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TITLE: Investigation of the Lobular Carcinoma in Situ, Using Molecular Genetic Techniques, for the Involvement of Novel Genes

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<td>Atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS), i.e. lobular neoplasia (LN), are lesions of significance in terms of risk to the patient in the development of invasive carcinoma. A correlation between the lobular histological type and inactivation of E-cadherin, a cell adhesion protein, has been reported. As well, mutations in CDH1 have been reported in invasive lobular carcinoma (ILC) and LCIS with adjacent ILC. Our study proposes to investigate LN lesions, lacking any adjacent invasive carcinoma, for alterations in and expression of known and novel genes/proteins with the goal of characterizing a molecular genetic profile. We have accrued 36 cases containing ALH/LCIS without adjacent invasive carcinoma. Previous studies have found negative E-cadherin, beta- and alpha-catenin protein expression in these lesions. Moreover, LCIS but not ALH cases were characterized by mutations and LOH at 16q was found to be an infrequent event. Recent studies have also demonstrated cytoplasmic (rather than membrane) localization of p120-catenin in LN lesions. As a mechanism for the inactivation of E-cadherin has yet to be elucidated in LN, we have used CGH microarrays to study 12 ALH and 14 LCIS lesions. Following validation by Real Time PCR it will be possible to describe events occurring at the earliest stages of breast cancer.</td>
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Introduction

Lobular neoplasia (LN) is a histological classification that includes atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS). These breast lesions are of epithelial origin and histologically show a proliferative gradation from ALH to LCIS. Although a finding of LN is usually incidental during breast tissue biopsy, the relative risk to the patient in the development of invasive breast cancer is noteworthy. In fact, a number of epidemiological studies have reported that ALH and LCIS lesions are high-risk indicators, conferring a respective 4 to 5 and 8 to 10 fold increase risk.

Previous work by our group (reported in Annual Summary Reports for 2002-2003 and 2003-2004) and others, investigated these lesions with respect to E-cadherin, a cell adhesion protein found on the membrane of epithelial cells. Membrane E-cadherin protein expression, as well as expression of beta- and alpha-catenin, have been found to be completely and simultaneously lost in ALH, LCIS and invasive lobular carcinoma (ILC). In studies investigating ILC, the loss of expression has been explained by the acquisition of inactivating mutations in the E-cadherin gene (CDH1) coupled with loss of heterozygosity (LOH) at the chromosome locus of 16q (where CDH1 is located). However our study, specifically investigating ALH and LCIS lesions not in the presence of an invasive cancer, was unable to provide an explanation for the loss of E-cadherin protein expression in the context of CDH1 mutation and LOH at 16q.

Body

Please note that the writing of this report is based on the revised SOW submitted to USARMC early in 2005. A copy of this SOW has been appended to the report (Appendix 1).

Our studies of lobular neoplastic lesions have used the cases collected through the Mount Sinai Hospital Pathology Department. These cases were accrued with the assistance of Dr. Frances O'Malley and as of April 2005 case accrual has concluded (Task 2a). All cases in the collection are formalin-fixed, paraffin-embedded archived breast tumor blocks containing lobular neoplastic lesions lacking adjacent invasive carcinoma and, at completion, the collection includes 36 cases of ALH/LCIS.

In previous work, we established that although LCIS lesions are characterized by mutation it is likely that a mechanism other than mutation/LOH is the cause of the loss of E-cadherin protein expression in early lobular neoplastic lesions, especially in cases containing ALH. Therefore it is necessary to evaluate other mechanisms by which CDH1 may be inactivated. To this end, evaluation of methylation of the CDH1 promoter had been proposed (Task 3c, 3d). However, optimization of the methylation-specific PCR (MSP) technique was unsuccessful. MSP requires a large quantity of sodium bisulfite modified DNA which was unable to be obtained from our cases of ALH/LCIS. The MethyLight technique was also attempted because it had greater sensitivity and required less DNA template. However despite these attributes, efficient and reproducible amplification of the target promoter area was unable to be optimized, again attributed to the DNA template. Given that there are no other template options for the cases in our collection (i.e. DNA from frozen tissue), this study has been discontinued.

The optimized protocol for p120-catenin immunohistochemistry (IHC) and staining of the ALH/LCIS cases with this antibody was previously reported to be complete (Task 5a-c, Annual Report 2003-2004). Subsequently, the scoring system has been developed to evaluate
the IHC for p120-catenin (Task 5d). The scoring system created assesses not only a lack of circumferential membrane staining but also the cytoplasmic localization of the protein. All LCIS lesions showed no membrane but diffuse cytoplasmic staining, as well as 10 of 11 ALH lesions, with only one case (A7) showing positive circumferential membrane staining. Together with the previously reported IHC results for E-cadherin, beta-catenin and alpha-catenin, we can conclude that without the presence of an invasive lesion the expression of the entire E-cadherin complex at the cell membrane is altered in both ALH and LCIS lesions.

Understanding the picture outside of the E-cadherin complex could be key to discerning what is altered in these early breast lesions. For this we had proposed using comparative genomic hybridization (CGH) microarrays (Task 4) to determine what other known or novel genes are altered at the lobular neoplastic stage. The collection of cases able to be studied by array-CGH (aCGH) includes 17 ALH and 15 LCIS lesions. For each lesion, four to six 8uM sections were microdissected by either laser capture microdissection or stereomicroscope-based needle dissection and DNA was extracted (Task 4a). The DNA was labeled with Cy3 and aCGH successfully performed on 12 ALH and 14 LCIS (using a female genomic DNA labeled with Cy5 as reference) (Task 4b). Each array was scanned (Applied Precision Arrayworx CCD scanner) and then the array images were loaded into the analysis software (Applied Precision Softworx) and overlaid with the spot grid. The raw data was exported from this program and subsequently visualized using SeeGH software.

