Germline mutations in *CDH1* are infrequent in women with early-onset or familial lobular breast cancers

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ABSTRACT

Background Germline mutations in *CDH1* are associated with hereditary diffuse gastric cancer; lobular breast cancer also occurs excessively in families with such condition.

Method To determine if *CDH1* is a susceptibility gene for lobular breast cancer in women without a family history of diffuse gastric cancer, germline DNA was analysed for the presence of *CDH1* mutations in 318 women with lobular breast cancer who were diagnosed before the age of 45 years or had a family history of breast cancer and were not known, or known not, to be carriers of germline mutations in *BRCA1* or *BRCA2*. Cases were ascertained through breast cancer registries and high-risk cancer genetic clinics (Breast Cancer Family Registry, the kConFab and a consortium of breast cancer genetics clinics in the United States and Spain). Additionally, Multiplex Ligation-dependent Probe Amplification was performed for 134 cases to detect large deletions.

Results No truncating mutations and no large deletions were detected. Six non-synonymous variants were found in seven families. Four (4/318 or 1.3%) are considered to be potentially pathogenic through in vitro and in silico analysis.

Conclusion Potentially pathogenic germline *CDH1* mutations in women with early-onset or familial lobular breast cancer are at most infrequent.

INTRODUCTION

CDH1 encodes the cell–cell adhesion molecule, E-cadherin. Loss of expression of E-cadherin contributes to the infiltrative and metastatic behaviours of cancers. Germline loss-of-function mutations in *CDH1* are associated with the autosomal dominant cancer-predisposition syndrome, hereditary diffuse gastric cancer (HDGC) (OMIM: +192090).[1 2] In HDGC, germline mutations in *CDH1* confer a high lifetime risk of DGC for male and female mutation carriers.[3 4] Additionally, female mutation carriers have a 39%–52% lifetime risk of breast cancer, although these estimates have wide confidence intervals.[3 4] Multiple reports have established the association of lobular breast cancer (LBC) with HDGC and germline mutations in *CDH1*.[4–7]

Previously, we identified one carrier of a germline truncating *CDH1* mutation among 23 women with LBC known not to carry germline *BRCA1* and *BRCA2* mutations.[8] This case series included women diagnosed with LBC at a young age (≦45 years) and women diagnosed with LBC at any age with a family history of breast cancer but not of gastric cancer (1/23 or 4.3%).[8] The same mutation was subsequently confirmed in a relative of the mutation carrier who also had LBC. This coincidence of *CDH1* mutations and hereditary LBC led us to assess the prevalence of *CDH1* mutations in a series of 318 women with early-onset LBC or a family history of breast cancer, consistent with hereditary LBC, ascertained through breast cancer registries and high-risk cancer genetic clinics (Breast Cancer Family Registry (Breast CFR), the kConFab and a consortium of breast cancer genetics clinics in the United States and Spain).

MATERIALS AND METHODS

Patient accrual, preparation of DNA and *CDH1* sequencing, deletion analysis, mutation validation, and protein structure and functional analyses are described in the online supplementary material.

RESULTS

Germline DNAs from 327 eligible patients with LBC were analysed for variants in *CDH1*, but for nine samples, several exons failed to amplify, yielding incomplete results. Sequence analysis for heterozygous variants in the 318 patients with complete results did not detect any protein-truncating mutations. Multiplex Ligation-dependent Probe Amplification analyses in 134 patients did not reveal any large deletions in *CDH1*.

