Use of laser capture microdissection allows detection of loss of heterozygosity in chromosome 9p in breast cancer

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Abstract. The present study was designed to determine whether loss of heterozygosity (LOH) in the p arm of chromosome 9 in invasive ductal carcinoma of the breast is detected during the neoplastic progression of the disease. Using laser capture microdissection (LCM) epithelial cells were isolated from 14 invasive ductal carcinoma cases (IDC), ductal carcinomas in situ (DCIS), normal mammary lobules, skin and/or lymph nodes of paraffin embedded tissue sections. LOH analysis of chromosome 9p was performed utilizing the microsatellite markers D9S199, D9S157, D9S171, D9S265 and D9S270. The highest frequency of LOH was observed in invasive ductal carcinomas, which reached a maximum at the 9p22-23 chromosomal location (D9S157). In addition, DCIS lesions presented a high frequency of LOH in 9p22-23 (D9S157), followed by 9p21 (D9S171), D9S199 and D9S265, which were similar in frequency to those observed in IDC. A novel finding was the intralesional heterogeneity in LOH within the same DCIS or IDC case. This is an indication that clones of cells that differ in genetic composition coexist in the same lesion. Notably, phenotypically normal breast tissues adjacent to IDC or DCIS exhibited LOH at D9S157 and/or D9S171. Together, these data indicate that LOH of chromosome arm 9p occurs very early in the progression of cancer and that different clones of cells co-exist within a single tumor.

Introduction

Human cancer arises through the accumulation of genetic alterations in multiple oncogenes and tumor suppressor genes. However, the exact timing of the majority of molecular genetic events during carcinogenesis and their correlation with defined histopathological stages are largely unknown. (1-7). Invasive ductal carcinoma (IDC) of the breast is the result of a multistep process, beginning with ductal hyperplasia and followed by atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS), invasive ductal carcinoma and metastatic disease (1-3). Previous studies in the literature (8-10) indicate that alterations in the p arm of chromosome 9 may be a common denominator in human cancer, and may have a role in the early stages of breast cancer, including ductal hyperplasia and DCIS (11-14). Of interest is the finding that loss of heterozygosity (LOH) in the p arm of chromosome 9 may be involved in the pathogenesis of breast cancer (15-19).

In the present study, laser capture microdissection (LCM) was used to analyze paraffin-embedded tissues of the normal breast, ductal hyperplasia, DCIS and IDC to obtain DNA from selected populations of cells for molecular genetic analysis (20-22). LCM was used in order to obtain cells with a high degree of purity in their phenotypes, without contamination of stromal, inflammatory or other cells that could interfere with final conclusions of molecular analysis. The isolated cells representing different stages of breast cancer progression were used for detecting LOH using five microsatellite markers: D9S199, D9S 157, D9S 171, D9S265 and D9S270. The present study was conducted in an attempt to investigate the intratumoral heterogeneity and to associate chromosomal alterations with morphologic findings and proliferation state of the tumor.

Materials and methods

Tissue samples. Paraffin blocks from fourteen primary breast IDC cases (mean age, 56; range, 27-86) that also contained areas of carcinoma *in situ* were selected for the present study. Paraffin blocks containing areas of normal tissue, including breast, skin and lymph nodes, were available from the same patients. Tissue blocks were obtained from the tumor bank of the Breast Cancer Research Laboratory of the Fox Chase Cancer Center (FCCC; Philadelphia, PA, USA). Six serial 5- μ m sections were obtained from each paraffin-embedded tissue block and stained with hematoxylin and eosin (H&E). The first section was coverslipped and the remaining five sections were dehydrated and air dried for their use in LCM and DNA extraction. Tissue sections containing IDC were selected on the basis that DCIS was also present in the same

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Figure 1. Relative map locations of chromosome 9p markers. Approximate genetic distances (cM) were obtained from Genome Interactive Data Bases. Right panel reveals the results of loss of heterozygosity analysis at five loci of chromosome 9p in 14 sporadic breast carcinomas. Three to ten different groups of cells obtained by laser capture microdissection were analyzed in each patient. Each group of cells consists of normal breast tissue (TDLU), DCIS and IDC. •, loss of heterozygosity; o, retention of heterozygosity. DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.





Figure 2. Representative microsatellite amplification (D9S157) relative to one patient (P1). Two foci of cells were analyzed from normal tissue surrounding tumor areas, DCIS and IDC. LOH was observed in different microdissections from IDC and DCIS and also from one foci of normal breast tissue. N, normal breast tissue; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.



Figure 3. Genomic DNA from microdissected normal skin does not display genetic alterations. N, normal breast tissue; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

section. The histopathological type of the carcinoma was classified according to previously described criteria (23). Control tissues consisted of phenotypically normal cells, which were obtained by LCM from: a) Type 1 lobules or terminal duct lobular units (TDLUs) (24); b) normal skin obtained from the mastectomy specimen; or c) lymph nodes free of metastases obtained from axillary dissection from the same patient. This study was approved by the Ethical Review Board (IRB 93-031) of the FCCC and informed consent was obtained from patients for use of their tissue.

