E-cadherin.<sup>7,8</sup> Here we carried out a retrospective study of oncogenic changes in the E-cadherin gene in gastric tumors from patients of different ethnic background, with a view toward discerning differences that may be related to ethnicity.

## METHODS

### DNA Isolation from Gastric Tumor Tissue

The margins of tumor tissue in paraffin sections obtained from surgical

resection were identified and demarcated, and small pieces of paraffin-embedded tumor from the corresponding paraffin blocks were scraped into microfuge tubes with a scalpel. The tissue was then deparaffinized, and DNA was purified from the tissue with DNeasy genomic DNA isolation kits (Qiagen, Valencia, Calif) according to the manufacturer's instructions.

### PCR Amplification of E-Cadherin Gene Segments and Sequencing

DNA at a final concentration of .5–.9 µg/mL was used for PCR with a standard three-segment cycling protocol for 35 cycles: 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 45 seconds and a final product extension segment of 72°C for five minutes with the following primer sets:

exon 7: 5'-ACCCAGTCCCCAAAGTGCAGC-3'

5'-GGGATTGAGCTAATACACATTTGTCC-3'

exon 8: 5'-GCTAGTGTTTCCTGGTCCTGACT-3'

5'-CCATGAGCAGTGGTGACACTTAG-3'

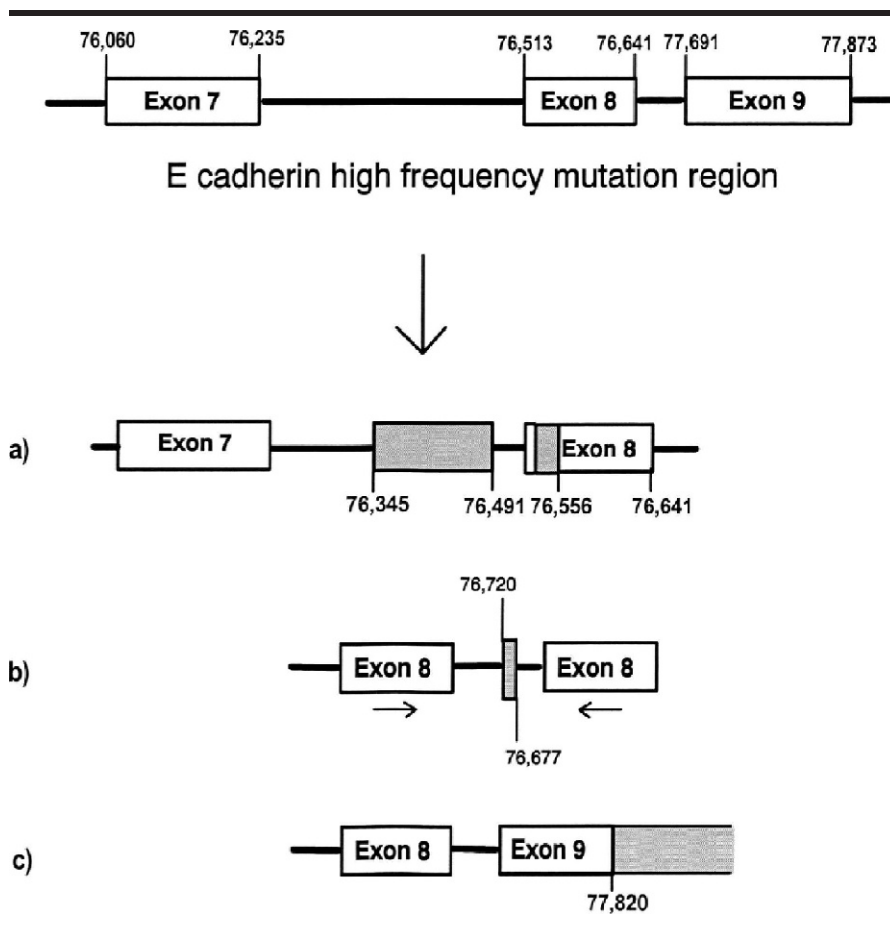
exon 9: 5'-CCAGCCTGGTGACAGTGAGAT-3'

5'-CAGCTGTGAGGATGCCAGTTTC-3'

For sequencing, residual primers and deoxynucleotides were eliminated from PCR reactions mixtures with ExoSAP-IT reagent (Amersham Biosciences, Piscataway, NJ), and PCR products were sequenced at a commercial sequencing facility (GeneWiz, South Plainfield, NJ). Sequences were compared to the E-cadherin reference sequence, DQ090940.1. Each PCR product found to contain putative sequence alterations was sequenced 2–4 times with forward and reverse primers for confirmation.

