Germline E-Cadherin mutations in familial lobular breast cancer

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Keywords: Lobular Breast Cancer, germline mutations, CDH1, familial breast cancer

Word Count: Abstract 250 words
Letter 2997 words
Abstract

Background: The cell surface glycoprotein, E-Cadherin (CDH1) is a key regulator of adhesive properties in epithelial cells. Germline mutations in CDH1 are well-established as the defects underlying the Hereditary Diffuse Gastric Cancer (HDGC) syndrome: an increased risk of lobular breast cancer (LBC) has been described in HDGC kindreds. However, germline CDH1 mutations have not been described in LBC patients outside of HDGC families. We sought to investigate the frequency of germline CDH1 mutations in LBC patients with early onset disease or family histories of breast cancer without DGC.

Methods: Germline DNA was analyzed in 23 women with invasive lobular or mixed ductal and lobular breast cancers who had at least one close relative with breast cancer or had themselves been diagnosed before age 45, had tested negative for a germline BRCA1 or BRCA2 mutation, and reported no personal or family history of diffuse gastric cancer. The full coding sequence of CDH1 including splice junctions was PCR amplified and screened for mutations using DHPLC and sequencing.

Results: A novel germline CDH1 truncating mutation in the extracellular portion of the protein (517insA) was identified in one subject who had lobular breast cancer at age 42 and a first degree relative with invasive lobular breast cancer.

Conclusions: Germline CDH1 mutations can be associated with invasive lobular breast cancer in the absence of diffuse gastric cancer. The finding, if confirmed, may have implications for management of individuals at risk for this breast cancer subtype, and compels clarification of the cancer risks in the syndrome.

Keywords: Lobular Breast Cancer, germline mutations, CDH1, familial breast cancer

Key points:
- Germline CDH1 mutation was found in a woman with Lobular Breast Cancer (LBC) and family history of breast cancer but not diffuse gastric cancer.
- LOH was demonstrated in tumor specimen from the mutation carrier.
- These results, if confirmed, have implications for the genetic basis of LBC and for the identification and management of individuals at risk.
Introduction

The existence of a strong hereditary predisposition to breast cancer has been recognized for more than a century. Germline mutations in \textit{BRCA1} and \textit{BRCA2} have been shown to account for approximately one-third of hereditary breast cancers, among young women with the disease. Mutations in other genes such as \textit{TP53}, \textit{PTEN}, \textit{STK11}, \textit{CHEK2} and \textit{ATM} account for a small proportion of hereditary breast cancer syndromes, often with distinct clinical features. However, in many breast cancer families, no predisposing gene mutation can be identified. Although the existence of other strongly predisposing genes is controversial, the search for additional breast cancer susceptibility genes remains an active area of investigation.

The \textit{CDH1} (epithelial cadherin, OMIM 192090) gene is composed of 16 exons located on chromosome 16q22.1. The calcium-dependent molecule E-cadherin, a key regulator of cell adhesion, is the protein product of \textit{CDH1}, and is commonly used in the immunohistochemical evaluation of breast cancers, discriminating between lobular and ductal histologies. Germline inactivating mutations in the \textit{CDH1} account for one third of hereditary diffuse gastric cancer (HDGC) kindreds, defined as having two or more cases of DGC in first degree relatives, with at least one documented case of DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50 or, multiple cases of gastric cancer of which at least one is confirmed as DGC before age 50. Germline \textit{CDH1} mutations are inherited in an autosomal dominant manner, and are highly penetrant, conferring a cumulative risk of DGC of 67% in men and 83% in women. Recently, an excess of invasive lobular (including mixed ductal and lobular histology) breast cancers has been reported in families with HDGC. Like DGC, LBC show histologic features consistent with loss of cell-to-cell adhesiveness, and the absence of E-cadherin by immunohistochemical techniques in a substantial majority. Moreover, as sporadic DGC, more than 50% of sporadic infiltrating LBC harbour inactivating somatic \textit{CDH1} mutations accompanied by loss of heterozygosity. We identified probands with invasive lobular or mixed ductal and lobular breast cancer and either early age at diagnosis or family history of breast cancer systematically from a breast cancer databank, and analyzed their peripheral lymphocyte DNA to assess possible germline mutations in the \textit{CDH1} gene.
Material and Methods