All arrays were analyzed using SeeGH and subsequently statistically analyzed to determine if the regions found were significant (Task 4c). The statistical analysis revealed regions of alteration also found by SeeGH. In general, a greater number of alterations were found in the ALH lesions compared to the LCIS. Regions specific to ALH or LCIS are identifiable, however, there appear to be no regions that are common to the paired lesions (cases containing both ALH and LCIS). Real Time PCR is currently being optimized to validate some areas of amplification found by aCGH (Task 4d). Primer/probe sets specific to genes at 1p11.2, 10p15.2, and 20q13.13 are being assessed.

**Training Experiences (2004-2005)**

Teresa Mastracci is receiving her research training in the laboratory of Dr. Irene Andrulis at the Fred A. Litwin Centre for Cancer Genetics in the Samuel Lunenfeld Research Institute (SLRI) of Mount Sinai Hospital. As with any laboratory, Dr. Andrulis’ lab has had turnover in the last year with the graduation of students and the acquisition of new associates. However, the laboratory continues to provide a rich research environment with 1 research associate, 2 postdoctoral fellows, 3 research technicians, 2 M.Sc. and 5 Ph.D. students. Moreover, the schedule of weekly lab meetings as well as monthly meetings with other groups in the Centre for Cancer Genetics has been maintained.

The Department of Laboratory Medicine and Pathobiology at the University of Toronto, to which Teresa is affiliated, requires the completion of 5 courses for students in the PhD program; a requirement that was fulfilled this year. Furthermore, the Department has maintained regular weekly Research Seminars which feature distinguished scientists from outside and within Toronto as well as weekly Departmental Student Seminars which give students the opportunity to present their research and receive input from the staff about their studies.
Both the University and SLRI hold yearly retreats where students and faculty present their work in a relaxed environment, facilitating discussion between laboratory groups. Teresa has presented at both of these events this year.

The Student Supervisory Committee monitors a student's progress regularly during his/her graduate career. It consists of the supervisor plus at least two other members of the Department, one of who should be from an area that is outside of the supervisor's immediate area of expertise. Committee meetings for Teresa have been consistently held every six months.

Dr. Andrulis' laboratory has also recently become involved in a weekly Molecular Medicine journal club held at the Hospital for Sick Children in Toronto. In addition, Teresa was invited to attend and present at the weekly Work In Progress Seminar Series hosted by Dr. Tak Mak at the Breast Cancer Research Institute at Princess Margaret Hospital. In the past, Teresa also has had the opportunity to attend and present at international conferences and this year has been invited to give a platform presentation at the Era of Hope DOD BCRP Meeting.

**Key Accomplishments (2004-2005)**

- Case accrual has been completed. The collection includes 36 cases of ALH/LCIS.

- Optimization of a methylation specific PCR protocol was unsuccessful due to the limited amount of DNA able to be obtained from each ALH/LCIS case and therefore this study has been discontinued.

- Twelve ALH and 14 LCIS lesions were successfully arrayed using the tiling BAC array generated in the laboratory of Dr. Wan Lam. The results from these microarrays have been analyzed and regions specific to only ALH or LCIS cases have been identified. Real Time PCR is currently being optimized to validate some of these areas including 1p11.2, 10p15.2, and 20q13.13.

- A scoring system for p120-catenin has been developed assessing cases for a lack of membrane staining as well as cytoplasmic localization of protein. The trend for LN cases is cytoplasmic, and not membrane, localization of p120-catenin in all ALH and LCIS.

**Reportable Outcomes (2004-2005)**

- **Papers (Appendix 3)**
  Mastracci TL, Tjan S, Bane AL, O'Malley FP, Andrulis IL. E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma *in situ*. Mod Pathol. 2005 Jan 14; [Epub ahead of print]
Abstracts (Appendix 2)

Presentations
Characterization of a molecular genetic profile for lobular neoplasia. Graduate Student Research Day, Department of Laboratory Medicine and Pathobiology, University of Toronto. March 2005

Lobular Neoplasia: an Array of discovery. Work-In-Progress Seminar Series, Breast Cancer Research Institute, Princess Margaret Hospital, Toronto. February 2005

Profiling Lobular Neoplasia. Annual Retreat, Samuel Lunenfeld Research Institute, Geneva Park Conference Center, Orillia. October 2004

Development of a Tissue Repository
No further cases have been added to the collection in 2004-2005 and the accrual of lobular neoplastic cases has concluded with a total of 36 ALH/LCIS cases.

Conclusions
ALH and LCIS lesions, without the presence of adjacent invasive disease, both show loss of the entire E-cadherin protein complex. Although we have shown that LCIS lesions are characterized by mutations, the genetic hits of mutation and loss of heterozygosity cannot explain this loss of E-cadherin protein expression (as well as the related loss of beta-, alpha- and p120-catenin). With the use of CGH microarray, we have found that ALH lesions appear to have a greater number of genetic alterations compared to LCIS lesions. Although many of these alterations are common to both neoplastic lesions, there appear to be regions that are specific to only ALH or LCIS. As the specific areas of amplification and deletion are currently being validated we cannot speculate yet as to the specific genetic events occurring in these lesions. However once complete, this study will define a genetic signature for lobular neoplasia that clearly describes events occurring at the earliest stages of breast cancer.
REFERENCES


characterize lobular breast carcinoma from preinvasive to metastatic lesions. Oncogene 2004, 23:3272-3283


Appendix 1: Revised Statement of Work (SOW)

MASTRACCI, TERESA L.

STATEMENT OF WORK

Investigation of lobular carcinoma in situ, using molecular genetic techniques, for the involvement of novel genes.