### Immunohistochemical Staining

Formalin-fixed, paraffin-embedded tissue blocks from gastric tumors were



**Fig 1. Gene rearrangements.** Diagrams of the gene rearrangements indicated in Table 1 above. Nucleotide sequence numbering according to the standard E-cadherin reference sequence (GenBank accession no. DQ090940.1) is shown. Letters (a, b, c) refer to footnotes in superscript in Table 1.

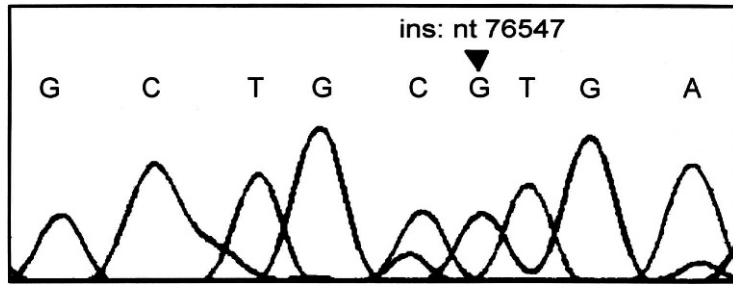
cut in 4- to 5-µm sections, placed on slides, and then baked at 55°C–60°C for ≥60 minutes. The slides were deparaffinized in xylene, followed by a graded series of xylene:ethanol washed, and rehydrated in water. Endogenous peroxidases were inactivated by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS) at room temperature for 15 minutes. The sections were incubated overnight at 4°C in a humidified chamber with monoclonal antibodies in 2% bovine serum albumin-PBS. Immunohistochemical staining was performed with an indirect biotin streptavidin diaminobenzidine method (<http://www.ihcworld.com/introduction.htm>). Slides were lightly counterstained with Mayer's hematoxylin, mounted in Permount Staining, and scored semiquantitatively

for intensity of membranous or nuclear staining of tumor suppressors by a trained technician. The β-catenin antibody (Becton Dickinson/Transduction Laboratories, San Jose, Calif) was used at dilution of 1:200; a colon adenocarcinoma was used as a positive control. E-cadherin antibodies (Zymed Laboratories/Invitrogen, Carlsbad, Calif) were used at a dilution of 1:2000; a breast lobular carcinoma was used as a positive control.

## RESULTS

### E-Cadherin Sequence Alterations

A retrospective study of alterations in the E-cadherin gene in gastric tumors was carried out by using paraffin-embedded gastric tumor tissue from



**Fig 2.** Sequence electropherogram showing a single nucleotide insertion in exon 8 in a tumor from a Caucasian patient.

the Memorial Sloan Kettering Cancer Center (MSKCC) histopathology archive to examine sequence alterations within the segment of the E-cadherin gene spanning exons 7–9, which has been shown to represent a high frequency mutational cluster region in gastric cancer.<sup>9</sup> The exonic region examined covers amino acid residues 279–439 that contains portions of the second and third tandem CA repeats within the E-cadherin extracellular domain that mediates Ca<sup>++</sup> dependent cell-cell adhesion.<sup>10</sup>