A group of subjects from the Dana Farber Cancer Institute were retrospectively identified from among women with breast cancer who had provided signed informed consent for an Institutional Review Board-approved banking protocol. The consent permitted collection, storage and analysis for research of medical records, peripheral blood and tumor specimens. Participants also completed a risk factor questionnaire including unconfirmed family cancer history information stored in a linked database. Specimens were stored in the annotated Dana-Farber/Harvard Cancer Center SPORE CORE Laboratory Blood Repository, which has been maintained since 2000. The criteria for the identification of the index cases were established at the beginning of the collection and included subjects who had documented invasive lobular or mixed ductal and lobular breast cancer at any age, no reported relatives with gastric tumors and either (1) family history with two or more cases of breast cancer in first or second degree relatives in the maternal and paternal lineage, including third degree relatives in the paternal lineage; or (2) lobular or mixed breast cancer diagnosed in the proband before 45 years of age independent of family history (Table 1). Because lobular breast cancers are observed in carriers of germline BRCA1 and BRCA2 mutations, the cohort was restricted to women whose germline BRCA1/2 status was known. Those with germline mutations in the BRCA1/2 breast cancer susceptibility genes were excluded from the analysis. BRCA1/2 rearrangement analyses (BART™, Myriad Genetic Laboratories, Salt Lake City, UT) had been performed clinically on only 2 probands: however, the prevalence of BART-detected mutations is no more than 3% in “severely-affected” kindreds (R. Wenstrup, Myriad Genetics, personal communication) so was not performed on our cohort.

Three hundred thirty women with invasive lobular or mixed ductal and lobular breast cancer were identified from the data bank, which has enrolled more than 2000 newly diagnosed breast cancer patients at Dana Farber Cancer Institute since 1999. Family history information provided by the patient at enrollment was available in more than 90% of cases, but could not be directly confirmed under the terms of the protocol which precluded further patient contact. Forty-eight of these women had had DNA analyzed for BRCA1 or BRCA2 germline mutations, identified in clinical testing or in the course of other research. Among these, five were excluded because of a positive BRCA1 (n=2) or BRCA2 (n=3) mutation identified by sequence analysis in the patient or close relatives. Among the forty-three subjects meeting histologic criteria who had tested negative for BRCA1 and BRCA2 mutations, nine subjects were excluded because there was no blood specimen for DNA extraction available from the core laboratory, and 11 were excluded because of failure to meet age or family history criteria. Therefore, the analysis was limited to 23 women with documented invasive lobular (9) or mixed ductal and lobular (14) breast cancers who had previously tested negative for germline BRCA1 and BRCA2 mutations (Figure 1). Nineteen of these women met the first eligibility criterion; four women met the second. The median age at breast cancer diagnosis was 45 years (range 36-66 years) for the entire group, 46 years (range 36-66 years) for women meeting the first criterion and 40.5 (range 36-42 years) years for those in the age-related category. Medical record documentation of histopathology was assembled. Family history was confirmed when it was possible with medical records. All pathology slides were reviewed at time of clinical evaluation at the Brigham and...
Women’s Hospital. After the cohort was finalized and clinical information linked to specimens, all identifiers were removed, in accordance with protocol stipulations. Genomic DNA was extracted from blood samples in the Dana Farber/Harvard Cancer Center Breast Cancer SPORE core laboratory at Dana Farber Cancer Institute using a Qiamp DNA Blood Midi kit (Qiagen, Valencia, CA). Analyses detailed below were performed at the Centre for Translational and Applied Genomics at the British Columbia Cancer Agency, Vancouver, B.C. (DH, director).

Table 1
Study criteria of subject’s inclusion for E-cadherin mutation analysis.

<table>
<thead>
<tr>
<th>Criteria for Inclusion</th>
<th>Cases (N)</th>
<th>Median Age</th>
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<tbody>
<tr>
<td>Proband with documented invasive lobular or mixed ductal and lobular breast cancers tested negative for germline BRCA1 and BRCA2 mutations and has:</td>
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<tr>
<td>1. Family history with two or more cases of breast cancer in first or second degree relatives in the maternal or paternal lineage, including third degree relatives in the paternal lineage; or</td>
<td>19</td>
<td>46 (range 36-66)</td>
</tr>
<tr>
<td>2. Proband diagnosed with lobular or mixed breast cancer before 45 years of age independent of family history.</td>
<td>4</td>
<td>40.5 (range 36-42)</td>
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**CDH1 analysis**

