Polymorphisms in the 3'-untranslated region of the *CDH1* gene are a risk factor for primary gastric diffuse large B-cell lymphoma

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Primary gastric B-cell lymphomas arise from mucosa-associated lymphatic tissue (MALT) in patients with chronic *Helicobacter pylori* infection. We investigated whether germline variants in the *CDH1* gene, coding for E-cadherin, genetically predispose patients to primary gastric B-cell lymphoma.

Design and Methods

Single marker analyses of the *CDH1* gene were conducted in patients with primary gastric B-cell lymphoma (n=144), in patients with primary gastric high-grade lymphoma (n=61), and in healthy blood donors (n=361). Twelve single nucleotide polymorphisms were genotyped by TaqMan® technology. Allelic imbalance was tested by pyrosequencing and clone direct sequencing of heterozygote genomic and cDNA. Mutation detection was conducted around the poly-A signal of the *CDH1* 3'-untranslated region. The influence of the 3'-untranslated region on protein translation was determined by a luciferase reporter assay.

Results

Single marker analyses identified two single nucleotide polymorphisms in strong linkage disequilibrium located in the *CDH1* 3'-untranslated region. One of them was significantly associated with primary gastric diffuse large B-cell lymphomas after correction for multiple testing and this association was confirmed in an independent sample set. Patients homozygous for the rare T allele (rs1801026) had a 4.9-fold increased risk (95% CI: 1.5-15.9) of developing primary gastric diffuse large B-cell lymphoma. Allelic imbalance and reporter gene assays indicated a putative influence on mRNA stability and/or translational efficacy.

Conclusions

We identified variants in *CDH1* as the first potential genetic risk factors for the development of primary gastric diffuse large B-cell lymphomas. One of the potentially causative variants affects allelic *CDH1* expression. These findings support the hypothesis that besides somatic alterations of B-cells, germline variants in the *CDH1* gene contribute to a predisposition to the development of primary gastric diffuse large B-cell lymphomas.

Key words: lymphoma, CDH1, *H. pylori*, allelic imbalance, E-cadherin.

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Introduction

In 1984, Isaacson and Wright first described four cases of extranodal malignant lymphoma arising from mucosa-associated lymphoid tissue (MALT).[1] *Helicobacter pylori* infection was found in 92% of extranodal marginal zone lymphomas of MALT type (MALT lymphomas). Isaacson and colleagues postulated that *H. pylori* infection provides the necessary background in which MALT lymphomas might develop.[2] Originally, primary gastric diffuse large B-cell lymphomas (high-grade lymphomas) were considered to develop independently of *H. pylori* infection, but prospective studies provide evidence that a subset of early stage diffuse large B-cell lymphomas may also regress completely after *H. pylori* eradication.[3-5] Fischbach[6] and Cavanna *et al.*[7] both reported and reviewed a number of cases of primary gastric diffuse large B-cell lymphomas successfully treated by *H. pylori* eradication and suggested that patients with primary gastric diffuse large B-cell lymphomas associated with *H. pylori* should first be managed with an anti-*H. pylori* treatment. In fact, primary gastric diffuse large B-cell lymphomas are often diagnosed in an advanced tumor stage and lead to death within a few months without treatment.[8] In comparison to extranodal marginal zone lymphomas of MALT type (MALT lymphomas, low-grade lymphomas), primary gastric diffuse large B-cell tumors are characterized by more aggressive tumor growth and worse prognosis.[9]

Besides the virulence of different *H. pylori* strains, which does not seem to play a decisive role in the development of gastric lymphoma,[10] the host genetic background influences the clinical course of *H. pylori* infection. In preceding studies, we were able to identify variants at *TNF* and *NOD2* gene loci as genetic risk factors for primary extranodal marginal zone lymphoma of MALT type or gastric lymphoma in general,[11-12] but a distinct genetic risk factor for primary gastric diffuse large B-cell lymphomas is lacking.

The *CDH1* gene, coding for the calcium ion-dependent cell adhesion molecule E-cadherin, plays a pivotal role in epithelial integrity and carcinogenesis. Reduced expression of E-cadherin is regarded as one of the main molecular events triggering cancer invasion and metastasis.[13-17] Inactivation is linked to different types of malignant tumors,[18-27] especially hereditary diffuse gastric cancer.[28-32] Primary gastric extranodal marginal zone lymphomas of MALT type and primary gastric diffuse large B-cell lymphomas express different patterns of adhesion molecules,[33] and B-cell tumor growth and clinical aggressiveness are related to the adhesive capacities of the tumor cells.[34] E-cadherin is expressed on lymphoma cells of patients with non-Hodgkin's lymphoma[35] and is released into the blood. Serum levels of E-cadherin are of prognostic significance in patients with multiple myeloma, another B-cell-associated hematologic malignancy.[36]

With the hypothesis that CDH1 could also have in important role in the etiopathogenesis of gastric lymphoma, we conducted a candidate gene association study in patients with primary gastric diffuse large B-cell lymphomas from a European multicenter study.

