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13. ABSTRACT (Maximum 200 Words)

Atypical lobular hyperplasia (ALH) and lobular carcinoma *in situ* (LCIS), i.e. lobular neoplasia (LN), are lesions of significance in terms of implication to the patient in the development of invasive carcinoma. A correlation between the lobular histological type and the 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 in order to characterize a profile for lobular neoplasia. We have obtained 22 LN cases (14 ALH and 14 LCIS lesions). LN cases have been found to be negative for E-cadherin (26/26), beta-catenin (25/26) and alpha-catenin (21/23) protein expression. CDH1 alterations have been found to characterize LCIS lesions (14/14) but not ALH lesions (1/14). Moreover, LOH at the 16q locus was found to be an infrequent event (3/25). Studies are now in progress to evaluate p120-catenin protein expression, E-cadherin promoter methylation, and the involvement of novel genes at the stage of LN by CGH microarray. The completion of these analyses will provide insight into the molecular genetic profile for lobular neoplasia.

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Introduction

Tumor development from an early lesion through to invasive disease is not a clearly defined progression in the breast. A continuum can be hypothesized for breast lesions of the lobular histological type, from hyperplasia through *in situ* carcinoma to invasive breast carcinoma (IBC). However, discovering the specific molecular genetic events that mark the transition from an early lobular lesion to an invasive tumor is necessary to both support and subsequently understand this potential lobular progression.

Lobular neoplasia is a histological classification that includes atypical lobular hyperplasia (ALH) and lobular carcinoma *in situ* (LCIS).[1] Both ALH and LCIS 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 ALH to LCIS that is also reflected in the relative risk to the patient in the development of IBC. A finding of ALH implies a four to five fold increased risk of subsequent carcinoma in either breast, and a finding of LCIS implies an eight to ten fold increased risk to the patient.[2-4]

A strong correlation between the lobular histological type and inactivation of the cell adhesion protein epithelial(E)-cadherin has been reported.[5-11] As well, mutations in the E-cadherin gene have been found in invasive lobular carcinoma (ILC) and LCIS with adjacent ILC.[5-11] Our studies look to investigate both ALH and LCIS 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.

Key Research Accomplishments

As stated in the annual summary report for 2002/2003, the type of lesion originally proposed to be investigated by this study has been expanded to include both types of lobular neoplastic lesions, i.e. ALH and LCIS. Tasks proposed in the original Statement of Work that looked solely to investigate LCIS lesions continue to be carried out on the accrued ALH lesions.

<u>**Task 1:**</u> Tissue Accrual

As reported last year, the original collection has been re-reviewed to identify all the lobular neoplastic lesions in each case. The collection contains 22 cases currently being studied, including 14 ALH lesions (A1-A14) and 14 LCIS (L1-L13) lesions. Five cases contain both ALH and LCIS lesions (A2/L1, A3/L3, A4/L5, A11/L12, A14/L13). Moreover, case A2/L1 was also characterized by multifocal LCIS (L1-1, L1-2) and the LCIS lesions were housed in separate blocks. A lesion containing low grade DCIS (P1) was included as a control for the IHC analyses. All cases in the collection are formalin-fixed, paraffin-embedded archived breast tumor blocks containing lobular neoplastic lesions lacking adjacent invasive carcinoma. The accrual of cases remains ongoing.

A recent collaboration has also resulted in 13 cases containing only ALH to be added to our collection (these cases are not included in the above collection of 22). As all of these cases generously came to us from other hospitals, we are limited by the number of sections able to be cut from each block. Therefore due to this finite amount of material, we have opted to use these cases solely for the CGH microarray study. Where possible, IHC will be carried out to evaluate the status of E-cadherin, beta-catenin, alpha-catenin and p120-catenin protein expression.

Task 2: Completion of the Analysis of E-cadherin in LCIS

The analysis has been completed for sequence alterations in the E-cadherin gene, CDH1. All cases of ALH and LCIS have been screened for alterations using single strand conformation polymorphism (SSCP) and manual DNA sequencing (in both techniques results were visualized by autoradiography). Sixteen polymorphisms (data not shown) and 15 mutations (Table 1) have been characterized. Three of the mutations (cases A12, L4, L8) are deletions causing a frameshift and a premature stop codon. The mutation found in case L13 is a nonsense mutation and the remaining 11 were missense mutations (cases L1-1, L1-2, L2, L3, L5, L6, L7, L9, L10, L11, L12). The mutations are located in exons 3, 6, 7, 9, 10, 11, 12, 13 and 15. With the exception of the missense mutation found in case L1-1 (previously reported by Rieger-Christ *et al.*(16)) all alterations found in this study are novel.