Mutational Analysis:

Samples with insufficient DNA for complete mutational analysis of CDH1 underwent whole genome amplification using the GenomiPhi DNA amplification kit (Amersham Biosciences) according to the manufacturer’s instructions. Briefly, 10 ng of DNA (10ng/uL) was mixed with 9uL of sample buffer containing random hexamer primers and heated to 95°C for 3min. After cooling, 9uL of reaction buffer and 1uL of enzyme (Phi29 DNA polymerase) was added to the sample and incubated at 30°C for 18hrs. The sample was then heated to 65°C for 10 min. to inactivate the enzyme. Amplified DNA was purified by ethanol precipitation prior to DHPLC (denaturing high performance liquid chromatography) analysis. The full coding sequence of CDH1 including splice junctions was PCR amplified and screened for mutations using DHPLC. Primer sequences and conditions are as previously described. Primer sequences and conditions are as previously described. Primer sequences and conditions are as previously described.

PCR products, which had shown a potential variant with DHPLC, were sequenced in both directions starting from a fresh PCR product. Prior to sequencing, the PCR products were purified using the Qiagen MinElute gel extraction kit (Qiagen, Mississauga, ON). Sequencing was then performed using the Big Dye Terminator V3.1 cycle sequencing kit and analyzed using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City CA).
**CDH1 promoter methylation analysis**

CDH1 promoter methylation analysis was performed in microdissected tumor material from the proband. DNA was extracted using the Invisorb Spin Tissue Mini Kit (Invitek) following manufacturer’s instructions. Approximately 200ng of DNA were treated with EpiTect Bisulfite Kit (Qiagen). Unmethylated Cytosines were converted to Uracil while methylated ones remained unmodified. Bisulfite treated DNA from white blood cells was *in vitro* methylated with M.SssI DNA MeTase and used as a positive control for methylation determination. The CDH1 promoter CpG island 3 was PCR-amplified using flanking primers (sequences available upon request), specifically designed for bisulfite treated DNA sequences without CpG sites, and sequenced for methylation status determination.

**LOH analysis**

Loss of Heterozygosity (LOH) analysis was performed using microdissected tumor and DNA extracted from adjacent normal tissue. The CDH1 promoter common polymorphism -160C/A as well as the CDH1 exon 4 mutation site were used as intragenic markers for LOH analysis in DNA extracted from tumor and normal material from the proband. Moreover, DNA was PCR amplified and sequenced for each site with the aim of determining whether the wild-type allele was under-represented in tumor DNA when compared to the sequencing profiles obtained from normal breast epithelia and constitutional DNA.
Results
Among 23 women with documented invasive lobular or mixed ductal and lobular breast cancer who had previously tested negative for germline BRCA1 and BRCA2 mutations, one novel mutation in the CDH1 gene was detected by DHPLC and confirmed by direct sequencing (Figure 2B). The 517insA mutation is located near the 5’ end of the CDH1 gene, and results in a premature stop codon, eliminating all of the transmembrane and intracellular domains and the majority of the extra-cellular domain of the protein.

The mutation 517insA was found in a woman whose LBC was diagnosed at age 42 and whose mother reportedly had lobular breast cancer at age 28. The diagnosis of the mother was confirmed with her doctor’s notes. No other breast or gastric cancers were reported in the family (Figure 2A). The proband’s breast cancer was negative for E-cadherin (CDH1) by immunohistochemistry indicating that a second molecular event, towards the complete inactivation of the CDH1 gene, had occurred (Figure 2C, D). We searched for the inactivation of the wild-type allele in a microdissected tumor sample from the proband. Promoter methylation analysis was performed and no methylated alleles were found (data not shown). Subsequently, LOH analysis was performed in the same tumor sample using CDH1 distal and proximal microsatellite markers as well as intragenic markers. No loss of genetic material was found using CDH1 flanking markers (data not shown).