Task 1: Completion of the Analysis of E-cadherin

a. Using the previously microdissected LCIS DNA, screen the remaining exons of the E-cadherin gene using Single Strand Conformation Polymorphism (SSCP).

b. Excise alterations, appearing as shifted bands, from the SSCP gel and extract the altered DNA.

c. Manually sequence the DNA to characterize each alteration.

Task 2: Tissue Accrual

a. Collaborate with Dr. Frances O'Malley to search/request new cases of LCIS and ALH from external hospitals and institutions.

b. Using a microtome, cut newly acquired formalin-fixed, paraffin-embedded (FFPE) blocks, mount sections on glass slides, and microdissect/extract the DNA.

Task 3: Evaluation of methods of inactivation of E-cadherin

a. Using the previously microdissected DNA from all cases, evaluate loss of heterozygosity (LOH) for chromosomal region 16q using five microsatellite markers (D16S421, D16S496, D16S503, D16S3095, D16S752) and a PCR-based method to determine the LOH status of each tumor/normal pair (the PCR products separated using 7% denaturing formamide gel and visualized with autoradiography.

b. Multiple independent observers evaluate each LOH marker, scoring each case as ‘LOH’, ‘no LOH’, or ‘uninformative’.

c. Optimization of methylation-specific PCR (MSP) technique using hypermethylated placenta DNA and methylated cell-line DNA, in order to assess the E-cadherin promoter for methylation.

d. Carry out MSP using DNA from all cases, and assess methylation status of the E-cadherin promoter for each case.

Task 4: Analysis of LCIS and ALH by CGH Microarray

a. Microdissect/extract DNA from all cases.

b. Carry out CGH microarray experiments using optimized protocol and tiling BAC array (DNA of interest labeled with Cy3, reference DNA labeled with Cy5).

c. Quantification and statistical analysis of microarray experiments using scanners and software available through collaboration with Dr. Wan Lam (B.C. Cancer Research Center, British Columbia, Canada) and Dr. Shelly Bul (SLRI, Toronto, ON, Canada)

d. Validation of results by Real Time PCR with custom primers.

Task 5: Analysis of LCIS and ALH by Immunohistochemistry

a. Using a microtome, cut FFPE LCIS blocks and mount sections on glass slides.

b. Optimize antibody (beta-catenin, alpha-catenin, p120-catenin) concentrations using any available FFPE breast tumor sections and FFPE cell lines.

c. Stain sections with antibodies for beta-catenin, alpha-catenin, and p120-catenin.

d. Collaborate with Dr. Frances O'Malley to evaluate the results of these immunohistochemical experiments.

INVESTIGATION OF LOBULAR NEOPLASIA, USING MOLECULAR GENETIC TECHNIQUES, FOR THE INVOLVEMENT OF NOVEL GENES

Teresa L. Mastracci(1,2), Suzanna Tjan(3), Ashleen Shadeo(4), Sarah Colby(5), Anita L. Bane(1,2,3), Shelley Bull(5), Wan Lam(4), Frances P. O'malley(2,3), Irene L. Andrulis(1,2)

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2 Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada;
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Breast cancer is one of the most prevalent human cancers and a leading cause of death among women. Lobular neoplasia is a classification of breast lesions that includes atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS). Both ALH and LCIS are premalignant lesions that are impalpable and mammographically silent. Though these lesions are only found incidentally during breast biopsy, ALH/LCIS lesions are significant in terms of implication of risk to the patient in the development of invasive carcinoma. A strong correlation between the lobular histological subtype and inactivation of E-cadherin, a protein involved in cell adhesion, has been reported. As well, mutations in the E-cadherin gene (CDH1) have been reported in invasive lobular carcinoma (ILC) and LCIS with adjacent ILC. We have been investigating lobular neoplastic lesions, lacking any adjacent invasive carcinoma, for alterations in and expression of known and novel genes/proteins with the goal of characterizing a molecular genetic profile for lobular neoplasia.

We have obtained 21 archived cases of which there are 13 ALH lesions and 13 LCIS lesions all without adjacent invasive carcinoma. E-cadherin, beta-, alpha-, and p120-catenin protein expression were assessed by immunohistochemistry (IHC). Sequence alterations in CDH1 were identified using Single Strand Conformation Polymorphism (SSCP)/manual sequencing and PCR-based loss of heterozygosity (LOH) analysis was carried out for the 16q locus. Following the characterization of the E-cadherin complex, array-CGH was used to determine gene copy number alterations in these cases.

By IHC, we have found negative E-cadherin (23/24), beta-catenin (23/24), and alpha-catenin (21/23) protein expression in these lesions. Cytoplasmic (rather than membrane) localization of p120-catenin was observed in 20 of 21 lesions. LCIS cases were characterized by mutations, however ALH cases were not. LOH at 16q was found to be an infrequent event. Array-CGH has been carried out on 11 LCIS and 9 ALH lesions and the data is currently being analyzed to determine the molecular genetic profiles.

Loss of the E-cadherin adhesion complex is an early event in lobular neoplasia affecting ALH as well as LCIS and occurs prior to progression to invasive disease. However, loss of E-cadherin protein expression is accompanied by DNA alterations in LCIS but not in ALH. Cases lacking both protein expression and gene alterations suggest that another mechanism is involved, possibly as early as at the hyperplastic stage, causing the silencing of the E-cadherin complex. Moreover, the array-CGH study may allow us to characterize a molecular genetic profile for lobular neoplasia, identifying events that mark the earliest stages in the development of cancer. This advance will significantly expand our ability to design strategies for the prevention of invasive carcinoma and could identify targets for therapeutic treatment.