The five cases (A2/L1, A3/L3, A4/L5, A11/L12, A14/L13) that contained both lobular neoplastic lesions were found to harbor a sequence alteration in the LCIS but not the adjacent ALH lesion. Furthermore case A2/L1, which contains ALH and two LCIS lesions (in separate blocks) was found to have a different missense mutation in each of the LCIS lesions, but no alteration in the ALH component. The complete list of alterations has been appended (Table 1).

Task 3: Analysis of LCIS by Chromosomal Comparative Genome Hybridization (CGH)

The analysis of ALH and LCIS lesions by chromosomal CGH has not yet begun.

Task 4: Analysis of LCIS by CGH Microarray

A CGH microarray protocol has been optimized for use with DNA extracted from formalin-fixed, paraffin-embedded material. The technique has been tested for efficiency and accuracy using cases containing both lobular neoplastic and invasive breast lesions. The statistical analysis from these experiments is currently in progress. Once complete, the ALH and LCIS cases in our collection will be arrayed to determine whether there are novel genes altered at either stage of lobular neoplasia.

Task 5: Analysis of LCIS by Immunohistochemistry (IHC)

IHC optimization and staining has been completed for all antibodies i.e. E-cadherin, beta-catenin, alpha-catenin, and p120-catenin. Subsequently, all ALH/LCIS cases have been stained for E-cadherin, beta-catenin, alpha-catenin and p120-catenin. A scoring system to evaluate the protein expression of each protein has been developed (in collaboration with Dr. Frances O'Malley) for E-cadherin, beta-catenin, and alpha-catenin. The scoring system for p120-catenin is currently being developed. To date, a complete lack of E-cadherin, beta-catenin and alpha-catenin protein expression has been found in all cases of ALH and LCIS evaluated by IHC (Table 1). Each lesion contains an adjacent internal positive control. Moreover, we have included a case of DCIS as a positive control for all IHC experiments, which has subsequently stained positive for all proteins.

Additional work: Evaluation of methods of inactivation of E-cadherin in lobular neoplasia.

As reported last year, the ongoing results from our studies have suggested that in lobular neoplastic lesions E-cadherin may be inactivated by means other than the presence of mutation(s). To address this, other methods of E-cadherin inactivation, namely loss of heterozygosity (LOH) and E-cadherin promoter methylation, were proposed to be evaluated.

The LOH analysis of chromosome 16q (the chromosomal area that houses the E-cadherin gene) has been completed. LOH was determined using five microsatellite markers and for each marked each case was evaluated paired with its corresponding normal. Four

microsatellite markers amplified regions of dinucleotide repeats (D16S421, D16S496, D16S503, D16S3095) and one amplified a region containing tetranucleotide repeats (D16S752). The markers used are located at chromosome locus 16q21-16q22.1, with the amplified PCR products ranging in size from 100 to 232 base pairs. A PCR-based method was used to determine the LOH status of each tumor/normal pair (the PCR products were separated using 7% denaturing formamide gel and visualized with autoradiography. Multiple independent observers evaluated each marker, scoring each case as 'LOH', 'no LOH', or 'uninformative'. We observed 80 to 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 22 showed no LOH. The LOH results to date have been compiled in Table 1.

The assessment of E-cadherin promoter methylation has also been proposed to be carried out on our lobular neoplastic lesions. Optimization of a methylation specific PCR protocol is in progress.

Reportable Outcomes

1. "E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma in situ". Manuscript is in preparation.
2. "Characterization of a molecular genetic profile for lobular neoplasia." (Oral presentation) United States and Canadian Academy of Pathology (USCAP) Annual Meeting, March 2004.
3. "Characterization of a molecular genetic profile for lobular neoplasia". Department of Laboratory Medicine and Pathobiology Graduate Student Research Day, University of Toronto, March 2004.
4. "Characterization of a molecular genetic profile for lobular neoplasia." (Poster presentation) San Antonio Breast Cancer Symposium, December 2003.
5. "Characterization of a molecular genetic profile for lobular neoplasia". Proceeding of the American Association of Cancer Research (1st Edition), volume 44, 2003.
6. "Characterization of a molecular genetic profile for lobular neoplasia". Department of Laboratory Medicine and Pathobiology Graduate Student Research Day, University of Toronto, March 2003.
7. "Analysis of E-cadherin in Lobular Carcinoma *in situ*". Proceedings of the American Association of Cancer Research, volume 43, 2002.
8. "Profiling Lobular Neoplasia". Center for Cancer Genetics Seminar Series, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, March 2002.
9. "Analysis of E-cadherin in Lobular Neoplasia". Department of Laboratory Medicine and Pathobiology Graduate Student Research Day, University of Toronto, March 2002.
10. "Analysis of E-cadherin in Lobular Neoplasia". Divisional Seminar Series, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, February 2002.