We did find 10 patients with non-synonymous variants. One non-synonymous change, c.1774G→A, p.A592T, was found in two patients and is a known germline variant that is not associated with risk of familial breast cancer or HDGC.[9 10] The variant, c.2494G→A, p.V832M, which had previously been identified in a patient with HDGC and was functionally characterised as a pathogenic mutation,[11 12] was found in a woman who was diagnosed as having LBC at the age of 43 years and had a family history of ductal breast cancer in a sister and unspecified breast cancer in a maternal aunt. Segregation analysis has not yet been performed. The remaining non-synonymous variants were novel and did not appear in any

public databases. These variants were c.8C→G, p.P3R; c.1223C→T, p.A408V; c.1297G→A, p.D433N; c.1813A→G, p.R695G and c.88 C→A, p.P30T, which were found in two patients not known to be related. There was no family history of gastric cancer for any of the patients who carried novel non-synonymous variants (table 1).

Nine unreported novel silent changes were identified: five synonymous variants in exons and four variants in introns. Two of these novel changes were found in more than one patient (data not shown).

We performed several tests to assess the likelihood that any of the non-synonymous variants resulted in a loss of normal function. Web-based software (Sorting Intolerant from Tolerant, SIFT) that predicts whether the amino acid change conferred by non-synonymous variants might alter protein structure, and thus possibly function, indicated that all but one variant, c.8C→G, p.P3R, which occurred in the signal peptide of the pre-protein, should be tolerated and therefore is unlikely to be pathogenic. Moreover, web-based software (Berkeley Drosophila Genome Project, Splice Site Prediction by Neural Network, Berkeley, Calif) did not predict alteration of splicing by any of the novel synonymous or non-synonymous variants or intronic variants identified.

The likely pathogenicities of the novel non-synonymous variants were further assessed by analysing the predicted effects of amino acid changes on the three-dimensional structure of E-cadherin. Because the coordinates of the three-dimensional structure of the ectodomain of E-cadherin were not available, we used the model of the closely related paralog, C-cadherin, to predict likely changes in the structure. One of the mutations, c.1223C→T, p.A408V, changes the alanine residue, which is well-conserved in this family of proteins, to bulkier valine and is located in calcium ion-binding extracellular domain 3. Surface modelling of the mutated protein indicated that this bulky valine could conceivably alter the binding pocket of one of three calcium ions that mediate homotypic cadherin domain interactions (Supplementary figure 1). Another mutation, c.1297G→A, p.D433N, was also found to be located in close proximity to this calcium-binding site (Supplementary figure 1). Because the c.8C→G, p.P3R variant occurs in the signal peptide of the precursor protein and had been predicted to be pathogenic, we hypothesised that this variant could result in mislocalisation or lack of expression of E-cadherin on the cell surface. To test this hypothesis, we expressed normal E-cadherin or each of the mutated versions of the protein in cells lacking endogenous E-cadherin. As seen in Supplementary figure 2, E-cadherin mutated with the c.8C→G, p.P3R variant did exhibit membrane localisation, indicating that protein localisation was not grossly affected by this variant. Additionally, the other novel non-synonymous variants also demonstrated membrane localisation (data not shown). However, because the levels at which we expressed E-cadherin were not physiological, it is possible that subtle effects of the mutations could have been missed.

Taking into account the in vitro and in silico analysis, four non-synonymous variants (c.8C→G, p.P3R; c.1223C→T, p.A408V; c.1297G→A, p.D433N and c.2494G→A, p.V832M) are considered potentially pathogenic (4/318 or 1.3%). If we only consider the subset of patients who have been tested and found not to carry *BRCA1* or *BRCA2* mutations, the prevalence of potentially pathogenic variants is 1.6% (4/246).

DISCUSSION

Germline mutations in *CDH1* are associated with a substantively increased risk of LBC.[8] This study found that the prevalence of potentially pathogenic *CDH1* variants is low in patients with early-onset or familial LBC who do not report a clear

Table 1 Clinical characteristics of patients with LBC with non-synonymous variants