The U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0498 supported this work.
E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma in situ of the breast

Teresa L Mastracci1,2, Suzanna Tjan3, Anita L Bane1,2,3, Frances P O'Malley2,3 and Irene L Andrulis1,2,4

1Fred A Litwin Centre for Cancer Genetics, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada; 2Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; 3Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada and 4Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada

Tumor development from an early lesion through to invasive disease is not a clearly defined progression in the breast. Studies of invasive lobular carcinoma have reported mutations, loss of heterozygosity (LOH) and loss of protein expression in epithelial (E)-cadherin, a protein involved in cell adhesion. Our study examines in situ lobular neoplastic lesions without concurrent invasive carcinoma for E-cadherin gene alterations and protein expression, beta-catenin, alpha-catenin and p120-catenin protein expression, and LOH at the chromosome 16q locus, with the goal of determining the events occurring at the stage of lobular neoplasia. In all, 13 atypical lobular hyperplasia lesions and 13 lobular carcinoma in situ lesions from archived cases were examined. E-cadherin sequence alterations were evaluated using single strand conformation polymorphism and DNA sequencing, and PCR-based LOH analysis was carried out for the 16q locus. Using immunohistochemistry, we assessed protein expression. A total of 23 of 24 lesions evaluated by immunohistochemistry were negative for both E-cadherin and beta-catenin protein expression, and 21 of 23 lesions were negative for alpha-catenin. Cytoplasmic (rather than membrane) localization of p120-catenin was observed in 20 of 21 cases. Lobular carcinoma in situ cases were characterized by mutations; however, atypical lobular hyperplasia cases were not. LOH at 16q was an infrequent event. From our study, we conclude that an altered E-cadherin adhesion complex is an early event affecting atypical lobular hyperplasia as well as lobular carcinoma in situ and occurs prior to progression to invasive disease. However, the loss of protein expression is accompanied by E-cadherin DNA alterations in lobular carcinoma in situ but not in atypical lobular hyperplasia. These cases lacking both protein expression and gene alterations suggest that another mechanism is involved, possibly as early as at the hyperplastic stage, causing silencing of the E-cadherin complex.

Keywords: atypical lobular hyperplasia; breast cancer; E-cadherin; lobular carcinoma in situ; lobular neoplasia

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Our study investigates in situ lobular neoplastic lesions that have not progressed to invasive disease with the goal of determining the molecular genetic events occurring at the stage of lobular neoplasia. Discovering the specific events that mark the transition from an early lobular neoplastic lesion to an invasive tumor is necessary to both support and subsequently understand this breast cancer progression.

Lobular neoplasia is a histological classification that includes atypical lobular hyperplasia and lobular carcinoma in situ. Lobular carcinoma in situ is a lesion of epithelial origin and is defined by a population of cells that are small, round, monomorphic and discohesive. The lesion is often
multicentric and bilateral, and greater than 50% of the acini in the affected terminal duct lobular unit are distended by the cellular proliferation. The cells that define atypical lobular hyperplasia are similar to those that characterize lobular carcinoma \textit{in situ}, however, the cellular proliferation does not completely occlude the lumen, and less than 50% of the acini in the affected terminal duct lobular unit exhibit distension. Both lobular neoplastic lesions are found incidentally during breast tissue biopsy due to their inability to be detected by palpation or mammography.

Histologically, there is a proliferative gradation from lobular hyperplasia to \textit{in situ} carcinoma, which is also reflected in the relative risk to the patient in the development of invasive disease. A number of epidemiological studies have reported that lobular neoplastic lesions are high-risk indicators.

A finding of atypical lobular hyperplasia has been reported to imply a four- to five-fold increased risk of subsequent carcinoma in either breast, and a finding of lobular carcinoma \textit{in situ} implies an eight- to ten-fold increased risk to the patient.

In sporadic breast cancers, histological type has been correlated with expression of the cell adhesion protein epithelial (E)-cadherin, the cadherin sub-type expressed in epithelial cells. Proteins that complex with E-cadherin at the cell membrane include beta-, gamma-, alpha- and p120-catenin. Reduced expression of E-cadherin has been reported in invasive ductal carcinoma whereas lobular carcinoma \textit{in situ} and invasive lobular carcinoma show complete loss of the protein. A report on invasive lobular carcinomas with adjacent lobular carcinoma \textit{in situ} demonstrated not only loss of E-cadherin expression but also the simultaneous loss of beta-, gamma- and alpha-catenin protein expression. More recently, Sarrio et al demonstrated that the loss of E-cadherin along with the cytoplasmic localization of p120-catenin characterizes lobular breast cancers and suggested that p120-catenin plays a role in mediating the oncogenic effects of E-cadherin loss in these cancers. Unquestionably there is evidence that an altered E-cadherin adhesion complex is characteristic of invasive lobular carcinoma and lobular carcinoma \textit{in situ} with adjacent invasive lobular carcinoma.

In light of these findings, several studies have investigated the E-cadherin gene, \textit{CDH1}, for alterations in lobular carcinomas. Mutations have been detected in invasive lobular carcinoma and lobular carcinoma \textit{in situ} with adjacent invasive, and the loss of chromosome 16q was detected in solitary \textit{in situ} and synchronous \textit{in situ}/invasive lesions. These investigations of lobular carcinoma and E-cadherin have provided some evidence that \textit{in situ} lobular carcinoma may be not only a risk indicator but also a precursor lesion to invasive carcinoma.