Conclusions

Cases lacking both expression of E-cadherin, beta-catenin, and alpha-catenin, as well as CDH1 gene alterations suggest that another mechanism involved at the stage of hyperplasia is causing the lack of protein expression. In light of these findings, studies were proposed to evaluate alternate mechanisms of gene silencing. As we have determined LOH to be an infrequent event in all cases of lobular neoplasia, we are currently investigating the possibility that promoter methylation is the means by which E-cadherin is silenced in these lesions. Furthermore, the involvement of novel genes at the stage of lobular neoplasia is an alternate explanation for the results we have found to date, and is currently being evaluated by CGH microarray. The completion of these analyses will ultimately provide insight into the molecular genetic profile for lobular neoplasia.

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Table 1: Summary of results to date for the Case Accrual, CDH1 Mutation Analysis, Immunohistochemistry (for E-cadherin, beta-catenin, alpha-catenin and p120-catenin) and LOH Analysis.

Case Accrual			Mutation Analysis			Immunohistochemistry[a]				LOH
Case	**Original**	**Lesion**	**Alteration**	**Exon**	**Effect**	**E-cad**	**β-ctn**	**α-ctn**	**p120-ctn**	**LOH**
A1	Yes	ALH	none			-[b]	-	-	not scored[c]	No
A2(L1)	Yes	ALH	none			-	-	-	not scored	No
A3(L3)		ALH	none			-	-	-	not scored	NR
A4(L5)		ALH	none			-	-	-	not scored	No
A5	Yes	ALH	none			-	-	-	not scored	No
A6	Yes	ALH	none			NR	NR	NR	NR	NR
A7	Yes	ALH	none			-	+	+	not scored	No
A8		ALH	none			-	-	-	not scored	No
A9		ALH	none			-	-	-	not scored	No
A10		ALH	none			-	-	-	not scored	Yes
A11(L12)		ALH	none			-	-	-	NR	No
A12		ALH	2410delC	15	Frameshift	-	-	-	not scored	No
A13		ALH	none			-	-	-	not scored	No
A14(L13)		ALH	none			-	-	NR	NR	No
L1-1(A2)	Yes	LCIS	856G>A	7	Ala > Thr	-	-	-	not scored	No
L1-2(A2)	Yes	LCIS	362A>G	3	His > Arg	-	-	-	not scored	Yes
L2	Yes	LCIS	274C>T	3	His > Tyr	-	-	-	not scored	No
L3(A3)	Yes	LCIS	2125G>A	13	Ala > Thr	-	-	-	not scored	No
L4	Yes	LCIS	1323_1333del	10	Frameshift	-	-	-	not scored	No
L5(A4)	Yes	LCIS	1366G>A	10	Val > Met	-	-	-	not scored	No
L6	Yes	LCIS	1676G>A	11	Ser > Asn	-	-	-	not scored	No
L7	Yes	LCIS	185G>A	3	Gly > Asp	-	-	-	not scored	Yes
L8	Yes	LCIS	1309_1310del	9	Frameshift	-	-	+	NR	No
L9		LCIS	760G>T	6	Asp > Tyr	-	-	NR	not scored	NR
L10		LCIS	989C>T	7	Thr > Ile	-	-	-	not scored	No
L11		LCIS	2075C>T	13	Ala > Val	NR	NR	NR	NR	No
L12(A11)		LCIS	1800A>G	12	Ile > Met	-	-	-	NR	No
L13(A14)		LCIS	1595G>A	11	Trp > amber	-	-	NR	NR	No
P1[d]	Yes	DCIS	none			+	+	+	not scored	NR

[a] - All cases contained adjacent normal epithelium that served as the internal positive control for the IHC analyses.
[b] -, negative membrane staining; +, positive membrane staining; NR, no result due to lack of tissue.
[c] - A pathologist has not yet evaluated/scored the p120-catenin IHC stained sections.
[d] Case P1 was used as a positive control for all IHC experiments as it contains no ALH/LCIS.