Design and Methods

Patients from a European multicenter study

We studied patients with gastric lymphoma (median age of 61 years, range 29-75 years; 56% males) participating in an intention-to-treat prospective multicenter study of the German-Austrian-Lymphoma Study Group.[9] Caucasian patients with newly diagnosed primary gastric B-cell lymphoma were recruited from March 1993 through March 1996 at 166 centers in Germany and at 13 Austrian centers in this European multicenter study (EMCS). The exclusion criteria were age above 75 years, primary nodal or human immunodeficiency virus-associated lymphoma of any type, and prior or concomitant malignancies including gastric collision tumors. The primary gastric origin was defined histopathologically for low-grade MALT lymphomas and primary gastric diffuse large B-cell lymphomas with evidence of a low-grade component (secondary high-grade lymphoma). Patients with secondary high-grade lymphoma (n=9) were excluded from the analyses due to the ambiguous phenotype. Patients with diffuse large B-cell lymphoma (high-grade lymphoma) without low-grade features were regarded as having primary gastric lymphoma if they met the criteria described by Lewin.[37] In all remaining 144 cases, the diagnosis was based on morphological and immunophenotypic analyses. The *H. pylori* status was assessed by the urease test on samples taken from the gastric antrum and corpus and histological examination of biopsy specimens. The work-up to determine disease stage included the patient's history and physical examination, blood cell count and serum chemistry, inspection of Waldeyer's tonsillar ring, chest and small bowel radiography, cervical and abdominal ultrasound, computed tomography of the chest and abdomen, bone-marrow aspirate and biopsy, ileocolonoscopy and upper gastrointestinal endoscopy with biopsies of visible lesions and macroscopically normal mucosa. The stage was defined according to the Ann Arbor staging system[38] with modifications described by Musshoff[39] and Radaszkiewicz[40] (*Online Supplementary Table S1*).

Patients from the Lymph Node Registry in Kiel

The Lymph Node Registry in Kiel, founded in 1965, serves as a nationwide reference center for hemato-lymphatic diseases in Germany. Using the registry's database, patients with a diagnosis of primary gastric diffuse large B-cell lymphoma according to the World Health Organization classification were identified retrospectively from 2005-2007. Biopsy samples of normal gastric mucosa, taken at the same time as the pathological biopsy samples, were acquired for 61 patients by contacting the relevant pathologists.

Healthy blood donor study population

The healthy blood donor study population was recruited in the clinical blood donation center of the University Hospital Schleswig-Holstein, Kiel, Germany by the PopGen biobank. Participants were allowed to donate blood if they were serologically negative for human immunodeficiency virus, hepatitis virus and cytomegalovirus and physical examination/history indicated no contraindications to blood donation (e.g. active infections or malignant disorders). Using these criteria, 361 Caucasian age- and sex-matched blood donors from Kiel were genotyped as healthy controls. Parts of the population have been used in other studies without any signs of systematic allelic bias due to the selection criteria.[41-44]

Data protection and ethics

The study was approved by the Data Protection Commission of Schleswig-Holstein and the ethical committee of the medical faculty of the Christian-Albrechts University of Kiel. Written informed consent was obtained from all individuals prior to enrollment. Personal information associated with medical information and blood samples was rendered anonymous.

Marker selection and genotyping

We prepared genomic DNA from 10 mL samples of fresh or frozen blood using the Blood Gigakit (Invitek, Berlin, Germany) or from paraffin-embedded non-neoplastic tissue using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturers' recommendations (Lymph Node Registry sample set).

Twelve single nucleotide polymorphisms (SNPs) were selected with the intention of genetically covering the *CDH1* locus and the downstream region. No high-density tagging information from the HapMap was available at the time of our studies, hence the 12 SNPs were selected based on other public resources (SNPBrowser from Applied Biosystems, Foster City, CA, USA). We also included the only HapMap-validated coding variant SNP 6 (rs1801552) within the *CDH1* gene. The relative localization of the genotyped SNPs on chromosome 16q22.1 is illustrated in Figure 1 (for HapMap CEU LD structure and marker position see *Online Supplementary Figure S1*). All SNPs were genotyped using functionally tested TaqMan® assays provided by Applied Biosystems. The probe sequences are listed in *Online Supplementary Table S2*. In brief, genomic DNA was arrayed and dried on 96-well and 384-well microtiter plates. A TaqMan® polymerase chain reaction (PCR) was set up with Genesis pipetting robots (Tecan, Männedorf, Switzerland) and performed as described previously.[45] We amplified samples with ABI9700 PCR machines (Applied Biosystems), and measured fluorescence with ABI7700 and ABI7900 fluorometers (Applied Biosystems). PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 10 s and 60°C for 1 min. All process data were written into and administered by a previously described database-driven laboratory information management system (LIMS).[46] Duplicate or related samples were identified and excluded from the analyses, using algorithms implemented in the laboratory information management system. Case and control samples were genotyped on separate bar-coded plates. Visual inspection of the raw intensity data confirmed the absence of batch effects.

