Germline mutations in \textit{CDH1} are infrequent in women with early-onset or familial lobular breast cancers


\section*{ABSTRACT}

\textbf{Background} Germline mutations in \textit{CDH1} are associated with hereditary diffuse gastric cancer; lobular breast cancer also occurs excessively in families with such condition.

\textbf{Method} To determine if \textit{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 \textit{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 \textit{BRCA1} or \textit{BRCA2}.

\textbf{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.

\textbf{Conclusion} Potentially pathogenic germline \textit{CDH1} mutations in women with early-onset or familial lobular breast cancer are at most infrequent.

\section*{INTRODUCTION}

\textit{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 \textit{CDH1} are associated with the autosomal dominant cancer-predisposition syndrome, hereditary diffuse gastric cancer (HDGC) (OMIM: +192090).\textsuperscript{1,2} In HDGC, germline mutations in \textit{CDH1} confer a high lifetime risk of DGC for male and female mutation carriers.\textsuperscript{3,4} Additionally, female mutation carriers have a 39\%–52\% lifetime risk of breast cancer, although these estimates have wide confidence intervals.\textsuperscript{3,4} Multiple reports have established the association of lobular breast cancer (LBC) with HDGC and germline mutations in \textit{CDH1}.\textsuperscript{4–7}

Previously, we identified one carrier of a germline truncating \textit{CDH1} mutation among 23 women with LBC known not to carry germline \textit{BRCA1} and \textit{BRCA2} mutations.\textsuperscript{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 (1/25 or 4.3\%).\textsuperscript{9} The same mutation was subsequently confirmed in a relative of the mutation carrier who also had LBC. This coincidence of \textit{CDH1} mutations and hereditary LBC led us to assess the prevalence of \textit{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 CCR), the kConFab and a consortium of breast cancer genetics clinics in the United States and Spain).

\section*{MATERIALS AND METHODS}

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

\section*{RESULTS}

Germline DNAs from 327 eligible patients with LBC were analysed for variants in \textit{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 \textit{CDH1}.

We did find 10 patients with non-synonymous changes. One non-synonymous change, c.1774G\textsuperscript{+}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.\textsuperscript{9,10} The variant, c.2494G\textsuperscript{+}A, p.V832M, which had previously been identified in a patient with HDGC and was functionally characterised as a pathogenic mutation,\textsuperscript{11,12} was found in a woman who was diagnosed as having LBC at the age of 45 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 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. One of the mutations, c.8C→G, p.P3R, which occurred 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.

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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 (4/518 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 substantially 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

<table>
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<tr>
<th>Non-synonymous variant</th>
<th>Criteria 1 or 2</th>
<th>BRCA1/2 mutation status</th>
<th>Age at diagnosis</th>
<th>Family history (age at diagnosis)</th>
</tr>
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<tbody>
<tr>
<td>c.8C→G, p.P3R</td>
<td>1</td>
<td>Negative</td>
<td>38 years</td>
<td>Maternal aunt= breast cancer (46 years)</td>
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<td></td>
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<td>Maternal aunt= breast cancer (67 years)</td>
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<td>Maternal cousin= breast cancer (42 years)</td>
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<td></td>
<td>Mother=retropertioneal tumour</td>
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<td></td>
<td></td>
<td>Paternal grandmother= breast cancer</td>
</tr>
<tr>
<td>c.88 C→A, p.P30T</td>
<td>1</td>
<td>Unknown</td>
<td>40 years</td>
<td>Paternal aunt= breast cancer (40 years)</td>
</tr>
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<td></td>
<td>2</td>
<td>Negative</td>
<td>47 years</td>
<td>Female paternal cousin= breast cancer (40 years)</td>
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<td></td>
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<td></td>
<td>Male paternal cousin= breast cancer (50 years)</td>
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<td></td>
<td></td>
<td>Female paternal cousin= breast cancer (47 years)</td>
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<tr>
<td>c.1223C→T, p.A408V</td>
<td>1</td>
<td>Negative</td>
<td>44 years</td>
<td>No cancers</td>
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<tr>
<td>c.1297G→A, p.D433N</td>
<td>1</td>
<td>Negative</td>
<td>41 years</td>
<td>Paternal grandmother= intestinal cancer</td>
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<td>Maternal grandmother= lung cancer</td>
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<td>Maternal grandfather= mouth cancer</td>
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<tr>
<td>c.1813A→G, p.R605G</td>
<td>1</td>
<td>Unknown</td>
<td>42 years</td>
<td>Mother= breast cancer (60 years)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Maternal uncle= pancreatic cancer (64 years)</td>
</tr>
<tr>
<td>c.2494G→A, p.V832M</td>
<td>1</td>
<td>Negative</td>
<td>43 years</td>
<td>Sister= ductal breast cancer</td>
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<td>Paternal aunt= breast cancer</td>
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<td>Paternal uncle= leukaemia</td>
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<td>Paternal grandmother= colon cancer</td>
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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.
a family history of gastric cancer. In such families, and in those with a reported
of gastric cancer. In such families, and in those with a reported
CDH1
mutations.13 14 We had previously
confirmed our in vitro and in silico studies to be pathogenic,
and familial breast cancers are not accounted for by germline
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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 across a history of abdominal cancer.

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Competing interests None declared.

Ethics approval This study was conducted with the approval of the British Columbia Cancer Agency.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES


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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 \textit{BRCA1} and \textit{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 \textit{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 \textit{CDH1} was performed (Table 1).

\textbf{Preparation of DNA and \textit{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
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-dependent 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’-CCCCAATACCCCAATGCAGTGAGGTGCTGTAT-3’; c.8C>G, P3R, 5’-CTTACCATGGGGCGTTGGAGCCGCAGC-3’; c.88 C>A, P30T, 5’-GGAGCCCTGCCCCACTGGCTTTGACGC-3’; c.88 C>A, P30T, 5’-GGAGCCCTGCCCCACTGGCTTTGACGC-3’; c.1813A>G, 5’-ACTATATTTCCTCTGTGAGGGGAATCCAAAGCCTCAGG-3’; c.1297G>A, D433N, 5’-CCACAAATCCAGTGAACAAATGGGCTTTTGGAAAACAGCA-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 10 min 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


