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Ductal Lavage Fluid

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**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**  
Reliable intermediate biological markers for breast cancer risk, that can be easily detected in both pre- and post-menopausal women, do not exist at the present time. For more than 20 years, the ability to access breast ductal fluid through the nipple has prompted initiatives to develop a PAP-like test for breast cancer. Yields were variable, not every woman yielded fluid, and there was no assurance of obtaining samples from the entire length of the ducts. In this proposal, we will use a 1) a facile ductal lavage (DL) technique using cannulating catheters which flushes each duct to yield thousands of ductal cells. 2) a panel of markers consisting of three genes, Cyclin D2, Twist and retinoic acid receptor  $\beta$ 2 (RAR $\beta$ 2), which are aberrantly hypermethylated in breast cancer cells. We will standardize the techniques using fluid from cancer patients, and then evaluate the frequency of cells positive by MSP assays in ductal lavage obtained from women with a high risk of developing breast cancer, such as patients with lobular carcinoma, patients with cancer in one breast, and those with mammographically suspicious lesions. Thus, we aim to develop a PAP test for the breast.

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## INTRODUCTION

Detected early, breast cancer is an eminently curable disease. However, reliable intermediate biological markers for breast cancer risk, that can be easily detected in both pre- and post-menopausal women, do not exist at the present time.

### *Methods to detect breast cancer cells.*

Conventional cytological examination of breast cells is a fairly reliable test to detect breast cancer cells. However, its sensitivity and specificity could be complemented and supplemented with molecular diagnostic tests.

*Ductal lavage:* Ductal lavage is not a new procedure. It was performed successfully several years ago by Sartorius (1), who performed contrast ductography and ductal lavage in 469 women. Cytological evaluation revealed cancer in 18 women. Ductal fluid cytology was the only indication of cancer in 7 of the 18 women. Recently, two studies have been published on the use of ductal lavage as a means of detecting breast cancer early, particularly in women who are at high risk of developing breast cancer. In a recently published study, cells obtained by ductal lavage from nearly 500 women at high risk of developing breast cancer (risk more than 1.7 by the Gail model) were examined. Cytological examination revealed abnormalities in ---. At least three new cases of breast cancer were detected in this population consisting of mammographically and clinically normal women. Cytology continues to remain a difficult assay, subject to varied interpretation, and depending, in large part, on the expertise of the cytologist. This would be immensely aided by the development of an objective, molecular test.

### *Genes aberrantly hypermethylated in breast cancer*

It is increasingly clear that silencing of gene expression by promoter hypermethylation is a common feature of cancer and is seldom seen in normal tissues except for imprinted genes and genes on the inactive X chromosome. Using a sensitive assay called methylation specific PCR, initial studies show that MSP can detect 1 methylated gene copy in 1000 unmethylated gene copies, attesting to the sensitivity of this approach. Importantly, the assays are highly specific in that no abnormal methylation was detected in serum DNA if the same alteration was not present in the primary tumor.

Promoter methylation has been reported in 15-50% of primary breast tumors for the following genes: 14.3.3 sigma (2,3), RAR-beta (4, 5), cyclin D2 (6), HOXA5 (7), Twist (8), RASSF1A (9, 10, 11), HIN-1 (12) and NES-1 (13), to name a few.

## HYPOTHESIS

We hypothesize that cytologic and molecular analysis of ductal lavage fluid can serve as a noninvasive technique to assess presence of malignant epithelial cells within the breast. Thus, it will serve to complement screening mammography in asymptomatic women or supplement information obtained from diagnostic mammography in women with suspicious findings. It is also conceivable that studies of this type could ultimately identify intermediate biomarkers that could some day be useful for assessment of risk or efficacy of prevention strategies.

## OBJECTIVES

This proposal is designed to test the idea that it is possible to develop a PAP-like test for breast cancer. If successful, it would provide the foundation for a separate application to undertake definitive prospective testing of these approaches in a larger cohort of women. Thus the aims of this application are:

1. To standardize the techniques required for the performance of MSP assays on cells obtained by ductal lavage from women with breast cancer.
2. To evaluate the frequency of cells positive by MSP assays in ductal lavage obtained from women with a high risk of developing breast cancer.

### **STATEMENT OF WORK AND ACCOMPLISHMENTS 2001-2002**

By the time funding began on this grant, all the specific aims of the previous SOW had been accomplished, and a paper describing these is appended (6). A new SOW was submitted and approved. We will now describe the accomplishments on the revised SOW from June 2001 to June 2002.