Statistical analysis

All SNPs had a high call rate (≥ 95% in cases or controls), were not monomorphic, and did not deviate from Hardy-Weinberg equilibrium in the control samples (P>0.05). The detailed call rates and Hardy-Weinberg equilibrium P values are given in Table 1. Two types of association analyses were performed: (i) patients with MALT lymphomas were compared against healthy blood donors; (ii) patients with primary gastric diffuse large B-cell lymphomas were compared against healthy blood donors.

Statistical analyses were performed using the Hardy-Weinberg equilibrium web interface *http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl* for case-control studies. Pearson's χ^2 tests were used considering a recessive model based on relative genotype frequency distribution in the groups of patients and controls. Due to the potential multiple testing problem given the small sample size we only considered this hypothesis-driven and powerful (one degree of freedom) approach. Odds ratios and 95% confidence intervals were calculated for carriership of the rare allele using the above-described web interface. Linkage disequilibrium values, using the measure r^2, were calculated using HAPMAX.[47]

Mutation detection

For mutation detection around the poly-A signal, primers were designed based on the *CDH1* RefSeq NM_004360.3 and the downstream genomic sequence, provided by UCSC Genome Browser (http://genome.ucsc.edu) using ABI Primer Express 2.0 software (Applied Biosystems): forward primer: 5'-CTCAAAGCCCAGAATCCCC-3'; reverse primer: 5'-ATGAGCCACCGCGCC-3'. The reaction contained 5 ng of genomic DNA and 50 μM of primer DNA in 50 mM salt and 1 mM Mg^{2+}. A three-step PCR was performed for 35 cycles: denaturation at 94°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. Fluorescent sequencing of amplified fragments (640 bp) was carried out using a BigDye Cycle Sequencing system (Applied Biosystems). Products were analyzed on an ABIPRISM 3700 automated DNA sequencer (Applied Biosystems).

Isolation of complementary DNA

Total RNA was extracted from 7.5 mL peripheral blood and lymphoblastoid cell lines of individuals heterozygous for rs1801026 (SNP 7) using a PAXgene Blood RNA kit (Qiagen, Hilden, Germany). Ten microliters of water containing 1 μg of

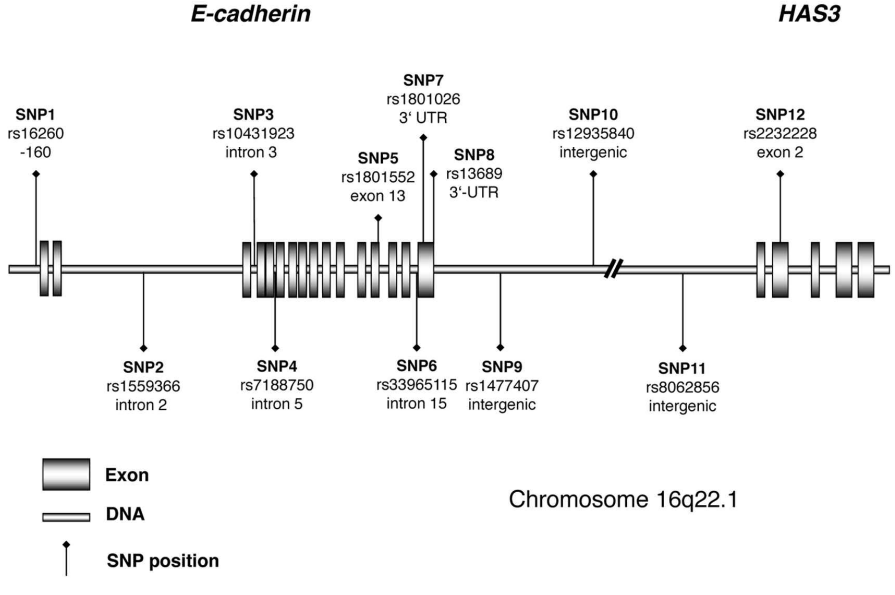

Figure 1. Schematic gene-model of *E-cadherin* (*CDH1*) and neighboring *HAS3* on chromosome 16q22.1 and relative position of the SNPs analyzed.

RNA together with 2.5 μL of RNasin plus RNase inhibitor (40 U/μL, Promega, Mannheim, Germany) and 5 μL of random hexamer primers (50 pmol/μL, Biotez, Berlin, Germany) were incubated for 10 min at 70°C. For the cDNA synthesis, 20 μL M-MLV reverse transcription buffer and 1 μL M-MLV reverse transcription enzyme (Promega), 5 μL dNTP (Amersham Biosciences, Buckinghamshire, UK) and 56.5 μL water were added to the RNA mix and incubated for 2 h at 42°C.