Non-synonymous variant	Criteria 1 or 2	*BRCA1/2* mutation status	Age at diagnosis	Family history (age at diagnosis)
c.8C→G, p.P3R	1	Negative	38 years	Maternal aunt=breast cancer (46 years) Maternal aunt=breast cancer (67 years) Maternal cousin=breast cancer (42 years) Mother=retroperitoneal tumour Paternal grandmother=breast cancer
c.88 C→A, p.P30T (two patients)	1	Unknown	40 years	
	2	Negative	47 years	Paternal aunt=breast cancer (40 years) Female paternal cousin=breast cancer (40 years) Male paternal cousin=breast cancer (50 years) Female paternal cousin=breast cancer (47 years)
c.1223C→T, p.A408V	1	Negative	44 years	No cancers
c.1297G→A, p.D433N	1	Negative	41 years	Paternal grandmother=intestinal cancer Maternal grandmother=lung cancer Maternal grandfather=mouth cancer
c.1813A→G, p.R605G	1	Unknown	42 years	Mother=breast cancer (60 years) Maternal uncle=pancreatic cancer (64 years)
c. 2494G→A, p.V832M (known missense mutation in HDGC)	1	Negative	43 years	Sister=ductal breast cancer Maternal aunt=breast cancer Paternal uncle=leukaemia Paternal grandmother=colon cancer

Clinical history of patients with LBC in whom potentially pathogenic variants were identified. There was no known family history of gastric cancer in these patients.

family history of DGC. The large sample size increases the likelihood that the results in this setting are precise. This study highlights the utility of publicly available registries as valuable resources of clinically and epidemiologically annotated families with accompanying germline DNA for future research in this field.

It remains possible that *CDH1* mutations are present in rare families with multiple LBCs even without gastric cancer. Although the patients in the present study had confirmed LBC, we were unable to confirm the pathology of the breast cancers in the relatives, which remained unspecified for most of the patients. Additionally, because 72 patients (23%) were not tested for mutations in *BRCA1* and *BRCA2* (table 2), it is possible that some *BRCA1* and *BRCA2* mutation carriers were included in this study. The likelihood, however, is low because most early-onset and familial breast cancers are not accounted for by germline mutations in *BRCA1* and *BRCA2*.[13 14] We had previously reported a pathogenic truncating *CDH1* mutation in a patient with LBC and her mother, who had both developed LBC before age 45 years.[8] However, our data suggest that *CDH1*-associated LBC without gastric cancer must be very rare because so few were identified in the present study among women highly selected for early-onset LBC or LBC with additional breast cancer in the family. It might still be prudent to consider germline *CDH1* testing in families with confirmed multiple cases of early-onset LBC, even in the absence of a family history of gastric cancer. In such families, and in those with a reported but unspecified history of abdominal cancer, the possibility of ovarian cancer would lead to *BRCA1* and then *BRCA2* testing, and the possibility of DGC should lead to consideration of *CDH1* testing. For women with LBC, it is important to look for a family history of gastric cancer so that HDGC families will be recognised and offered appropriate management for their risk of DGC.

In our study, the pathogenic germline variant, p.V832M, was identified in a patient with LBC without a family history of gastric cancer. This variant was initially found to segregate with disease in a Japanese family where the proband had DGC at age 61 years and four of seven siblings, the mother and a niece all had unspecified gastric cancer. Functional characterisation in Chinese hamster ovary cells demonstrated reduced cell aggregation and increased invasive properties of the mutant compared with wild-type E-cadherin.[12] Although this effect was not reproduced in functional characterisation undertaken in human squamous epithelial cells,[15] further work has demonstrated a mechanism by which this mutation might confer a pathogenic effect, through loss of type Iγ phosphatidylinositol phosphate kinase binding, causing abnormal E-cadherin trafficking and adherens junction formation.[16]

The novel non-synonymous variants in this study were not confirmed by our in vitro and in silico studies to be pathogenic, although further investigation needs to be done on the suggestive evidence that the variants c.1223C→T, p.A408V and c.1297G→A, p.D433N might interfere with calcium-dependent homophilic binding. Also, a novel, presumably rare variant (c.88 C→A, p.P30T) was shared by two patients with LBC from one of the high-risk breast cancer clinics: this could imply that this variant is linked to the disease and that these two women are distantly related. Alternatively, this may represent a rare variant not associated with LBC, whose distribution in the normal population frequency will become known as the genomes of more people are sequenced. Data from the 1000 Genomes Project may also be helpful in the interpretation of the significance of these variants, through demonstration of the full profile of