Genomic DNA derived from the tumors was used as template for PCR, and exonic segments were amplified and PCR products sequenced by using primer sets for exons 7, 8, or 9 as described in Methods. Genomic DNA from 58 tissue specimens from 17 Asian, 11 Black, 20 Caucasian, and 10 Hispanic patients were used for the preliminary study. As shown in Table 1, most of the sequence abnormalities found were frame-shift single nucleotide insertions (Figure 2), but one example of a transversion (G→T) in exon 7 in a Caucasian patient brought about a Pro→His substitution at amino acid residue 28 in the exon 7 coding region. There were two cases in which oligonucleotide insertions were seen in tumors in similar locations within exon 8 in one Asian and one African American patient (nt 76,691→76,709 and 76,680→76,708 respectively). An insertion of 32 bp was found in exon 7 of one Caucasian patient, while separate insertions of 146 and 19 bp

were found in exon 8 of another (Figure 1). A rearrangement involving a duplication of exon 8 and a 43-bp intronic insertion was found in another Caucasian patient (Table 1 and Figure 1). Although nucleotide sequence alterations were found in all three exons that comprise the hypermutable region, most of the changes occurred in exon 8; of the 19 sequence alterations found, 13 occurred within, or involved, exon 8.

**Immunohistochemical Staining**

Nuclear localization of β-catenin is an indicator of altered Wnt signaling.<sup>11</sup> Paraffin sections of tumors were stained

for E-cadherin and β-catenin to examine patterns of localization within the cellular compartment (nucleus vs cytoplasm) in tumors from the different ethnic groups (Table 2). Overall, most tumors showed strong membranous staining for E-cadherin and little nuclear localization of β-catenin; significant nuclear localization of β-catenin was observed in 9 of the 42 tumors examined. Compared to the other ethnic groups, tumors from Hispanic patients showed the greatest tendency toward nuclear localization of β-catenin; 4 out of the 12 tumors examined showed strong nuclear localization, and another showed less, but distinct, localization. Complete absence of staining for E-cadherin in the membrane was observed in only 2 of the 42 sections examined, although 3 others showed weak staining. Significantly less membrane staining of E-cadherin was observed in the African American group in which the average staining score was 2.1 vs 2.7, 2.7, and 2.5 for Caucasians, Hispanics, and Asians, respectively.

Of the patient tumors for which E-cadherin gene sequence data (exons 7–9) was obtained, immunohistochemical

**Table 1. Summary of sequence alterations in exons 7, 8, and 9\* of the E-cadherin gene in gastric tumors from patients of different ethnic/racial groups**

Ethnicity	Exon 7	Exon 8	Exon 9
African American (n=28)	• ins: 76151 :A ins: 76144: G	• ins 76598: C • ins 76598: C • ins:76680→76708	NC
Asian (n=25)	NC	• ins 76691→76709 • trunc/ins: 76567	NC
Caucasian (n=36)	• G→T subst:76153 • ins:76120→76152	• ins: 76547:G • ins: 76560:G ins: 76597:C ins:76345→76491 <sup>a</sup> ins:76537→76556 <sup>a</sup> • exon 8 duplication <sup>b</sup> ins 76677→76720 <sup>c</sup>	• ins: 77703:C • ins: 77789
Hispanic (n=21)	NC	• ins: 76604: T • ins: 76596: C ins: 77064 :C	NC

NC = no changes observed, ins = insertion, subst = substitution, trunc = truncation. Results for individual patients are indicated by (•); multiple alterations found in the same patient are shown without bullets. Total number of exons sequenced in each racial/ethnic group is shown for each ethnic group.

\* Alterations in exon 7 and 8 in the same patient.

<sup>a,b,c</sup> Examples of duplications and insertions observed in tumors from Caucasian patients, as diagrammed in Figure 1.

Table 2. Immunohistochemical staining of paraffin sections

Patient ID	Ethnicity	Staining Intensity*	
		E-Cadherin	$\beta$ -Catenin
04-44995	Caucasian	0	0
04-45425	Caucasian	3	0
04-1902	Caucasian	3	0
04-15895	Caucasian	3	0
04-38503	Caucasian	3	0
04-34891	Caucasian	3	0
05-15918	Caucasian	3	0
05-26448	Caucasian	2	2
04-39656	Caucasian	3	0
05-13523	Caucasian	3	0
05-329	Caucasian	3	0
05-4146	Caucasian	3	3
05-42820	Black	2	0
05-17647	Black	3	0
05-42902	Black	2	3
05-10318	Black	2	3
06-766	Black	1	0
03-38179	Black	3	0
04-39117	Black	0	0
04-15758	Black	3	0
02-8761	Black	2	0
02-2129	Black	3	0
05-7092	Hispanic	3	3
05-20319	Hispanic	3	3
05-32384	Hispanic	2	0
05-45626	Hispanic	3	0
05-7611	Hispanic	2	3
04-19256	Hispanic	2	0
04-11201	Hispanic	3	3
04-23210	Hispanic	3	0
03-20865	Hispanic	3	0
03-31367	Hispanic	2	0
03-33073	Hispanic	3	1
03-20940	Hispanic	3	0
05-42110	Asian	1	0
05-22625	Asian	1	0
04-35866	Asian	3	0
04-34816	Asian	3	0
04-1758	Asian	3	3
04-26887	Asian	3	0
04-30952	Asian	3	0
03-17572	Asian	3	0