Amplification

PCR was performed using primers for *CDH1* (CDHf: 5'-GCTTCAAGAAGCTGGCTGAC-3'; CDHr: 5'-TTTGGACATCACCACCATGT-3') with 10 ng of cDNA or genomic DNA, respectively. Amplification was done in 96-well PCR plates (ABgene, Epsom, UK) containing 25 μL reaction mix/well using ReadyToGo PCR beads (Amersham Biosciences) according to the manufacturer's recommendation. PCR primers were used as sequencing primers for the direct sequencing of PCR amplicons in Sanger sequencing using the dye terminator chemistry of ABI (BigDye). All primers were from Metabion (Munich, Germany). Individual PCR products were ligated by the topoisomerase reaction into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) following the manufacturer's recommendations. Aliquots of ligated samples were used for transformation of *E. coli* to obtain clones and as templates for PCR in order to prepare a biotinylated amplicon for pyrosequencing.

Cloning

Cells of *E. coli* strain TOP10 were transformed with the respective ligation products and plated onto LB-Amp agar. White colonies were picked and individually grown overnight at 37°C in 5 mL of LB broth supplemented with 100 μg/mL ampicillin. Plasmids were isolated and their insertion sequenced with vector primers M13f (5'-GTAAAACGACGGCCAG-3') and M13r (5'-

Table 1. Genotype counts and frequencies of all markers in patients with primary gastric diffuse large B-cell lymphomas (high-grade gastric lymphoma) from the European multicenter study (EMCS) and healthy blood donors. Alleles are coded according to the plus strand (5'→3' orientation of *CDH1*). Differences between n per category and sum of genotype scores per category and SNP are due to missing genotypes (call rate ≥ 95%). *P* values calculated for carriership of the rare allele. $P_{corr.}$ is the *P* value adjusted for multiple testing using Bonferroni's correction (n=12). OR: odds ratio; 95% CI: 95% confidence interval. HWE: Hardy-Weinberg equilibrium (Pearson), CR: call rate.

SNP	Locus	Geno-type	Healthy controls (n=361)	High-grade gastric NHL (n=56)	CR control	CR cases	HWE control	*P* value	OR (95% CI)
1 rs16260	*CDH1* -160	A/A A/C C/C	26 – 7.3% 142 – 39.8% 189 – 52.9%	3 – 5.3% 22 – 39.3% 31 – 55.4%	100%	99%	0.924	0.600	0.72 (0.2-2.5)
2 rs1559366	*CDH1* intron 2	A/A A/G G/G	64 – 18.0% 177 – 49.7% 115 – 32.3%	8 – 14.5% 25 – 45.5% 22 – 40.0%	98%	99%	0.774	0.533	0.77 (0.4-1.7)
3 rs10431923	*CDH1* intron 3	G/T G/G T/T	87 – 24.6% 169 – 47.7% 98 – 27.7%	10 – 17.8% 29 – 51.8% 17 – 30.4%	100%	98%	0.405	0.272	0.67 (0.3-1.4)
4 rs7188750	*CDH1* intron 5	A/A A/G G/G	7 – 2.0% 97 – 27.0% 255 – 71.0%	4 – 7.1% 15 – 26.8% 37 – 66.1%	100%	99%	0.523	0.024 ($p_{corr.}$ 0.293)	3.86 (1.1-13.7)
5 rs1801552	*CDH1* exon 13	T/T T/C C/C	65 – 18.5% 171 – 48.7% 115 – 32.8%	6 – 10.7% 27 – 48.2% 23 – 41.1%	100%	97%	0.919	0.153	0.53 (0.2-1.3)
6 rs33965115	*CDH1* intron 15	A/A A/G G/G	4 – 1.1% 41 – 11.5% 311 – 87.4%	0 – 0.0% 4 – 7.1% 52 – 92.9%	100%	99%	0.056	0.425	0.69 (0.0-13.0)
7 rs1801026	*CDH1* 3'UTR	T/T T/C C/C	7 – 2.0% 97 – 27.3% 251 – 70.7%	5 – 8.9% 12 – 21.4% 39 – 69.7%	100%	98%	0.500	0.004 ($p_{corr.}$ 0.049)	4.88 (1.5-15.9)
8 rs13689	*CDH1* 3'UTR	C/C C/T T/T	11 – 3.1% 107 – 29.6% 243 – 67.3%	6 – 10.7% 16 – 28.6% 34 – 60.7%	100%	100%	0.851	0.007 ($p_{corr.}$ 0.083)	3.82 (1.4-10.8)
9 rs1477407	intergenic	A/A A/G G/G	9 – 2.5% 114 – 32.3% 230 – 65.2%	3 – 5.3% 22 – 39.3% 31 – 55.4%	100%	98%	0.242	0.247	0.66 (0.4-1.2)
10 rs12935840	intergenic	G/G G/A A/A	5 – 1.4% 58 – 16.4% 291 – 82.2%	1 – 1.8% 9 – 16.1% 46 – 82.1%	100%	98%	0.288	0.829	1.27 (0.1-11.1)
11 rs8062856	intergenic	T/T T/C C/C	71 – 20.2% 179 – 50.8% 102 – 29.0%	6 – 10.7% 35 – 62.5% 15 – 26.8%	100%	98%	0.639	0.093	0.48 (0.2-1.2)
12 rs2232228	*HAS3* exon 2	G/G G/A A/A	64 – 18.4% 169 – 48.6% 115 – 33.0%	9 – 16.1% 35 – 62.5% 12 – 21.4%	100%	96%	0.890	0.675	0.85 (0.4-1.8)