Table 2 Criteria for ascertainment for *CDH1* mutation analysis

Breast Cancer Genetics Consortium							
Criteria for ascertainment	Patients (n=120)	Sex	Median age (years)	Age range (years)	Novel non-synonymous variants in criteria	*BRCA1/2* negative (n=66)	*BRCA1/2* unknown (n=54)
Criteria 1	66	F	40	(28–44) (n=66)	2	36	34
Criteria 2	54	F	54	(45–72) (n=54)	1	30	20
Breast CFR							
Criteria for ascertainment	Patients (n=165)	Sex	Median age (years)	Age range (years)	Novel non-synonymous variants in criteria	*BRCA1/2* negative (n=147)	*BRCA1/2* unknown (n=18)
Criteria 1	142	F	40	(31–44) (n=51)	3	130	12
Criteria 2	23	F	57	(45–79) (n=20)	0	17	6
kConFab							
Criteria for ascertainment	Patients (n=33)	Sex	Median age (years)	Age range (years)	Novel non-synonymous variants in criteria	*BRCA1/2* negative (n=33)	*BRCA1/2* unknown
Criteria 1	6	F	40	(37–43) (n=6)	0	6	0
Criteria 2	27	F	57	(45–77) (n=28)	0	27	0
All samples							
Criteria for ascertainment	Patients (n=318)	Sex	Median age (years)	Age range (years)	Novel non-synonymous variants in criteria	*BRCA1/2* negative (n=246)	*BRCA1/2* unknown (n=72)
Criteria 1	214	F	40	(28–44) (n=123)	5	172	46
Criteria 2	104	F	56	(45–79) (n=101)	1	74	26

The criteria for ascertainment were a patient with a history of lobular or mixed ductal and lobular pathology whose *BRCA1* and *BRCA2* mutation status was negative or unknown and either diagnosed before age 45 years or diagnosed at any age but with two or more cases of breast cancer in first- or second-degree relatives.

normal variation within *CDH1* and their distribution in and across populations.

Although a combination of LBC and DGC is strongly indicative of germline mutations in *CDH1*, in the absence of a history of DGC, *CDH1* mutations appear to be extremely rare. It is possible that *CDH1* mutations would be more often identified in families with multiple documented invasive lobular or mixed ductal/lobular breast cancers in the absence of DGC, but such families are uncommon. Therefore, a history of early-onset or familial LBC should trigger specific questions around a history of abdominal cancer.