To date, most molecular genetic studies of lobular carcinoma \textit{in situ} have focused on lesions with adjacent invasive carcinoma. However, to study events occurring specifically at the hyperplastic and \textit{in situ} stages, it is necessary to examine cases where the neoplastic lesion is not contaminated by an invasive lesion. Cases containing atypical lobular hyperplasia and lobular carcinoma \textit{in situ} without adjacent invasive carcinomas are known to occur in only 0.5–3.8% of breast cases that are otherwise benign. These lesions have rarely been studied at the molecular level. We have accrued a collection of cases fitting these criteria, making it possible to determine protein expression and gene alterations occurring at the stage of lobular neoplasia.

Materials and methods

Tissue Accrual

The study population consisted of 21 formalin-fixed, paraffin-embedded, archived cases accrued through the Mount Sinai Hospital Pathology Department (Toronto, ON, Canada). The cases were accessed from 1988 to 2003. The study pathologist (FOM) reviewed each neoplastic lesion from hematoxylin and eosin (H&E)-stained sections. Previously described histological characteristics were used to classify the lobular lesions. The collection included 13 atypical lobular hyperplasia lesions (A1-A13) and 13 lobular carcinoma \textit{in situ} lesions (L1-L12) lacking adjacent invasive carcinoma. Four of these cases contained both hyperplasia and \textit{in situ} lesions (A2/L1, A3/L3, A4/L5, A11/L12). More specifically, case A2/L1 was also characterized by multifocal lobular carcinoma \textit{in situ} (L1-1, L1-2) and these \textit{in situ} lesions were housed in separate blocks.

Two cases of interest, supplementary to our collection, were also included in all analyses. Case A14/L13 contained atypical lobular hyperplasia and lobular carcinoma \textit{in situ} lesions as well as a focus of invasive lobular carcinoma in a separate block; the lobular neoplastic lesions were analyzed. Moreover, case P1 contained a lesion of low-grade ductal carcinoma \textit{in situ} that was used as a control for the immunohistochemistry analyses. All cases were coded to prevent bias and maintain confidentiality.

To determine the robustness of the histological classification of the lesions, all cases were reviewed independently by a second pathologist (ALB) blinded to the results of the molecular analyses. There were discrepancies in two cases. Both cases had been called lobular carcinoma \textit{in situ} by the first pathologist (FOM) and 'atypical lobular hyperplasia with duct involvement by cells of atypical lobular hyperplasia' by the second pathologist (ALB). A consensus diagnosis of lobular carcinoma \textit{in situ} was reached for both cases by the pathologists following rereview at a multiheded microscope. Ultimately, the pathology review confirmed that our collection contained 13 atypical lobular hyperplasia and 13 lobular carcinoma \textit{in situ} lesions without adjacent invasive carcinoma.
Microdissection, DNA Extraction and DNA Amplification

From each case, serial sections (8 μm) were cut from the block containing the lesion of interest and mounted on glass slides. Following deparaffinization, the lesion was removed from the section using either a stereomicroscope-based microdissection technique or laser-capture microdissection (PixCell II, Arcturus, CA, USA). The microdissection technique used was determined by the size of the lesion. The majority of the in situ lesions (L1-1, L1-2, L2, L4, L6–L11) were microdissected using the stereomicroscope-based technique due to large lesion area. However, all hyperplasia lesions (A1–A14) were small in size and required laser-capture microdissection in order to accurately remove the lesion from the surrounding tissue. Cases L3, L5, L12 and L13 were also microdissected by laser-capture microdissection because they contained both neoplastic lesions in close proximity in the same section. Figure 1 depicts a case containing atypical lobular hyperplasia from our collection and demonstrates the lesion before and after laser-capture microdissection, as well as the degree of cellularity of a typical atypical lobular hyperplasia lesion for our collection. Whether by stereomicroscope or laser-capture, the use of a microdissection technique allowed for the isolation of a population of lobular neoplastic cells from each atypical lobular hyperplasia or lobular carcinoma in situ lesion that was assured to contain no greater than 15–20% contamination of non-neoplastic cells. Following microdissection, the tissue was incubated for 48 h and DNA was extracted using the QiaAMP DNA Mini Kit (Qiagen, Canada).

As neoplastic lesions are small in size, the amount of DNA available per case was limited. To increase the quantity of DNA available for mutation detection, we used the whole genome amplification technique degenerate oligonucleotide-primed polymerase chain reaction (DOP PCR) Random degenerate oligonucleotide primers and a modified PCR cycle were used to amplify the added DNA template. Each DOP PCR reaction contained 2 μl of microdissected DNA template and was amplified as per the manufacturer’s instructions (DOP PCR Kit, Roche Biomolecular, Canada).

Sequence Alteration Detection and Characterization

To detect sequence alterations, we used single strand conformation polymorphism (SSCP) followed by manual DNA sequencing to characterize each alteration. For the initial screening, each exon of CDH1 was individually amplified using PCR. Exon-specific PCR conditions were optimized for all primer pairs (exons 1–3, 6–16; exons 4–51). Amplification was performed in a volume of 30 μl containing 10 μl of DOP PCR product template, 1× High Fidelity PCR Buffer, 2 mM MgSO₄, 0.2 mM of each dNTP, 0.3 μM of forward and reverse primers, 1 U of PLATINUM Taq DNA polymerase High Fidelity (GIBCO BRL, Life Technologies, Canada), and 0.1 μCi of ³²P(Phosphorus-33)-labeled dATP (Perkin-Elmer, USA). Following an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 15 s, 50°C (exons 2, 3, 7–10, 16), or 53°C (exon 6), or 55°C (exons 4 and 5), or 58°C (exons 11–15), or 68°C (exon 1) for 15 s, and 72°C for 20 s were performed.