*Month 1 to 6: Optimize conditions for duplex or multiplex assays for the 5 markers RAR $\beta$ , RASSF1A, Twist, cyclin D2, and NES1 using fluid spiked with varying numbers of tumor cells.*

1. Conditions were optimized for RAR $\beta$ , RASSF1A, Twist, Cyclin D2 and HIN-1. The incidence of HIN-1 methylation is very high (60-70%) in primary breast cancers compared to NES-1 (30%). Therefore HIN-1 was substituted as a marker in this panel.
2. Multiplex PCR assays were developed for all five genes. Sensitivity was very high, specificity was very good as well. However, the method is not quantitative. Calling a reaction positive versus negative was also subjective and dependent on a number of factors. This was deemed unsatisfactory.
3. Therefore, we have now standardized quantitative methylation specific PCR for all five genes. Here the readout is of the percentage of methylation present in each sample, and one can calculate how many microgram equivalents of methylated DNA are present in the particular sample. This method is able to detect down to 20 picogram of methylated DNA. Efforts are underway to determine whether the same level of sensitivity will be achieved in the presence of excess normal ductal cells. We will use Q-MSP for all further analysis.

*Then test the MSP markers on ductal lavage from tumor-containing breast of 25 women just prior to surgery for known lesion.*

We have just received all the necessary IRB clearances needed to embark on this part of the study. Ductal lavage will be performed on 25 women within the next 2-3 months and the Q-MSP procedure will be applied to sodium bisulfite treated DNA.

*Months 7 to 12: If sample is limiting, perform RAR $\beta$ , RASSF1A, Twist, cyclin D2, and then NES1 in a stepwise fashion, going from the highest (85%) to the lowest (30%) incidence markers. Samples may need pre-amplification to enable use of all 5 markers on the varying numbers of tumor cells obtained by ductal lavage. Standardize this methodology. Then test the MSP markers on ductal lavage from tumor-containing breast of 25 additional women just prior to surgery for known lesion.*

We have made progress on this specific aim as well. Knowing that cells from ductal lavage will always remain a limiting factor, we are devising methods to amplify the DNA in such a way that fidelity is maintained, and a large quantity is made available.

1. We have been able to do this. Starting with 20 picogram of methylated DNA, we can now generate 30 micrograms of DNA. Whether this DNA retains its ability to be amplified by Q-MSP remains to be tested.
2. Also, mixtures of unmethylated and methylated DNA need to be tested to determine if 2-10 tumor cells equivalents can be amplified in the midst of 1000 to 10,000 normal cells.

*Months 12-18: Test fluid from contralateral ducts (tumor-free by mammogram and clinical exam) of 50 patients with breast cancer. Complete MSP assays on the fluid obtained from both breasts of a total of 50 cancer patients. If recovery of cells is not satisfactory, optimize conditions, add patients to the study to get results from approximately 200-300 samples of ductal fluid (2-3 ducts per breast X 50) from 50 individuals. Compare MSP results with cytopathological data, and histopathology of the resected tumor, on each sample.*

*Months 18-30: Approach high-risk, tumor free women who attend the BOSS (breast ovarian surveillance service) clinic in Johns Hopkins, and other high risk individuals to undergo this procedure. Accrual will be slower in this category until the minimal discomfort involved and potential benefit becomes a publicized fact. Enter 50 individuals into the study. Perform MSP on cells obtained from each ductal lavage, on a total of approximately 200 samples.*

*Months 30 to 36: Complete comparison of MSP results to cytopathologic and histopathology data, and data obtained by DNA analysis of tumor tissue obtained after surgery. Write and communicate papers.*

**KEY RESEARCH ACCOMPLISHMENTS:** The work proposed in the grant was completed (6).

- 1) We found that cells retrieved from the ducts of cancer patients by endoscopy were positive for one or more of the three markers-cyclin D2, RAR-b and Twist.
- 2) We found that some women at high risk of developing breast cancer were positive for the markers. When combined with a cytological finding of marked atypia, 2 women were found to harbor breast tumors that were not discovered by mammography.
- 3) A quantitative methylation specific PCR assay has been developed for all five marker genes, that include RASSF1A and HIN-1
- 4) A method for amplification of DNA has been standardized and will be applied to ductal cells.

## **REPORTABLE OUTCOMES:**

### Publications

Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, Soito AB, Hung DT, Ljung B-M, Davidson, NE, Sukumar S. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *The Lancet* 357:1335, 2001

Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjan B, Buluwela L, Weitzman SA, Marks J, and Sukumar S. Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res.* 61:2782-2787, 2001.