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REFERENCES

1. **Guilford P,** Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998;**392**:402—5.
2. **Oliveira C,** Bordin MC, Grehan N, Huntsman D, Suriano G, Machado JC, Kiviluoto T, Aaltonen L, Jackson CE, Seruca R, Caldas C. Screening E-cadherin in gastric cancer families reveals germline mutations only in hereditary diffuse gastric cancer kindred. *Hum Mutat* 2002;**19**:510—17.
3. **Pharoah PD,** Guilford P, Caldas C. International Gastric Cancer Linkage Consortium. Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. *Gastroenterology* 2001;**121**:1348—53.
4. **Kaurah P,** MacMillan A, Boyd N, Senz J, De Luca A, Chun N, Suriano G, Zaor S, Van Manen L, Gilpin C, Nikkel S, Connolly-Wilson M, Weissman S, Rubinstein WS, Sebold C, Greenstein R, Stroop J, Yim D, Panzini B, McKinnon W, Greenblatt M, Wirtzfeld D, Fontaine D, Coit D, Yoon S, Chung D, Lauwers G, Pizzuti A, Vaccaro C, Redal MA, Oliveira C, Tischkowitz M, Olschwang S, Gallinger S, Lynch H, Green J, Ford J, Pharoah P, Fernandez B, Huntsman D. Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. *JAMA* 2007;**297**:2360—72.
5. **Keller G,** Vogelsang H, Becker I, Hutter J, Ott K, Candidus S, Grundei T, Becker KF, Mueller J, Siewert JR, Höfler H. Diffuse type gastric and lobular breast carcinoma in a familial gastric cancer patient with an E-cadherin germline mutation. *Am J Pathol* 1999;**155**:337—42.
6. **Brooks-Wilson AR,** Kaurah P, Suriano G, Leach S, Senz J, Grehan N, Butterfield YS, Jeyes J, Schinas J, Bacani J, Kelsey M, Ferreira P, MacGillivray B, MacLeod P, Micek M, Ford J, Foulkes W, Australie K, Greenberg C, LaPointe M, Gilpin C, Nikkel S, Gilchrist D, Hughes R, Jackson CE, Monaghan KG, Oliveira MJ, Seruca R, Gallinger S, Caldas C, Huntsman D. Germline E-cadherin mutations in hereditary diffuse gastric cancer: assessment of 42 new families and review of genetic screening criteria. *J Med Genet* 2004;**41**:508—17.
7. **Suriano G,** Yew S, Ferreira P, Senz J, Kaurah P, Ford JM, Longacre TA, Norton JA, Chun N, Young S, Oliveira MJ, Macgillivray B, Rao A, Sears D, Jackson CE, Boyd J, Yee C, Deters C, Pai GS, Hammond LS, McGivern BJ, Medgyesy D, Sartz D, Arun B, Oelschlager BK, Upton MP, Neufeld-Kaiser W, Silva OE, Donenberg TR, Kooby DA, Sharma S, Jonsson BA, Gronberg H, Gallinger S, Seruca R, Lynch H, Huntsman DG. Characterization of a recurrent germ line mutation of the E-cadherin gene: implications for genetic testing and clinical management. *Clin Cancer Res* 2005;**11**:5401—9.
8. **Masciari S,** Larsson N, Senz J, Boyd N, Kaurah P, Kandel MJ, Harris LN, Pinheiro HC, Troussard A, Miron P, Tung N, Oliveira C, Collins L, Schnitt S, Garber JE, Huntsman D. Germline E-Cadherin mutations in familial lobular breast cancer. *J Med Genet* 2007;**44**:726—31.
9. **Salahshor S,** Haixin L, Huo H, Kristensen VN, Loman N, Sjoberg-Margolin S, Borg A, Børresen-Dale AL, Vorechovsky I, Lindblom A. Low frequency of E-cadherin alterations in familial breast cancer. *Breast Cancer Res* 2001;**3**:199—207.
10. **Keller G,** Vogelsang H, Becker I, Plaschke S, Ott K, Suriano G, Mateus AR, Seruca R, Biedermann K, Huntsman D, Döring C, Holinski-Feder E, Neutzling A, Siewert JR, Höfler H. Germline mutations of the E-cadherin(CDH1) and TP53 genes, rather than of RUNX3 and HPP1, contribute to genetic predisposition in German gastric cancer patients. *J Med Genet* 2004;**41**:e89.