A stop solution (95% formamide, 0.5 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to the SSCP reactions, which were subsequently heat denatured and subjected to electrophoresis on an 8% nondenaturing polyacrylamide gel (including 10% glycerol). Two gels were run simultaneously under different conditions: (i)
4°C, 8 W for 12 h, and (ii) room temperature, 12 W for 16 h. Results were obtained following autoradiography. If an aberrantly migrating band was observed, the SSCP reaction was repeated for that case using 5 μl of microdissected DNA template that had not been subjected to DOP PCR amplification (PCR/SSCP conditions as previously stated). If the abnormal banding pattern could be duplicated in this second independent SSCP experiment, then the alteration was characterized using manual DNA sequencing.

To characterize CDH1 alterations, aberrantly migrating bands were excised from the dried SSCP gel and DNA was extracted using a serial freeze–thaw technique. In all, 5 μl of the extracted DNA was used as template in an exon-specific PCR reaction using the conditions previously outlined. The Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Canada) was used to manually sequence the DNA, according to the manufacturer's instructions. Results were obtained following autoradiography. Alterations that were found were confirmed by repeating the sequencing using microdissected DNA (not previously preamplified by DOP PCR) as template from both the forward and reverse direction for each exon.

**Loss of Heterozygosity (LOH)**

LOH was evaluated for the E-cadherin gene located on chromosome 16q. Five microsatellite markers, located at chromosome locus 16q21–16q22.1, were used (D16S421, D16S496, D16S503, D16S3095, D16S752). Microdissected DNA from each lesion (not preamplified by DOP PCR), paired with DNA from an adjacent area of normal tissue, was used as template to examine LOH. PCR amplification was performed in a volume of 30 μl containing 5 μl of microdissected DNA template, 1× High Fidelity PCR Buffer, 2 mM MgSO₄, 0.2 mM of each dNTP, 0.3 μM of forward and reverse primers, 1 U of PLATINUM Taq DNA polymerase High Fidelity (GIBCO BRL, Life Technologies, Canada), and 0.1 μCi of [³²P]dATP (Perkin-Elmer, USA). Following an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 15 s, 54°C (D16S503) or 57°C (D16S421, D16S496, D16S752, D16S3095) for 15 s, and 72°C for 20 s were performed. A stop solution (as previously described) was added and each reaction was subsequently heat denatured and subjected to electrophoresis on a 7% denaturing formamide gel, which was run at 80 W for 3 h. Results were obtained following autoradiography. Multiple independent observers evaluated each marker, scoring each case as 'LOH', 'no LOH', or 'uninformative'. To evaluate each marker, LOH was defined as a relative decrease in band intensity greater than 50%. For each case, a minimum of three of the five markers with observed LOH was required for an overall classification of LOH.

**Immunohistochemistry**

Following sectioning for microdissection, 4 μm sections were cut for each formalin-fixed, paraffin-embedded block, and mounted on glass slides. Each section was deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Following heat antigen retrieval, the primary antibodies to E-cadherin (HECD-1, Monoclonal Mouse anti-E-cadherin 2nd Gen Predilute Antibody, Zymed Laboratories Inc., USA), beta-catenin (Monoclonal Mouse anti-beta-catenin, 1:6000 dilution, Transduction Laboratories, USA), alpha-catenin (NCL-A-CAT, Monoclonal Mouse anti-alpha-catenin, 1:50 dilution, Santa Cruz Biotechnology, Inc., USA) were applied. The Ultra Streptavidin Detection System (Vector Laboratories (Canada) Inc., Canada) was developed with the chromogen diaminobenzidine and sections were counterstained in hematoxylin.

Some cases have insufficient material to carry out immunohistochemistry due to sectioning order. Sections were cut from each case for immunohistochemistry analyses only after sectioning was complete for microdissection. In some cases, the lesion of interest was no longer present in the immunohistochemistry section and therefore no result could be obtained.

Immunohistochemical staining was reviewed and scored by the study pathologist. To evaluate the immunohistochemistry for E-cadherin, beta-catenin and alpha-catenin protein expression, a positive stain was determined to be complete circumferential membrane staining of the lobular neoplastic cells. The case of low-grade ductal carcinoma in situ (P1) was used as a positive control for E-cadherin, beta-catenin and alpha-catenin staining as it expressed these proteins at the membrane. Evaluation of the immunohistochemistry for p120-catenin required assessment of the circumferential membrane staining as well as the cytoplasmic staining pattern. A formalin-fixed, paraffin-embedded breast cancer cell line (MCF7) was used as the positive control for the p120-catenin immunohistochemistry as it contained membrane localized p120-catenin.

**Results**

**CDH1 Mutation Analysis**

Using the manual DNA sequencing technique, 16 polymorphisms (data not shown) and 15 mutations (Table 1) were characterized. Three mutations (cases A12, L4, L8) were deletions causing a frameshift and a premature stop codon. In all, 11 sequence alterations were classified as missense mutations (cases...
Table 1 Summary of CDH1 mutation, immunohistochemistry and LOH results

<table>
<thead>
<tr>
<th>Case accrual</th>
<th>Mutation analysis</th>
<th>Immunohistochemistry</th>
<th>LOH</th>
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<tr>
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</tr>
<tr>
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<td>ALH</td>
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<td>-</td>
</tr>
<tr>
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<td>ALH</td>
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<td>-</td>
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<td>A5</td>
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</tr>
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</tr>
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ALH = atypical lobular hyperplasia; LCIS = lobular carcinoma in situ; DCIS = ductal carcinoma in situ; (-), negative membrane staining; (+), positive membrane staining; NR = no result due to insufficient material; cyto = diffuse cytoplasmic staining.

All cases contained adjacent normal breast acini that served as the internal positive control for the immunohistochemical analyses.

Case A14/L13 contained atypical lobular hyperplasia and lobular carcinoma in situ lesions as well as a focus of invasive lobular carcinoma in a separate block. The lobular neoplastic lesions were analyzed.

