

Enhanced Touch Preps Improve the Ease of Interpretation of Intraoperative Breast Cancer Margins

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Positive margins and the resulting multiple operations are a major problem for breast conservation therapy. Accurate assessment of intraoperative tumor margins can limit multiple re-excision procedures. Intraoperative touch preparations have been used in the past but can be difficult to interpret without an experienced cytopathologist. The objective of this study is to examine the reliability of enhanced intraoperative touch preps (EIOTP) compared with final pathologic margins. We prospectively performed EIOTP on 20 tumors in women undergoing breast conservation therapy. Six margins and the main tumor were touched onto poly-L-lysine coated slides. The slides were stained with anti MUC-1 and anti-E-cadherin antibodies, and Hoechst nuclear stain. A parallel set of slides were stained with hematoxylin and eosin for comparison. The EIOTP results were compared with pathologic interpretation of paraffin embedded permanent sections. A total of 120 margins underwent EIOTP in 20 patients. We found a sensitivity equal to 80 per cent, specificity 100 per cent, positive predictive value 100 per cent, and negative predictive value 99 per cent. EIOTP in conjunction with MUC-1 and E-cadherin by immunofluorescence is a sensitive and highly specific mechanism to identify cancer cells at breast tissue margins. The immunofluorescence stains may help the pathologist to identify cancer cells in fresh breast tissue and limit breast re-excisions in the future.

MULTIPLE RANDOMIZED prospective trials with greater than 10 years of follow-up have proven that breast conservation therapy (BCT) has equal survival efficacy compared with mastectomy in treating early stage breast cancer and is the current standard of care. Many studies show that local recurrence is significantly higher in patients with a positive margin excision.¹⁻⁵ Therefore, achieving negative surgical margins during BCT is an essential goal in breast cancer treatment.⁶ Despite improved preoperative imaging techniques, such as breast MRI and ultrasound, many studies report positive margin rates of 25 to 50 per cent for partial mastectomy, even in early stage breast cancer patients.⁷⁻⁹ The consequences of a positive margin are significant for patients, the surgeons, and the healthcare system.

The most reliable method to achieve tumor-negative margins is to evaluate multiple intraoperative frozen sections of the excised tumor margin. However, the technique has many limitations. Technically, freezing the adipose tissue of the breast and making adequate and representative sections are difficult.¹⁰ Frozen section evaluations are labor intensive, time consuming, and may compromise permanent sections. Pathologists have used touch preparations of the margins to limit the tissue used. However, the utility of this technique alone is limited by the pathologist's expertise in cytology and technical difficulties related to artifacts produced by the air drying process.¹¹

There is no unique marker to all breast cancer cells, thus hindering the discrimination of breast cancer cells from normal epithelium. In our study, we used two breast epithelial membrane markers: MUC-1 and E-cadherin to identify potential tumor cells, in addition to visualization by traditional hematoxylin and eosin stains. They bind approximately 60 to 80 per cent of breast cancers and significantly less in benign breast epithelial cells. Increased MUC-1 expression correlates with high metastatic potential and poor survival,¹² whereas changes in E-cadherin function are

However, traditional immunostains are time consuming and limited by the paucity of cells at the margins. In this study, we propose to develop a real-time and sensitive intraoperative detection of enriched cells by touch preparation using a combination of cytology and immunofluorescent staining.

Methods

We obtained Institutional Review Board approval from the University of California at San Diego to study breast cancer and non-cancer tissue from patients. Patients underwent their planned procedure for breast surgical treatment and the specimen was removed and sent to pathology. The fresh specimen was gently imprinted onto poly-lysine coated glass slides in six margins (anterior, superior, inferior, medial, lateral, and deep) and a cross-section of the tumor. Two sets of slides were obtained. One set was fixed in 95 per cent ethanol and stained with hematoxylin and eosin. A separate set of slides was incubated with human IgG (Invitrogen, Carlsbad, CA) in PBS and 5 per cent fetal bovine serum for 10 minutes at room temperature to block Fc receptors and avoid nonspecific binding of the antibody. The slides were washed twice in PBS and fixed in 4 per cent paraformaldehyde for 10 minutes at room temperature. The antibodies were pre-labeled using the Alexa Fluor labeling kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and added together as a cocktail: MUC-1 (10 $\mu\text{g}/\text{mL}$) (Abcam, Cambridge, MA) and E-cadherin (20 $\mu\text{g}/\text{mL}$) in PBS + 5 per cent FBS. The slides were incubated for 30 minutes at room temperature in the dark, and then washed twice in PBS. For nuclear staining, the slides were incubated in Hoechst solution (10 $\mu\text{g}/\text{mL}$) in PBS for 30 minutes at room temperature in the dark. Slides were washed twice in PBS, mounted in Gelvatol (gift from Dr. Pestonjamas, University of California San Diego Cancer Center Digital Imaging Shared Resource) and stored at 4° C until they were analyzed using ImageJ software (NIH). A pathologist reviewed the touch prep slides and identified positive margins on touch prep. We correlated these findings to the final pathologic examination of the entire specimen.