11. **Yabuta T,** Shinmura K, Tani M, Yamaguchi S, Yoshimura K, Katai H, Nakajima T, Mochiki E, Tsujinaka T, Takami M, Hirose K, Yamaguchi A, Takenoshita S, Yokota J. E-cadherin gene variants in gastric cancer families whose probands are diagnosed with diffuse gastric cancer. *Int J Cancer* 2002;**101**:434—41.
12. **Suriano G,** Mulholland D, de Wever O, Ferreira P, Mateus AR, Bruyneel E, Nelson CC, Mareel MM, Yokota J, Huntsman D, Seruca R. The intracellular E-cadherin germline mutation V832 M lacks the ability to mediate cell-cell adhesion and to suppress invasion. *Oncogene* 2003;**22**:5716—19.
13. **Mann GJ,** Thorne H, Balleine RL, Butow PN, Clarke CL, Edkins E, Evans GM, Fereday S, Haan E, Gattas M, Giles GG, Goldblatt J, Hopper JL, Kirk J, Leary JA, Lindeman G, Niedermayr E, Phillips KA, Picken S, Pupo GM, Saunders C, Scott CL, Spurdle AB, Suthers G, Tucker K, Chenevix-Trench G. Kathleen Cuningham Consortium for Research in Familial Breast Cancer. Analysis of cancer risk and BRCA1 and BRCA2 mutation prevalence in the kConFab familial breast cancer resource. *Breast Cancer Res* 2006;**8**:R12.
14. **Neuhausen SL,** Ozcelik H, Southey MC, John EM, Godwin AK, Chung W, Iriondo-Perez J, Miron A, Santella RM, Whittemore A, Andrulis IL, Buys SS, Daly MB, Hopper JL, Seminara D, Senie RT, Terry MB. Breast Cancer Family Registry. BRCA1 and BRCA2 mutation carriers in the Breast Cancer Family Registry: an open resource for collaborative research. *Breast Cancer Res Treat* 2009;**116**:379—86.
15. **Curtis MW,** Ly QP, Wheelock MJ, Johnson KR. Evidence that the V832M E-cadherin germ-line missense mutation does not influence the affinity of alpha -catenin for the cadherin/catenin complex. *Cell Commun Adhes* 2007;**14**:45—55.
16. **Ling K,** Bairstow SF, Carbonara C, Turbin DA, Huntsman DG, Anderson RA. Type Igamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin. *J Cell Biol* 2007;**176**:343—53.

SUPPLEMENTARY MATERIALS AND METHODS:

Patient accrual:

327 LBC cases were identified through three different sources. The Breast Cancer Family Registry (Breast CFR) is an NCI-sponsored resource, which includes six population-based and clinic-based family registries and a collection of samples and data from more than 12,500 families with and without breast cancer [1]. Samples (n=168) were obtained from the Northern California, New York, Australia, Philadelphia, and Ontario sites of the Breast CFR. The Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab) provided specimens (n=33) from families with a strong history of breast cancer, recruited from family cancer clinics in Australia and New Zealand [2]. The 126 remaining samples were collected through a Breast Cancer Research Foundation (BCRF)-funded Breast Cancer Genetics Consortium, a group of high-risk cancer clinics which included Dana-Farber Cancer Institute, Baylor College of Medicine, Beth Israel Deaconess Medical Center, Georgetown University, Massachusetts General Hospital, Memorial Sloan Kettering Cancer Center, Stanford University, the University of Chicago, the University of Pennsylvania, and the Hospital Vall d'Hebron, Barcelona, Spain. The inclusion criteria for the identification of the eligible LBC cases differed slightly between these groups. For cases from the Breast CFR and kConFab, eligibility for this study required a female case with documented invasive lobular or mixed (lobular and ductal) breast cancer, not known to carry germline *BRCA1* and *BRCA2* mutations, and either: (1) diagnosed before age 45 years, or (2) at any age but with two or more cases of breast cancer in first- or second-degree relatives. For cases from the BCRF-funded Breast Cancer Genetics Consortium, eligible women had a

diagnosis of invasive lobular or mixed (lobular and ductal) breast cancer, were not known to carry germline *BRCA1* and *BRCA2* mutations, and were either: (1) diagnosed before age 45 years, or (2) diagnosed at any age but with at least two or more cases of breast cancer in first- or second-degree relatives, and third-degree relatives in the paternal lineage, and with no reported family history of gastric cancer. The characteristics of the LBC cases screened for mutations in *CDH1* are summarized in Table 1. All cases had provided written informed consent and the study protocols were approved by the institutional review board at each participating center. DNA was extracted at the molecular laboratories for some of the collaborating centers using standard procedures (Qiamp DNA Blood Midi kit; Qiagen, Valencia, CA, USA) and anonymized genomic DNA samples were sent to the Centre for Translational and Applied Genomics (CTAG) at the British Columbia Cancer Agency (BCCA) where the analysis of *CDH1* was performed (Table 1).