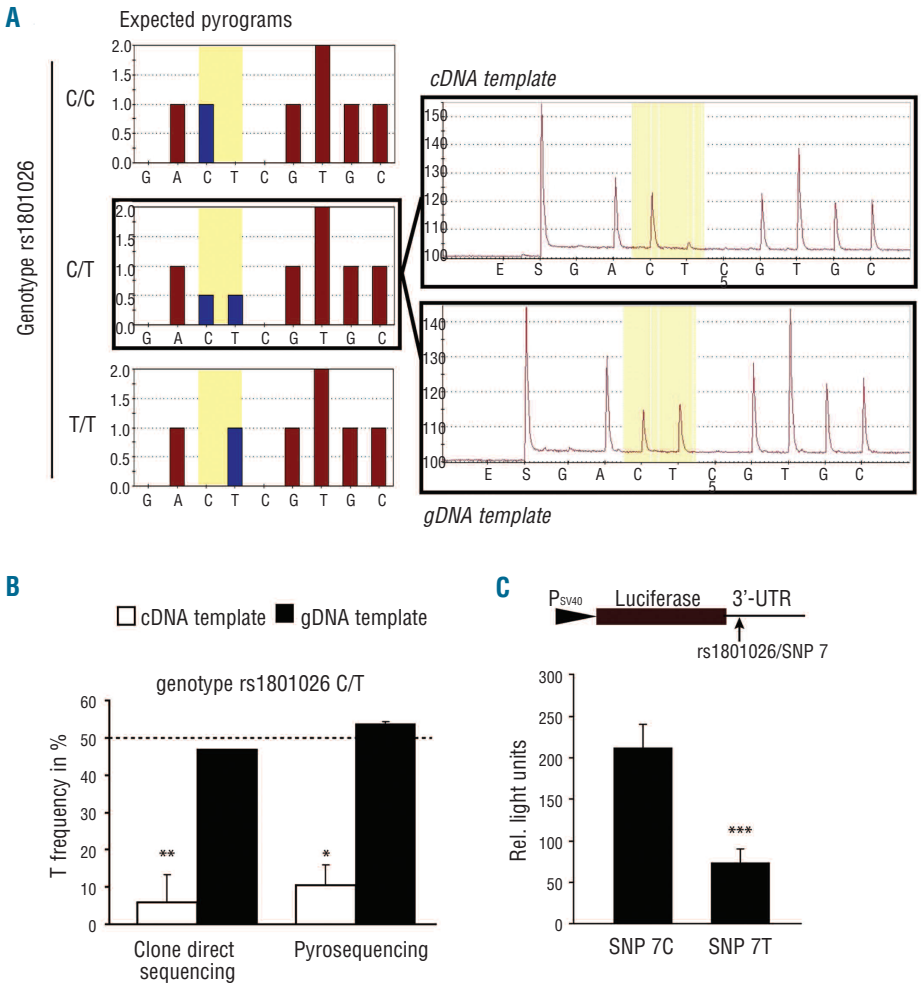

Figure 2. SNP 7 (rs1801026) is associated with allelic imbalance of *CDH1* mRNA expression and decreased allelic reporter gene activity. (A) Expected and observed pyrograms for SNP 7 for genomic DNA (gDNA) and complementary DNA (cDNA). In contrast to the expected pyrograms found in the gDNA template, the cDNA template revealed a pronounced allelic imbalance in rs1801026 heterozygous cells. (B) Allelic imbalance was confirmed by clone direct sequencing (see *Design and Methods*). The dashed line indicates the expected T frequency in heterozygous individuals (50%). (C) HeLa cells were transfected with luciferase reporter gene constructs containing the 3'-UTR of *CDH1* driven by a constitutive SV40-promoter. Luciferase activities were determined by a dual luciferase assay (n=3). **P*<0.05; ***P*<0.01; ****P*<0.005.

CAGGAAACAGCTATGAC-3'). Bases at SNP positions were called by manual inspection.

Pyrosequencing

Ligation products were diluted 1:100 and used as templates in a PCR with biotin-labeled primers (btM13r: 5'-CAGGAAACAGCTATGAC-3'; CDHfs: 5'-GTGCTGGGAAATGCAGAAATC-3'). PCR products were immobilized on streptavidin-coated paramagnetic beads (Dynabeads® M280, Dynal AS, Oslo, Norway) by mixing 20 µL of the PCR product with 10 µL Dynabeads (10 µg/µL) and 30 µL 2x BW buffer II (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.6). The samples were incubated with shaking at 43°C for 30 min using a thermal mixer. For denaturation and preparation of single-stranded DNA, the samples were transferred into 50 µL NaOH (0.5 M, 1 min) using the multi-magnet PSQ96 Sample Prep Tool (Pyrosequencing AB, Uppsala, Sweden). The samples were washed in 100 µL 1x annealing buffer (20 mM Tris, 5 mM magnesium acetate, pH 7.6) for 1 min. Subsequently, the immobilized templates were transferred to 42 µL 1x annealing buffer plus 3 µL primer CDHfs (5 µM in water) and heated with continuous shaking on a thermal mixer at 80°C for 10 min. After equilibration to room temperature, the sequencing reaction was performed with a PSQ96 SNP reagent kit (Pyrosequencing AB), according to the manufacturer's recommendation. The nucleotide dispensation order is illustrated in Figure 2.