Loss of Heterozygosity

LOH was evaluated with five microsatellite markers and each case was evaluated paired with its corresponding normal for each marker (Table 1).
We observed 80–100% agreement between observers and questionable cases were repeated and reevaluated. Three cases had insufficient material to carry out LOH analysis (A3, A6, L9). Of the remaining cases, three were found to have LOH (A10, L1–2, L7) and 20 showed no LOH (Figure 2).

E-cadherin, Beta-catenin, Alpha-catenin and p120-catenin Protein Expression

All lobular carcinoma in situ and 11 of 12 atypical lobular hyperplasia lesions were negative for E-cadherin and beta-catenin staining (Table 1, Figure 3); case A7 showed positive staining of the lobular neoplastic cells despite its identical morphologic appearance to the other cases of atypical lobular hyperplasia. The lesions in case A14/L13 had an identical staining pattern to all the lobular neoplastic cases, with negative E-cadherin and beta-catenin protein expression. For alpha-catenin, 10 of 11 lobular carcinoma in situ and 11 of 12 atypical lobular hyperplasia lesions were scored as negative, and two cases (A7, L8) were positive. The case of low-grade ductal carcinoma in situ (P1) stained positive for E-cadherin, beta-catenin and alpha-catenin. Moreover, all cases contained adjacent normal epithelium that served as an internal positive control and in all cases showed complete circumferential membrane staining (Figure 4).

The formalin-fixed, paraffin-embedded breast cancer cell line (MCF7) showed membrane localization of p120-catenin, and therefore positive circumferential membrane staining. Conversely, all lobular carcinoma in situ lesions showed no membrane, but diffuse cytoplasmic staining, as did 10 of 11 atypical lobular hyperplasia lesions, and only one case (A7) showed complete circumferential membrane staining.

Discussion

We investigated atypical lobular hyperplasia and lobular carcinoma in situ lesions for E-cadherin gene alterations and protein expression, beta-, alpha-, and p120-catenin protein expression, and LOH at the chromosome 16q locus. Unlike most studies, our atypical lobular hyperplasia and lobular carcinoma in situ cases were specifically selected without adjacent invasive carcinoma. The information to be gained from studying these lobular neoplastic lesions is substantial when considering the ambig-
E-cadherin and lobular neoplasia

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Figure 4 Normal breast acini stained by immunohistochemistry. All cases of atypical lobular hyperplasia and lobular carcinoma in situ contained areas of normal nonproliferating acini, adjacent to the lesion of interest, which served as internal positive control (Intermediate power, × 20). (a) Case L2 stained for E-cadherin protein expression, with complete circumferential membranous staining in the area of normal epithelium. (b) Case L2 stained for beta-catenin, showing adjacent normal acini with complete circumferential membranous staining. (c) Case A2/L12 stained for alpha-catenin, with adjacent normal acini showing complete circumferential membranous staining. (d) Case A5 stained for p120-catenin demonstrates cytoplasmic localization of the protein in the lesion and complete circumferential membranous staining in the adjacent normal acini (inset: high power, × 40).

Figure 4

Figure 4 Normal breast acini stained by immunohistochemistry. All cases of atypical lobular hyperplasia and lobular carcinoma in situ contained areas of normal nonproliferating acini, adjacent to the lesion of interest, which served as internal positive control (Intermediate power, × 20). (a) Case L2 stained for E-cadherin protein expression, with complete circumferential membranous staining in the area of normal epithelium. (b) Case L2 stained for beta-catenin, showing adjacent normal acini with complete circumferential membranous staining. (c) Case A2/L12 stained for alpha-catenin, with adjacent normal acini showing complete circumferential membranous staining. (d) Case A5 stained for p120-catenin demonstrates cytoplasmic localization of the protein in the lesion and complete circumferential membranous staining in the adjacent normal acini (inset: high power, × 40).

A number of studies have shown that E-cadherin is completely inactivated in invasive lobular carcinoma. Definitively, from our study of neoplastic lesions, we can conclude that lobular lesions, whether hyperplasia or carcinoma in situ, lack E-cadherin membrane staining. These immunohistochemistry results support the previously reported correlation between protein inactivation and histological type. Furthermore, the data indicate that this correlation is not restricted to lobular carcinoma in situ and invasive lobular carcinoma but can be extended to atypical lobular hyperplasia as well.

In addition, a complete lack of beta-catenin and alpha-catenin protein expression as well as cytoplasmic localization of p120-catenin was characteristic to the lobular neoplastic cells in all but one case. We postulate that this one exceptional case (A7), with membrane localization of all proteins of the E-cadherin complex, has not yet undergone the molecular genetic events that cause inactivation of the E-cadherin complex. Irrespective of case A7, the results of our immunohistochemical analyses demonstrate that without the presence of an invasive lesion the expression of the entire E-cadherin complex at the cell membrane is altered in both atypical lobular hyperplasia and lobular carcinoma in situ lesions.

The use of a whole genome amplification technique to increase the quantity of the DNA template obtained from the lobular neoplastic lesions made it possible to complete the screening of CDH1 for sequence alterations. DOP PCR has been used
previously in combination with SSCP and is sensitive with respect to amplifying small quantities of DNA. According to us, \(CDH1\) mutation analysis by DOP PCR-SSCP was reliable, as aberrantly migrating bands found by using this technique could be duplicated by SSCP using microdissected DNA, without prior amplification by DOP PCR, as template.