Preparation of DNA and *CDH1* sequencing:

To accommodate the limited amount of DNA available, genomic DNA samples were subjected to whole-genome amplification using the GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences Inc., Quebec, Canada) as performed in our earlier study [3]. Exons and intron-exon boundary splice junctions of half the study cohort were amplified and screened for heterozygous base changes by the denaturing high pressure liquid chromatography (DHPLC) [3]. The primer sequences and conditions used have previously been described [4]. Exons displaying DHPLC changes consistent with a heterozygous variation were reamplified and PCR products were purified (Qiagen

MinElute; Qiagen, Mississauga, ON). Bidirectional sequencing was then performed (Big Dye Terminator V.3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, California, USA) and analysed (ABI Prism 310 Genetic Analyzer). Germline mutations in *CDH1* appear as heterozygous sequence changes, with the exception of large deletions, which are not detected by sequencing. Sequencing of all exons and intron-exon boundaries were carried out on the remaining samples using validated primer sets [5], at the Genome Sciences Centre on a service basis or in our laboratory.

Deletion Analysis:

Multiplex Ligation-dependant Probe Amplification (MLPA), previously used to identify large-scale deletions in *CDH1*, has been described previously [6]. MLPA could only be performed on 134 samples for which sufficient germline DNA was available.

Mutation Validation:

Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare sample sequences to the NCBI cDNA NM_004360.3 and the genomic DNA NG_008021.1, GI:190341080. Web-based software programs were used to look for predicted effects on splicing (Splice Site Prediction by Neural Network hosted by the Berkeley Drosophila Genome Project) and the predicted effects of amino acid changes on protein structure (Sorting Intolerant From Tolerant, SIFT, software version 2, Fred Hutchinson Cancer Research Center, Seattle, Wash).

Protein structure analysis:

Protein structure analysis and preparation of structural models was performed using the PyMol software package (DeLano Scientific). Structural co-ordinates were obtained from X-ray crystal structures of the ectodomain of C-cadherin (PDB code 1L3W) [7].

Functional Characterization:

Site directed mutagenesis:

To examine the effects of the missense mutations on protein localization, wild-type E-cadherin-WTpcDNA3.1 plasmids [8] were mutated using QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, Texas) as per the manufacturer's instructions to create each of the novel non-synonymous variants we identified. The corresponding forward primer sequences are included following each variant: c.1223C>T, A408V, 5'-CCCCAATACCCCAGTGTGGGAGGCTGTAT-3'; c.8C>G, P3R, 5'-CTTACCATGGGCCGTTGGAGCCGCAGC-3'; c.88 C>A, P30T, 5'-GGAGCCCTGCCACACTGGCTTTGACGC-3'; c.1813A>G, 5'-ACTATATTCTTCTGTGAGGGGAATCCAAAGCCTCAGG -3'; c.1297G>A, D433N, 5'-CCACAAATCCAGTGAACAACAATGGCATTTTGAAAACAGCA-3'). The mutated plasmids were validated by DNA-sequencing.

Cell culture:

The immortalized ovarian surface epithelial cell-line, IOSE-80pc, that does not express E-cadherin [9] and the ovarian carcinoma cell line, OVCAR-3, that highly expresses E-cadherin [9], were maintained in a 1:1 mixture of MCDB 105 medium and Medium 199

(Sigma, St. Louis, MO, USA), supplemented with 5% fetal bovine serum (FBS) (Gibco BRL).