\*  $\beta$ -catenin: 0 = normal membranous staining; positive nuclear staining: 1 = <5%, 2 = 5%–50%, 3 = >50%. E-cadherin: 0 = complete loss of membranous staining; 3 = normal membranous staining.

staining results were available for 6 Asians, 11 Caucasians, 8 African Americans, and 7 Hispanics. Strong nuclear localization of  $\beta$ -catenin (score 2 or 3) was seen only in one Asian patient, two Caucasian patients, two African American patients, and four Hispanic patients. Of these, nuclear localization of  $\beta$ -catenin was correlated with inactivating sequence alterations in three patients: an Asian patient found to have a

truncation of exon 8 (ID 1758), an African American patient with a polynucleotide insertion in exon 8 (ID 42902), and a Hispanic patient with a single nucleotide insertion in exon 8 (ID 7092). Only three patients exhibited low or absent (scores of 1 or 0) E-cadherin membranous staining (ID 44995, 42110, 22625). Neither of these was correlated with any of the nucleotide sequence alterations found so far.

## DISCUSSION

Although oncogenic mutations in E-cadherin genes have been widely characterized in a variety of cancers, few studies of alterations in E-cadherins relate to racial genetics. Here we have described the results of a retrospective study of gastric tumors that was carried out with the goal of identifying sequence alterations in the E-cadherin gene with a view towards characterizing possible biomarkers of oncogenicity in different ethnic subpopulations.

A number of nucleotide sequence changes were found that may be potential biomarkers of gastric cancer in the groups studied. One of the single nucleotide insertions was found in two African American patients (nt 76598 in exon 8; Table 1). Since identical changes are unlikely to have arisen independently during tumor development in different individuals, this mutation may turn out to be a germline biomarker associated with gastric cancer. In contrast, several polynucleotide insertions of unknown origin, seen mainly in exon 8 in Asians and Caucasians, are likely to have arisen during tumorigenesis and may therefore be markers of tumor progression.

Interestingly, CDH1 mutation in exon 8 may be correlated with nuclear localization of  $\beta$ -catenin, since changes in exon 8 were observed in three cases where nuclear localization was also observed in conjunction with E-cadherin alterations. Of these, two showed extensive changes in exon 8: 1) multiple insertions along the segment 76680→76708 in an African American patient and 2) an exonic truncation at nt 76567 in an Asian patient (Table 1). There was also a lack of correlation between nuclear  $\beta$ -catenin and membrane staining of E-cadherin. This is not unexpected since nuclear localization  $\beta$ -catenin is linked more directly to Wnt pathway function<sup>11–14</sup> rather than E-cadherin. Five of the patient tumors examined showed little or no staining of



membrane E-cadherin, which suggests that these tumors may carry inactivating alterations in the E-cadherin transmembrane domain. Our finding that nuclear localization of  $\beta$ -catenin was not seen in any of these tumors parallels the findings of Koppert et al<sup>10</sup> with respect to correlation of nuclear localization and loss of function of Wnt components. This may reflect changes in  $\beta$ -catenin steady-state cytosolic levels that could accompany perturbation of the Wnt pathway or, alternatively, may involve other signaling pathways effected by E-cadherin.

### Implications for Improving Health Disparities

The goal of this study, as well as future studies based on a registry of gastric cancer patients currently being developed at MSKCC, is to identify risk biomarkers of gastric cancer specific to ethnic groups. It is anticipated that in the long term, biomarkers will have utility not only for identifying at-risk individuals for early intervention but will also have implications for tailoring therapies.

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