Nuclear morphology was performed by selecting the cells for acquisition by a technician and a pathologist. Cells were excluded for analysis if they had 1) overlapping nuclei, 2) incomplete staining, and 3) stripped nuclei. We measured the area, largest diameter, and circularity of the nucleus.

Results

A total of 120 margins underwent EIOTC in 20 patients. Three patients had ductal carcinoma *in situ*, 1

Eight out of 10 (80%) tumors were MUC-1 and 6 out of 10 were E-cadherin positive (60%).

Nuclear morphology: We found that the area of the nucleus in the tumor cells was significantly larger than the normal breast ductal cells (75.0 ± 11.0 vs 41.0 ± 3.0 μm^2 ; $P < 0.01$). Likewise, the diameter of the nucleus in the tumor cells was significantly larger than the normal breast ductal cells (12.0 ± 0.6 vs 9.4 ± 0.2 μm ; $P < 0.01$). However, the shape of the nucleus was not significantly different between the two groups (0.71 ± 0.03 vs 0.69 ± 0.01 ; $P = 0.11$).

In these consecutive 120 margins, 4 were positive on final pathology. The enhanced intraoperative touch prep (EIOTP) had one false-negative and no false-positives. We identified three out of four positive margins (Fig. 1). The overall sensitivity was 80 per cent; specificity 100 per cent. (Table 1).

Discussion

The technique of using touch preps to detect tumor cells at breast cancer surgical margins has been studied over the last 20 years.^{11, 14, 15} The largest study by Klimberg et al.¹¹ examined 428 patients with breast lesions and had a sensitivity of 96 per cent and specificity of 100 per cent. However, most institutions have not embraced this technique. Artifacts associated with drying and surface cautery can affect touch prep interpretation and the availability of an experienced cytopathologist limits its widespread use.¹⁰ Therefore, positive margins in BCT continue to be an issue.

With this data in mind, we set out to design a system which could detect malignant cells at surgical margins quickly, that would be less labor intensive for the pathologist. We aimed to determine the most reliable way to separate malignant and benign cells based on single cell appearance. Nuclear morphology has been used in analysis of fine needle aspirations to distinguish malignant and benign single cells, either manually or by computer-aided technology. Our study confirmed these reports that cancer cells had significantly larger nuclear area and diameters compared with benign cells. An automated system that separates cells by nuclear morphology could eventually be used for this purpose.¹⁶

Furthermore, other investigators have used quantitative fluorescence image analysis to confirm this separation, showing that fluorescence image analysis can be used to detect malignant cells in fine needle aspirations and ductal lavage fluid when used on archival material. These investigators used staining of G-actin, P53, and DNA content.^{17, 18} Our data confirmed that fluorescent staining can identify 80 per cent of cancer cells using known membrane markers