LCM. Serial 5- μ m thick sections containing IDC, DCIS and normal tissue were utilized for microdissection. Areas containing IDC, DCIS or normal tissue were identified in the slide that had been stained with H&E and coverslipped. Preferentially, areas containing microscopically homogeneous cells of each type of lesion were selected. Tissues containing areas with dense stroma, inflammatory cells, vascular or lymphatic vessels, muscle or adipose tissue were avoided. Uncoverslipped serial 5-µm sections slides were carefully matched with the respective area identified in the coverslipped stained slide for verifying the accuracy of the type of lesion selected for dissection. Tissue sections were microdissected using a PixCell laser capture microdissection apparatus (Arcturus Engineering, Mountain View, CA, USA) fitted with cap in which a transparent thermoplastic film (ethylene vinyl acetate polymer) was bonded to the underside. A cap was placed on the specific lesion or normal tissue selected for dissection under visual inspection by the operator. Then an infrared laser pulse was activated and selected cells were transferred to the undersurface of the cap, which was lifted off the tissue; the cells obtained at each one of these laser shots were termed 'a capture'. This process was repeated successively in adjacent areas of the same lesion twenty times using a $30-\mu m$ diameter laser beam. The caps containing the captured tissues were placed into a 500- μ l microcentrifuge tube for molecular processing. Multiple foci from three to ten different areas of



Figure 4. Results of representative microsatellite amplifications, loss of heterozygosity and heterogeneity of the marker D9S157 is observed in different microdissections from the same patient (P3) and detected by the complete absence of one of the two alleles present in constitutional normal DNA. Black arrows indicate the positions of the major allelic bands. The same DNA samples also retain heterozygosity at D9S270. N, normal breast tissue; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

in situ cancer, invasive carcinoma and 'normal' tissue were individually microdissected and separately analyzed (Fig. 1). Finally, direct visualization of the transferred tissue by light microscopy of the capsule verified that the desired cells had been captured.

DNA extraction. DNA extraction from the selected tissues was performed following the protocol provided by PixCell IITM (Arcturus Engineering, Inc., Mountain View, CA, USA) Selected tissues were digested for 16 h at 42°C in buffer containing: 10 mM Tris-HCL (pH 8.8), 1 mM EDTA, 1% Tween-20 and 0.05% Proteinase K. The lysate was heated at 96°C for 8 min to inactivate Proteinase K and aliquots of 2 μ l of this lysate were used directly as templates for PCR.

Polymerase chain reaction (PCR) amplification and microsatellite analysis. Five microsatellite markers mapped to the short arm of chromosome 9 (D9S199, D9S157, D9S171, D9S265 and D9S270) were used for LOH analysis. Primers for PCR amplification were obtained from Research Genetics Inc. (Huntsville, AL, USA) and all primer sequence position of the markers, their levels of heterozygosity and distances were obtained from Genome Database version February 2000 (Research Genetics, Inc.). PCRs were carried out according to published study (25). The samples were denatured for 5 min at 94°C and loaded onto a 6% polyacrylamide gel. Electrophoresis was performed at room temperature at 1,400 V for 2-3 h, depending on the length of the marker. Following electrophoresis, gels were transferred to a 3 mm Whatman paper, dried and autoradiographed using Kodak X-OMAT 35x43 film. Films were developed after a 48 to 72-h exposure. Autoradiograms were analyzed following the guidelines of published work (8).

Results

Invasive ductal carcinomas exhibited LOH for the five markers tested, and the marker at 9p22-23 (D9S157) was the most frequently identified, whereas the markers D9S171, D9S199, D9S265 and D9S270 (Fig. 1) were less frequently detected. LOH in the DCIS samples was found with 4/5 of the markers tested. D9S157 locus was also present in the majority of the samples, followed by 9p21 (D9S171), D9S199 and D9S265. There are several reports in the literature indicating that other tumor suppressor gene(s) may reside within different 9p loci, namely 9p22-23 (8,15,17,26-28). Notably, phenotypically normal breast tissues that were adjacent to IDC and DCIS also exhibited LOH at D9S157 (Fig. 2) and/or D9S171. The finding that LOH at these loci is also present in the normal tissue adjacent to either DCIS or IDC is an indication that microsatellite instability is an early event in the pathogenesis of breast cancer, and occurs even earlier than any morphological changes are able to be identified. The present study pursued further the validation of these observations by performing LCM of normal skin and lymphocytes from lymph nodes free



of metastatic disease from 7 of the patients and was unable to detect LOH in these other normal tissues. (Fig. 3). This data supports previous observations reported in the literature (29). It is notable that the practical implications of these observations are of major importance in the evaluation of the resected margins of conservative breast surgery.

A novel finding was that LOH was heterogeneous in its distribution, as it was exhibited in certain foci, but not in all of the tumor foci studied (Fig. 4), suggesting that clones of cells with varied genetic composition co-exist in the same lesion.

Discussion

The present data indicate that LOH at locus 9p22-23, (D9S157) and to a lesser degree at 9p21 (D9S171), occurs during the process of cancer initiation. More notably, clones of cells co-exist within a single tumor, indicating that they do not share a clonal origin and only those cells that have LOH at those loci may progress. The monoclonal origin of cancer has been suggested in the literature (30-32), and cytogenetic analyses have revealed that breast cancers are polyclonal (33-35). The use of LCM (36-38) has allowed the identification of more chromosomal aberrations than is possible using DNA isolated from tumor sections (39). By contrast to previous reports (7,40-42) that sustained clonal derivation from *in situ* cancer, the data presented in the current study support the findings of Fujii *et al* (8), who reported LOH heterogeneity in multiple foci of individual DCIS lesions.

In conclusion, the present study demonstrated that more than one clone of cells may exist in a simple lesion and that genetic divergence occurs during cancer initiation and progression.

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