All primers were from Metabion. All amplifications were done in 96-well PCR plates (ABgene) containing 25 µL reaction mix/well using ReadyToGo PCR beads (Amersham Biosciences) according to the manufacturer's recommendations with the following conditions: 1 cycle of 94°C for 3 min, followed by 45 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 45 s and a final elongation step (72°C for 10 min).

Luciferase reporter gene assay

To analyze the effect of the SNP rs1801026 (SNP 7) on mRNA stability, the 3'-untranslated region (3'-UTR) of *CDH1* was amplified and cloned directly to the 3' side of the luciferase gene of the vector pGL3-control (Promega). SNP variants were introduced by site-directed mutagenesis following the instructions in the manufacturer's manual (QuickChange site-directed mutagenesis kit; Stratagene, La Jolla, CA, USA). After sequence verification, plasmids were transfected into HeLa S3 cells by using FuGene6 (Roche). Twenty-four hours after transfection, cells were lysed and luciferase activity was measured by a dual luciferase assay (Promega) in a GeniosPRO plate reader (Tecan) and results normalized against pRL-TK (Clontech, Saint-Germain-en-Laye, France).

Results

Association analyses

In total, 12 SNPs were genotyped across the *CDH1* gene locus (8 SNPs) and the downstream region (Figure 1).

Single marker analyses identified SNP 7 (rs1801026), located in the 3'-UTR, as the SNP significantly associated with primary gastric diffuse large B-cell lymphomas. Patients homozygous for the rare T allele of SNP 7 in the 3'-UTR had a 4.9-fold (95% CI: 1.5-15.9) increased risk of developing high-grade lymphoma ($P_{corr.}$=0.049) (Table 1). This association signal was confirmed in the independent cohort of primary gastric diffuse large B-cell lymphomas from the Lymph Node Registry in Kiel (*P*=0.039, OR 3.5, 95% CI: 1.0-12.3) (Table 2) and findings were robust when corrected for multiple testing. SNP 8 (rs13689), a second marker in the 3'-UTR, was also associated with high-grade lymphomas (*P*=0.007, OR 3.8, CI 95%: 1.4-10.8), but the association did not remain statistically significant after correction for multiple testing. It should be pointed out that we used an overly conservative test to correct for multiple testing which shows, given the still significant findings, the robustness of our association findings. Bonferroni's correction, the method employed, assumes that all observations are independent from another. This is certainly not the case as shown by the correlation between the different SNPs. In order to exclude any neighboring candidate gene in linkage disequilibrium, three intergenic SNPs located more telomeric of the 3'-UTR of *CDH1* and one SNP in the neighboring hyaluronan synthase 3 (*HAS3*) gene (Figure 1) were genotyped, but did not show any association with lymphoma (Table 1). No significant associations were identified at any SNP between (low-grade) MALT lymphomas and healthy blood donors (*Online Supplementary Table S3*).

Pairwise linkage disequilibrium between SNP 7 and SNP 8 in the 3'-UTR was strong (r^2=0.82). SNP 4 was also in moderate linkage disequilibrium with SNP 7 and 8 (r^2=0.78 and 0.65, respectively), which explains its weak association with gastric diffuse large B-cell lymphomas in the single marker analysis. Linkage disequilibrium between other *CDH1* SNPs and the 3'-neighbouring intergenic markers or the *HAS3* SNP (rs2232228) was less than 0.5 (Table 3). The association signal is, therefore, most likely confined to the 3'-UTR of the *CDH1* gene.

Table 2. Genotype counts and frequencies of SNP 7 (rs1801026) in patients with primary gastric diffuse large B-cell lymphomas (high-grade gastric lymphoma) from the European multicenter study (EMCS) and the Lymph Node Registry and healthy controls. Alleles are coded according to the plus strand (5'→3' orientation of CDH1). Single marker analysis identified rs1801026 (SNP 7) in the 3'-UTR associated with high-grade gastric lymphoma. Homozygosity for the rare allele was tested in cases and controls assuming a recessive model of inheritance. *P* values calculated for carriership of the rare allele. OR: odds ratio; 95% CI: 95% confidence interval.