Transient transfections:

Aliquots of IOSE-80pc cells were transfected in parallel with the E-cadherin variants under investigation; c.1223C>T, A408V; c.8C>G, P3R; c.88 C>A, P30T; c.1813A>G, R695G; c.1297G>A, D433N and control plasmids. These controls included the empty vector LacZ as a control for the transfection procedure, wild-type (wt) E-cadherin and known loss of function E-cadherin mutants (c.1018A>G and c.2494G>A) that cause HDGC. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines.

Fluorescence microscopy:

E-cadherin staining was performed on subconfluent cell monolayers cultured on glass coverslips. Cells were washed once in media alone and then fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were washed with phosphate-buffered saline (PBS) before and after permeabilization with 0.2% Triton X-100 for 10min at room temperature. To decrease non-specific background, cells were incubated with serum-free protein block (Dako, Carpinteria, CA) for 30 minutes prior to incubation with a mouse monoclonal antibody [1:500] to the extracellular domain (HECD-1, 205601 Calbiochem) at room temperature. Primary antibody was detected using Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) [1:500] for 60 minutes at room temperature. Cells were counterstained with DAPI [1:1000] prior to

mounting with glycerol. Slides were analyzed using the Axioplan 2, Zeiss (MetaSystems, Isis) camera VAC-30054 and pictures were obtained at 40x magnification.

References

[1] John EM, Hopper JL, Beck JC, Knight JA, Neuhausen SL, Senie RT, et al. The Breast Cancer Family Registry: an infrastructure for cooperative multinational, interdisciplinary and translational studies of the genetic epidemiology of breast cancer. *Breast Cancer Res* 2004;**6**(4):R375-89.

[2] Mann GJ, Thorne H, Balleine RL, Butow PN, Clarke CL, Edkins E, et al. Analysis of cancer risk and BRCA1 and BRCA2 mutation prevalence in the kConFab familial breast cancer resource. *Breast Cancer Res* 2006;**8**(1):R12.

[3] Masciari S, Larsson N, Senz J, Boyd N, Kaurah P, Kandel MJ, et al. Germline E-Cadherin mutations in familial lobular breast cancer. *J Med Genet* 2007;**44**(11):726-31.

[4] Suriano G, Yew S, Ferreira P, Senz J, Kaurah P, Ford JM, et al. Characterization of a recurrent germ line mutation of the E-cadherin gene: implications for genetic testing and clinical management. *Clin Cancer Res* 2005 Aug 1;**11**(15):5401-5409.

[5] Mullins FM, Dietz L, Lay M, Zehnder JL, Ford J, Chun N, et al. Identification of an intronic single nucleotide polymorphism leading to allele dropout during validation of a CDH1 sequencing assay: implications for designing polymerase chain reaction-based assays. *Genet Med* 2007 Nov;**9**(11):752-760.

[6] Oliveira C, Senz J, Kaurah P, Pinheiro H, Sanges R, Haegert A, et al. Germline CDH1 Deletions in Hereditary Diffuse Gastric Cancer Families. *Hum Mol Genet* 2009 May 1;**18**(9):1545-55.

[7] Boggon TJ, Murray J, Chappuis-Flament S, Wong E, Gumbiner BM, Shapiro L. C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* 2002 May 17;**296**(5571):1308-1313.

[8] Suriano G, Mulholland D, de Wever O, Ferreira P, Mateus AR, Bruyneel E, et al. The intracellular E-cadherin germline mutation V832 M lacks the ability to mediate cell-cell adhesion and to suppress invasion. *Oncogene* 2003 Aug 28;**22**(36):5716-5719.

[9] Wu C, Cipollone J, Maines-Bandiera S, Tan C, Karsan A, Auersperg N, et al. The morphogenic function of E-cadherin-mediated adherens junctions in epithelial ovarian carcinoma formation and progression. *Differentiation* 2008 Feb;**76**(2):193-205.