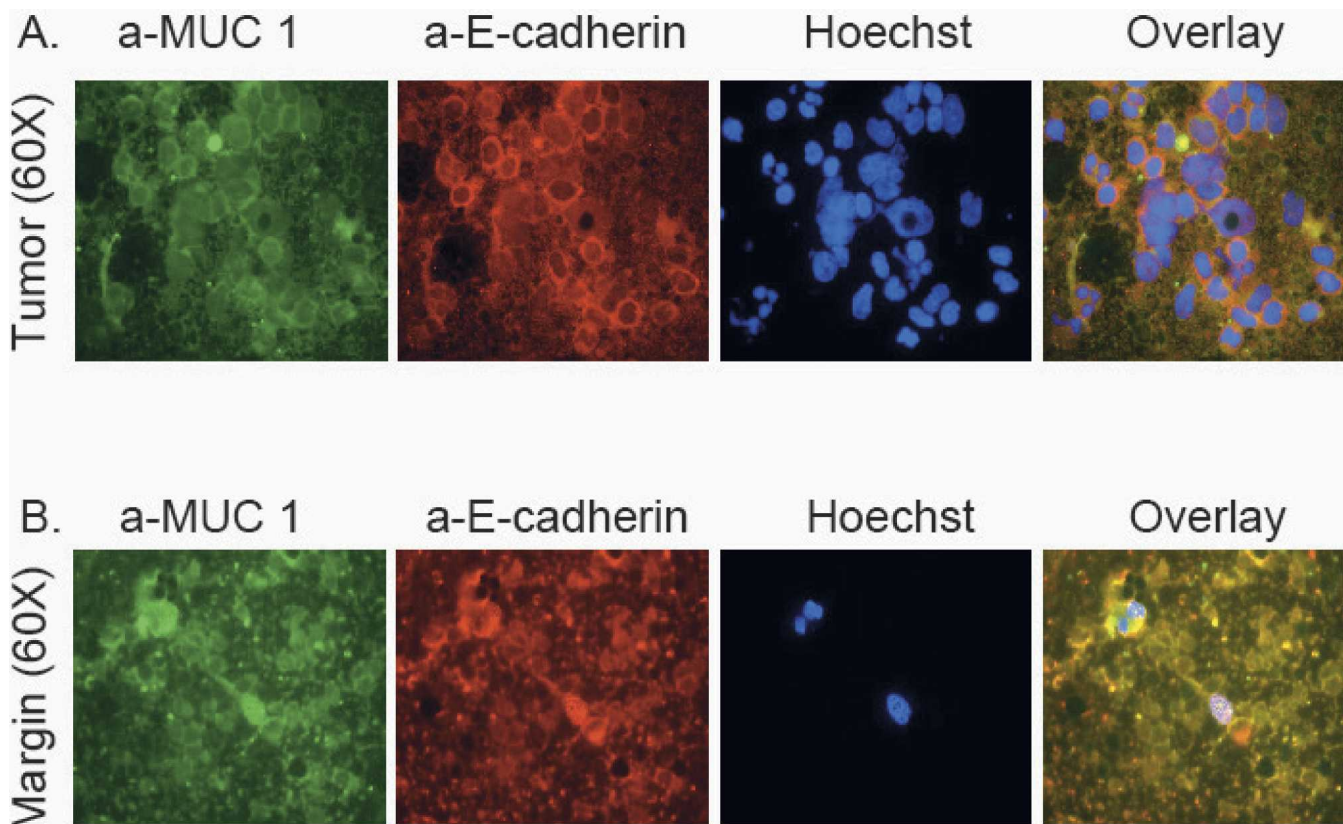


FIG. 1. Immunofluorescent staining for MUC-1 and E-cadherin and with Hoechst nuclear stain. (a) Cross section touch preparation of the breast tumor stained for MUC-1, E-cadherin and Hoechst shown at 60x magnification. (b) Touch preparation of positive margin. The cells are stained for MUC-1, E-cadherin and Hoechst and shown at 60x magnification.

TABEL 1. Results of Enhanced Intra-Operative Touch Preparation (EIOTP)

	EIOTP +	EIOTP -
Permanent Section +	3	1
Permanent Section -	0	115

such as MUC-1 and E-cadherin even when studied prospectively. Other surface markers for neoplastic cells are currently being evaluated to increase the sensitivity of the method. The time required for the stains were within 50 minutes in this study, which demonstrates the potential to offer real time results intraoperatively. We are improving our staining techniques with the goal to visualize the images within 30 minutes. At our institution, frozen sections are supposed to be completed within 30 minutes and we plan to refine our techniques to be completed in 30 minutes as well. Furthermore, we plan to automate our system so it will be less labor intensive for the pathologist.

This data demonstrates we were able to detect cancer cells at surgical margins that were collected in real time in the operating room, with 80 per cent sensitivity and 100 per cent specificity with the aid of immunofluorescence staining. The advantage of using im-

muno-staining, in conjunction to traditional hematoxylin and eosin, is that it offers greater information on tumor cell immunophenotype and potentially other prognostic characteristics, such as ER, PR, or Her2/neu positivity, that can be available to the treating physicians immediately after the tumor is excised. Combined with elimination of the second surgery to excise tumor positive margins, immunophenotyping of the tumor cells allows greater flexibility in treatment and management of the patients.

Realizing some of the limitations of traditional touch cytopreparations, such as poor quality of the cells with excessive artifacts, we have to identify clear-set criteria on the cells that are acceptable for analysis. Another challenge is the paucity of the cells obtained by touch preps that can give insufficient results. We have chosen poly-L-lysine coated slides and achieved satisfactory success in cell quantity. Additional studies to improve on our techniques include multicolor, multiparameter antibody optimization in the least amount of time, as well as exploring automation of the image analysis.

Acknowledgments

We would like to acknowledge: DOD grant W81XWH-06-1-0522.

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