Cohort	Genotype	Healthy controls	High-grade gastric NHL	*P* value	OR (95% CI)
European Multicenter Study	T/T	7-2.0%	5-8.9%		
	T/C	97-27.3%	12-21.4%	0.004	4.9
	C/C	251-70.7%	39-69.6%		(1.5-15.9)
Lymph Node Registry Kiel	T/T	7-2.0%	4-6.6%		
	T/C	97-27.3%	13-21.3%	0.039	2.5
	C/C	251-70.7%	44-72.1%		(1.0-12.3)
Combined	T/T	7-2.0%	9-7.7%		
	T/C	97-27.3%	25-21.4%	0.003	4.1
	C/C	251-70.7%	83-70.9%		(1.5-11.4)

Mutation detection

We conducted mutation detection around the poly-A signal to identify a possible functional variant that could be in linkage disequilibrium with SNP 7 and 8. An amplicon of 640 bp was screened by genomic re-sequencing in 46 unrelated individuals (15 patients with low-grade MALT lymphoma, 15 patients with high-grade lymphoma and 16 *H. pylori*-infected patients) without detection of any new variants. We, therefore, performed further functional investigations of the strongest associated 3'-UTR SNP 7 (rs1801026).

mRNA instability/translational efficacy

To determine whether the lymphoma-associated SNP 7 (rs1801026) could potentially lead to reduced CDH1 mRNA stability, we investigated the balance of the C and T alleles in cDNA *versus* genomic DNA. Genomic DNA and total RNA extracted from a panel of lymphoblastoid cell lines and peripheral blood were isolated and mRNA was reverse-transcribed to cDNA using oligo(dT). We identified five heterozygous cDNA samples for rs1801026 (C/T), suitable for allelic expression analysis. Using two different methods, clone sequencing and pyrosequencing, we detected a significant allelic imbalance with an allelic mRNA ratio (T/C), which was consistent between the two methods 0.06±0.07 (by clone sequencing) and 0.1±0.05 (by pyrosequencing) (Figure 2). Control experiments with genomic DNA showed values close to the expected ratio of 0.5 (Figure 2). The putatively lower mRNA stability of the T allele containing 3'-UTR could be confirmed by a luciferase reporter gene assay. The 3'-UTR of *CDH1* was cloned directly behind the open reading frame of a luciferase gene with a constitutive promoter (SV40).

Table 3. Pairwise linkage disequilibrium coefficient r^2 of all markers calculated using HAPMAX. Darker gray shading indicates a higher linkage disequilibrium coefficient.

	CDH1 SNP1	CDH1 SNP2	CDH1 SNP3	CDH1 SNP4	CDH1 SNP5	CDH1 SNP6	CDH1 SNP7	CDH1 SNP8	intergenic SNP9	intergenic SNP10	intergenic SNP11	HAS3 SNP12
CDH1 SNP1												
CDH1 SNP2	0.46											
CDH1 SNP3	0.35	0.52										
CDH1 SNP4	0.05	0.04	0.03									
CDH1 SNP5	0.01	0.02	0.04	0.11								
CDH1 SNP6	0.09	0.04	0.04	0.01	0.09							
CDH1 SNP7	0.03	0.05	0.04	0.78	0.10	0.01						
CDH1 SNP8	0.04	0.07	0.07	0.65	0.11	0.01	0.82					
intergenic SNP9	0.01	0.00	0.02	0.27	0.02	0.00	0.31	0.22				
intergenic SNP10	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.01	0.02			
intergenic SNP11	0.00	0.01	0.07	0.02	0.02	0.01	0.03	0.06	0.02	0.08		
HAS3 SNP12	0.00	0.02	0.08	0.02	0.04	0.04	0.03	0.05	0.12	0.11	0.68	

Comparison of the relative expressions/luciferase activities of the constructs containing T and C alleles showed that the normalized luciferase activity of the T allele-containing construct was 2.5 to 3-fold lower than that of the C allele (Figure 2), likely corresponding to reduced mRNA stability or translational efficacy and subsequent diminished protein levels compared to the C allele.

Discussion

H. pylori is the first formally recognized bacterial carcinogen and is one of the most successful human pathogens, as over half of the world's population is colonized with this Gram-negative bacterium. *H. pylori* infection is a key factor in the etiology of various gastrointestinal diseases, ranging from chronic active gastritis without clinical symptoms to peptic ulceration, gastric adenocarcinoma, and gastric MALT lymphoma.[48] Besides a chronic inflammatory response, down-regulation of the cell adhesion molecule E-cadherin can be found in gastric biopsy samples from *H. pylori*-infected stomachs.[49] More precisely, *H. pylori* has been found to target adherens junction regulatory proteins resulting in increased rates of migration in human gastric epithelial cells. At the same time, E-cadherin is translocated from the plasma membrane to intracellular vesicles.[50] *CDH1* methylation was found in 85% of gastric cancer specimens and was associated with depth of tumor invasion and regional nodal metastasis. Since normal mucosa did not show *CDH1* methylation, gene-silencing by promotor methylation is an early event in gastric carcinogenesis, and is initiated by *H. pylori* infection.[51]

In general, partial or total loss of CDH1 is associated with changes or loss of differentiation characteristics, acquisition of cell invasiveness, increased tumor grade, metastasis and poor prognosis[52-56] and is linked specifically to hereditary diffuse gastric cancer.[28-32] Given its suppressive effect on tumor cell invasion and metastasis, *CDH1* is often termed a "metastasis suppressor" gene.[55]