The LOH using intragenic markers revealed a different scenario: the sequencing of CDH1 -160C/A polymorphism showed equal peak heights for both alleles in tumor material from the proband, suggesting that LOH is not occurring at this specific 5’-end of the CDH1 gene. In contrast, the sequencing analysis for CDH1 exon 4 performed in tumor DNA shows a clear reduction of the peak heights corresponding to the wild-type when compared with the mutant allele. This reduction could not be observed in the sequencing analysis of constitutional DNA or in DNA from normal breast epithelia. This result is suggestive of LOH, downstream of the promoter region of the gene and encompasses at least exon 4.
Discussion
In this report, we describe the finding of a germline CDH1 mutation in a woman with lobular breast cancer whose family history includes additional lobular breast cancer but no gastric cancer. Germline mutations in CDH1 have been previously associated with marked risk of diffuse gastric cancer (67-83%), the dominant tumor in the Hereditary Diffuse Gastric Cancer syndrome. Recent observations have noted an excess of invasive lobular breast cancers in some HDGC kindreds. The estimated cumulative lifetime risk of breast cancer in women with germline CDH1 mutations calculated among 11 DGC families is 39%. A penetrance analysis of 4 families with a founder CDH1 mutation confirmed the increased risk for breast cancer, with a cumulative risk of breast cancer of 52% (95% CI, 29%-94%).

Previous efforts to identify germline CDH1 mutations in familial breast cancer patients have not been very forthcoming. In a Swedish study, 19 patients with familial breast cancer whose tumors showed loss of heterozygosity at the CDH1 locus tested negative for germline CDH1 mutations. The majority of the cases (10 of 19), however, were ductal carcinomas and one was medullary, a ductal subtype. Of the remainder, two were lobular and one was mixed ductal and lobular breast cancer; information on the other five tumors was not included. Since loss of E-cadherin characterizes more than 90% of lobular breast cancers and only 5-10% of ductal histologies, this distribution of histologic subtypes is unexpected. Lei et al did not identify a germline CDH1 mutation in 13 patients with familial LBC. However, in this small cohort, a positive family history was not clearly defined either for degree of relation or the number of family members with breast cancer. The search for CDH1 germline mutations in a series of 65 LCIS patients also yielded negative results. One study has proposed that the CDH1 missense mutation 1774G>A (A592T) is a risk factor for comedo-type carcinoma, a pathologic variant of ductal carcinoma in situ. A second germline missense mutation (1876G>A (F626V)) has been reported in an individual with LBC; no family cancer history is included in the report. The pathogenicity of these two missense mutations is not known.

The CDH1-encoded protein E-cadherin is a calcium dependent cell to cell adhesion glycoprotein comprised of an extracellular domain, a transmembrane region that bridges the plasma membrane, and the highly conserved cytoplasmic tail. It is one of the key molecules for the establishment of the intercellular junction complex and for the adhesive properties among epithelial cells. The cytoplasmic domain of E-cadherin directs the β-catenin mediated interaction with actin cytoskeleton and p120 controls the strength of cell-to-cell adhesion by regulating cadherin stability and retention at the cell surface. It acts in a zipper-like fashion at the tight junctions of adjacent epithelial cells. Down-regulation of CDH1 leads to the disruption of the tissue architecture and the increase of invasive properties of the malignant cells of epithelial origin. The loss of CDH1 expression can occur as a result of various genetic mechanisms. For example, in sporadic DGC somatic mutations target preferentially exons 7 and 9 and promoter hypermethylation account for bi-allelic silencing of CDH1 expression in more than 50% of this type of tumors, while in most sporadic LBC, CDH1 complete silencing is
achieved by mutations scattered along the gene accompanied by either CDH1 promotor methylation or LOH\textsuperscript{10, 22}.

The 517insA mutation described in this report is located near the 5’ end of the CDH1 gene. LOH at the mutation site was found in the analysis of two separate samples extracted from the proband’s lobular breast cancer. In contrast to gastric cancers from germline CDH1 mutation carriers, in which promotor methylation is the most common second hit\textsuperscript{21, 23}, in this case of LBC we found that the second hit is through LOH. Interestingly, in the present study LOH was not identified using CDH1 flanking LOH markers, but using polymorphic intragenic markers, namely the mutation site in exon 4, which revealed an intragenic deletion that encompasses at least exon 4 of the CDH1 gene. This mechanism was previously reported in a tumor from a HDGC CDH1 mutation carrier\textsuperscript{21, 23}.