Only one (A12) of 13 atypical lobular hyperplasia lesions contained an alteration. On the other hand, every case of lobular carcinoma \(in\ situ\) analyzed has been found to harbor a sequence alteration (13/13). To date, there have been no mutations reported in cases of lobular carcinoma \(in\ situ\) that lacked adjacent invasive carcinoma; somatic mutations have only been found in invasive lobular carcinoma or lobular carcinoma \(in\ situ\) with adjacent invasive disease. Although the data do not allow us to speculate as to whether these \(in\ situ\) lesions are precursors to invasive carcinoma, we can conclude that somatic alterations in \(CDH1\) appear to occur predominantly at the \(in\ situ\) stage.

The study design, the sensitivity of the techniques used and the high cellularity of the lesions of atypical lobular hyperplasia ruled out the possibility of false-negative results with respect to \(CDH1\) mutation analysis. The microdissection techniques allowed for the isolation of each lesion from the surrounding tissue/adjacent lesions with no greater than 15–20% contamination of non-neoplastic cells (Figure 1). In addition, the lack of sequence alterations found in the hyperplasia cases was reproduced in independent experiments using microdissected DNA (not previously preamplified by DOP PCR) from each atypical lobular hyperplasia lesion. Altogether, the trend that lobular carcinoma \(in\ situ\) but not atypical lobular hyperplasia cases carried alterations was prominent. Furthermore, this trend supports previous reports of a precursor role for \(in\ situ\) lesions as it demonstrates an increase in genetic hits from hyperplasia to carcinoma \(in\ situ\) characteristic of progression.

Moreover, in the cases that contained both lobular neoplastic lesions, the atypical lobular hyperplasia and lobular carcinoma \(in\ situ\) were microdissected separately and in all cases the \(in\ situ\) component was found to harbor a sequence alteration, whereas hyperplasia did not. Even case A14/L13, noted in the pathology review as containing adjacent atypical lobular hyperplasia and lobular carcinoma \(in\ situ\) lesions as well as invasive lobular carcinoma in a separate block, was found to contain a mutation in the \(in\ situ\) stage but not hyperplasia. These cases further substantiate our hypothesis that mutations are first detected at the \(in\ situ\) stage.

\(CDH1\) sequence alterations reported to date in studies investigating progression from lobular carcinoma \(in\ situ\) to invasive lobular carcinoma have been inactivating mutations. The nonsense mutation found in case A14/L13 was predicted to cause protein truncation. As this case (A14/L13) has an adjacent invasive lesion, we suggest that the presence of an inactivating \(CDH1\) mutation could be an event that distinguishes lobular carcinoma \(in\ situ\) lesions that are precursors from those that are not.

The frameshift mutations found in cases L4, L8, and A12 are likely to have an effect on protein function. The bioinformatics tool, SIFT, clarified to some extent the functional significance of the missense mutations we detected. As only three missense mutations were predicted to affect protein function, it is likely that lobular carcinoma \(in\ situ\) lesions found to harbor only missense mutations do not progress to invasive carcinoma. The presence of a missense mutation could simply indicate an environment amenable to genetic alteration, as observed in case A2/L1 where adjacent \(in\ situ\) lesions were found to harbor different missense mutations. Moreover, since the loss of protein expression was not always associated with a sequence alteration, as in the cases containing atypical lobular hyperplasia, we conclude that mutation alone could not cause the lack of E-cadherin protein expression that we have observed.

Very little is known about the molecular genetic events occurring at the stage of atypical lobular hyperplasia. To our knowledge, this study represents the first investigation of alterations in E-cadherin in atypical lobular hyperplasia. The overwhelming absence of mutations in cases of atypical lobular hyperplasia, coupled with a loss of E-cadherin protein expression, suggests that in these lesions, E-cadherin may be inactivated by means other than the presence of mutation. To address this, we evaluated these lesions for LOH.

LOH has been studied in lobular breast cancers and the chromosomal region of 16q, the location of \(CDH1\), has been found to have a high degree of LOH. These previous studies have found LOH to accompany mutations in cases of invasive lobular carcinoma or lobular carcinoma \(in\ situ\) with adjacent invasive lobular carcinoma. However, in the present study, all cases of lobular carcinoma \(in\ situ\) were found to harbor mutations but LOH was found in only two of these 13 cases. In both instances, LOH was detected in cases that harbored missense mutations. The classic pattern of an inactivating mutation coupled with LOH does not appear to be characteristic of the lobular carcinoma \(in\ situ\) lesions in our collection.

Methylation of the E-cadherin promoter has been reported in studies investigating invasive lobular carcinoma, breast carcinomas, and breast cancer cell lines that lack E-cadherin expression. In the case of sporadic gastric carcinomas, promoter methylation has been described as a second hit leading to inactivation of the E-cadherin gene. We hypothesize that epigenetic mechanisms acting at the hyperplastic stage could provide an explanation for the loss of E-cadherin that we have observed in both atypical lobular hyperplasia and lobular
carcinoma in situ. Further research efforts will address this possibility.

Although many epidemiological studies have clarified the risk associated with atypical lobular hyperplasia and lobular carcinoma in situ, at present there are no morphological or clinical features that help identify those individuals who have the greatest risk of developing invasive disease. From our study, we conclude that an altered E-cadherin adhesion complex, including alpha-, beta- and p120-catenin, is characteristic of lobular neoplastic lesions and occurs prior to progression to invasive disease. Furthermore, somatic mutations appear to be an event characteristic of lobular carcinoma in situ and not atypical lobular hyperplasia lesions. However, the reported molecular data, that is, mutations, LOH, chromosomal gains/losses, and loss of protein expression, coupled with the findings of our investigation, still leave questions regarding the progression from lobular neoplasia to invasive breast cancer. Further research into the molecular events occurring at the hyperplastic and in situ stages is essential to understanding and identifying this subset of lobular neoplastic lesions that have the highest risk of progressing to invasive carcinoma.

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