The influence of *CDH1* on the etiopathogenesis of lymphoproliferative disorders is less clear. It is tempting to speculate that loss of epithelial integrity *per se* - even in the absence of *H. pylori* - may contribute to malignant transformation of lymphocytic cells. However, the *CDH1* gene was found to be expressed in a subset of human bone marrow mononuclear cells and plays a role in hematopoiesis.[57] Hypermethylation of *CDH1* is associated with leukemia.[55] E-cadherin is expressed on lymphoma cells of patients with non-Hodgkin's lymphoma[35] and is released into the blood. Serum levels of (soluble) E-cadherin are of prognostic significance in patients with multiple myeloma, another B-cell-associated hematologic malignancy.[36] Recently, it was shown that E-cadherin-expressing monocyte-derived inflammatory dendritic cells promote intestinal inflammation and are, therefore, putative therapeutic targets for the treatment of inflammatory bowel diseases.[58]

The *CDH1* gene (OMIM 192090) maps to chromosome 16q22.1 and comprises 16 exons spanning approximately 100 kb of genomic DNA. Exon 16 spans 2269 bp and codes almost entirely for an unusually long 3'-UTR. In this study, we genotyped 12 SNP in order to cover the broad linkage disequilibrium structure of the region of *CDH1* (*Online Supplementary Figure S1*) and identified two polymorphisms in high linkage disequilibrium in the 3'-UTR of the *CDH1* gene as the first detected genetic risk factors for primary gastric diffuse large B-cell lymphomas using DNA isolated from blood and tissue samples. The genotyping results (SNP 7, Table 2) of the EMCS and the Lymph Node Registry sample sets showed very similar allele frequencies and, therefore, strongly argue for no differences at our genetic locus of interest between blood- or tissue-derived DNA. As with most other association findings, we cannot, of course, exclude other rare private variants also contributing to the disease risk at this locus. Meanwhile, several additional rare coding variants have been added to the dbSNP database, which were identified in the 1000 Genomes Project.[59] Our functional *in vitro* experiments suggest that one of the identified variants (SNP 7) may affect mRNA stability. A simultaneous effect on mRNA levels with *CDH1* promotor hypermethylation *in vivo* cannot be excluded. Lower CDH1 expression levels could be detected by immuohistochemistry in biopsies from patients with MALT lymphoma (C/C *versus* T/T; *data not shown*). Although the evidence for mRNA instability is suggestive, it cannot be completely excluded that the SNP also affects translational efficacy or contributes to differential nuclear RNA processing or export.

Many clinically relevant mRNAs are regulated by differential RNA stability,[60] and the aberrant control of RNA stability has been implicated in the development of cancer and chronic inflammatory responses.[61] AU-rich elements of the 3'-UTR have the ability to promote rapid deadenylation of mRNA transcripts. AU-rich elements are grouped into three classes according to their sequence features and decay characteristics.[62] Whereas group I and II are characterized by the pentamer AUUUA and its reiteration, group III contains AU-rich regions lacking the hallmark AUUUA. Given the large sequence variation of AU-rich elements, a plethora of AU-rich element-binding proteins have been identified. Binding of these factors can have positive or negative effects on stability, translation and localization of the mRNA.[61,63] The 3'-UTR of the *CDH1* transcript is characterized by an abundance of AU-rich elements and one could speculate that the SNP identified may interfere with AU-rich element-binding proteins (*Online Supplementary Table S4*).

Annotation of SNP 7 (rs1801026) and SNP 8 (rs13689) in the genomic sequence of *CDH1* shows that SNP 7 is located 141 bp upstream of the poly-A signal and SNP 8 918 bp downstream (*Online Supplementary Table S4*). Genetic variations in the vicinity of the poly-A signal may directly interfere with RNA-polymerase binding sites and thus impair the polyadenylation of the 3' end of the mRNA.

Keirsebilck *et al.* investigated the influence of the 3'-UTR of *CDH1* on gene expression *in vivo*.[64] The *CDH1*$^{-/-}$ mouse mesenchymal tumor cell line MO4 was transfected with several plasmids expressing mouse *CDH1* cDNA. These plasmids differed from each other by the extent of *CDH1*-specific 3'-UTR sequences. Transfectants were isolated which expressed functional E-cadherin in a homogeneous way. In syngenic mice, such MO4-Ecad transfectants invariably produced malignant fibrosarcoma-like tumors, which were completely E-cadherin-negative at the protein level. Northern blotting revealed that *CDH1* mRNA expression was down-regulated in some but not all MO4-Ecad tumors. Down-regulation was caused by mRNA instability triggered by particular 3'-UTR sequences.[64]

As we demonstrated a significant association of SNP 7 homozygosity with gastric lymphoma (under a recessive

model), it is conceivable that, despite the allelic imbalance of CDH1 expression in the heterozygous state, the mRNA levels of subjects carrying the wild-type allele are sufficient to compensate for the functional defect.

In conclusion, our findings support the hypothesis that besides somatic alterations of B cells, germline variation in the *CDH1* gene contributes to a predisposition to primary gastric diffuse large B-cell lymphoma.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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