Our finding suggests that genetic heterogeneity may also characterize familial invasive lobular breast cancer. Lobular breast cancers comprise 9% of the breast cancer in carriers of germline BRCA2 mutations and only 3% in BRCA1 mutation carriers\textsuperscript{24}. The present report demonstrates that CDH1 germline mutations occur in 4.3% (1/23) of lobular breast cancer probands. Therefore, if these results are confirmed in larger series CDH1 testing may become part of the evaluation of women with lobular breast cancer, in whom features suggesting the presence of hereditary predisposition are present. In other known cancer syndromes, histopathologic information defines subsets of cancers linked to particular genes. For example, medullary thyroid cancer is associated with activating germline mutations in the RET proto-oncogene, but follicular thyroid cancer occurs excessively in Cowden’s syndrome with germline PTEN mutations. Clear cell renal carcinoma is the most frequent histology observed in the Von Hippel Lindau syndrome, while papillary renal cell carcinoma is associated with germline mutations in the proto-oncogene c-MET\textsuperscript{25}. Among breast cancers, medullary, atypical medullary and basal-like tumors are more frequently observed in individuals with germline BRCA1 mutations\textsuperscript{26, 27}. If confirmed, the association between invasive lobular breast cancer and germline CDH1 mutations may help to guide the genetic evaluation of affected individuals and families.

The finding raises questions for the clinical management of CDH1 carriers. Further study will be necessary to more clearly determine the penetrance of germline CDH1 mutations, and the proper management of women with germline CDH1 mutations. Although LBC represent only 8-14% of all breast cancers\textsuperscript{28}, they account for a disproportionate number of breast cancers undetectable by screening mammogram. The role of breast MRI has not been defined in this cohort. Challenges already exist in the management of the DGC risk inherent in previously identified CDH1 mutation carriers. We have shown that several detection methods have low sensitivity for detecting early gastric cancer in HDGC patients, including endoscopy, endoscopic ultrasound, chromoendoscopy, and PET scanning, which failed to detect early DGC in all six patients one week prior to prophylactic gastrectomy\textsuperscript{29, 30}.

In summary, we report a novel germline CDH1 mutation in a woman with lobular breast cancer and family history of lobular breast cancer in the absence of DGC. Additional research can now focus on reliable estimates of the mutation frequency, spectrum,
penetrance, and range of malignancies associated with germline \textit{CDH1} mutations. Further work to identify appropriate and effective surveillance and prevention strategies for individuals at hereditary risk of lobular breast cancer with and potentially without risk of DGC will also be critical.

\textbf{Acknowledgments:} This research was funded in part by the BC Yukon Breast Cancer Foundation to David Huntsman and by the Breast Cancer Research Foundation (BCRF) to Judy E Garber. Serena Masciari is supported by a Charles A. King Trust, Bank of America Fellowship, Co-Trustee (Boston, MA) and The Humane Society of the Commonwealth of Massachusetts Postdoctoral Research Fellowship. Nina Larsson was supported for this work by a Patterson Fellowship.

\textbf{Competing interests:} All the authors have reviewed the manuscript and none has competing interests for this publication.

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Figure 1
Flow chart of study participants.

Figure 2A: Pedigree of the family displaying a new deleterious CDH1 insertion in exon 4 (517insA) and family history of breast cancer; 2B: 517insA (1C) CDH1 mutation screening results demonstrating dHPLC results (top) and sequencing results (bottom). The dHPLC shows the wildtype sequence in black and the mutated sequence in red. Heteroduplexes that form in PCR samples having internal sequence variation display reduced column retention time relative to homoduplexes found in control samples. Mutation is indicated by arrows in sequencing results.
2C: Hematoxylin and Eosin (H and E) staining for the infiltrating lobular carcinoma; 2D: E-cadherin stain of the invasive lobular carcinoma from the proband: the epithelium of a normal duct is E-cadherin positive whereas the tumor cells are E-cadherin negative.
REFERENCES


breast cancers by truncation mutations throughout its extracellular domain. 


Figure 1

Data-Bank

330
Lobular or Mixed, Ductal/Lobular BC

48
BRCA1/BRCA2 results reviewed

9
No specimens available

11
Did not meet the criteria

25
EXCLUDED

5
BRCA1/BRCA2 mutation carriers

23
Eligible Subjects
Figure 2

A

517insA, exon 4

Legend:

▼ Proband
□ Male unaffected
〇 Female unaffected
● Breast Cancer/BR

B

dhplc results

 sequencing results

C

D
Germline E-Cadherin mutations in familial lobular breast cancer

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*J Med Genet* published online July 27, 2007

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