D.M. Loeb, E. Evron, C.B.Patel, P.M.Sharma, B. Niranjana, L.Buluwela, S.A. Weitzman, D.Korz, and S.Sukumar. WT1 is expressed in primary breast tumors despite tumor-specific promoter methylation. **Cancer Res.**, 921-925, 2001.

Li, B, Goyal J, Dhar S, Dimri G, Evron E, Sukumar, S, Wazer D.E. and Band V. CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. **Cancer Res.** 61:8014-8021, 2001.

C.B. Umbricht, E. Evron, E.Gabrielson, J. Marks, and S. Sukumar.Hypermethylation of 14.3.3 $\alpha$  (Stratifin)is an early event in breast cancer. **Oncogene** 20: 3348-3353, 2001

Fackler MJ, Mc Veigh M, Evron E, Mehrotra J, Sukumar S, Argani P. Methylation profiling of early breast cancer (manuscript submitted to **Cancer Res.**), May 2002.

M. Vali, McVeigh M, Ming-Zhou Ren, Nicoletta Sacchi, Argani P, and S. Sukumar. Increased incidence of hypermethylated genes in breast cancer metastasis to the bone, brain and lung. (manuscript in preparation)

Presentations:

Breast Spore meeting at Dana Farber Cancer Center, Boston, MA, October 2001

Breast Cancer Research Meetings, - December. 10-13, 2001, San Antonio, TX -

10<sup>th</sup> SPORE Investigators' Workshop –Early detection of breast cancer cells in ductal lavage fluid – quantitative assessment of cyclin D2, RAR- $\beta$ , Twist, RASSF1A, and HIN-1 by real time methylation specific PCR (abstract). July 13-16, 2002.

Annual Meeting of Society of Gynecology and Obstetrics, April 3-5, Innsbruck, Austria

Association of Investigative Pathologists, April 20, 2002

American Radium Society Meeting – April 27-30, 2002, Las Croabas, Puerto Rico

CYTYC Health Corporation – May 23, 2002, Boxborough, MA.

Abbott Laboratories – May 10, 2002, Abbott Park, IL

Patent application – Aberrantly Methylated Genes as Markers of Breast Malignancy (Docket # JHU1630; Ref. # DM-3729)

**CONCLUSIONS:** Cells obtained by ductal lavage will prove to be valuable resource for detecting breast cancer cells early. Recognizing the paucity of the cells, methods need to be developed that will increase the specificity and sensitivity of detection methods. This method may prove to be a PAP test for the breast.

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**APPENDICES:**

Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjana B, Buluwela L, Weitzman SA, Marks J, Sukumar S. Loss of Cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res.* 61(6):2782-7, 2001.

Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, Soito AB, Hung DT, Ljung B, Davidson NE, Sukumar S. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *Lancet.* 357(9265):1335-6, 2001.

Research letters

## Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR

Ella Evron, William C Dooley, Christopher B Umbricht, Dorothy Rosenthal, Nicoletta Sacchi, Edward Gabrielson, Angela B Saito, David T Hung, Britt-Marie Ljung, Nancy E Davidson, Saraswati Sukumar

If detected early, breast cancer is curable. We tested cells collected from the breast ducts by methylation-specific PCR (MSP). Methylated alleles of *Cyclin D2*, *RAR-β*, and *Twist* genes were frequently detected in fluid from mammary ducts containing endoscopically visualised carcinomas (17 cases of 20), and ductal carcinoma in situ (two of seven), but rarely in ductal lavage fluid from healthy ducts (five of 48). Two of the women with healthy mammograms whose ductal lavage fluid contained methylated markers and cytologically abnormal cells were subsequently diagnosed with breast cancer. Carrying out MSP in these fluid samples may provide a sensitive and powerful addition to mammographic screening for early detection of breast cancer.

The recent decline in breast cancer mortality rate is due, in part, to early diagnosis by screening mammography. However, given the well-recognised limitations of mammography,<sup>1</sup> further advances for early breast cancer detection are clearly needed.

We previously identified a number of genes that had lower expression in breast cancer than in healthy mammary epithelial cells using serial analysis of gene expression (SAGE) and microarray analysis of primary breast cancers. Many of these genes were silenced by hypermethylation of promoter sequences.<sup>2,3</sup> Sensitive methods of detection of methylated alleles have now enabled non-invasive detection of small numbers of cancer cells.<sup>4</sup> We searched for genes that were hypermethylated in more than 30% of breast cancers,<sup>3,4</sup> but unmethylated in healthy mammary epithelial cells, mammary stroma, and white blood cells. Three genes fulfilled this criteria: *Cyclin D2*,<sup>3</sup> *RAR-β*,<sup>3</sup> and *Twist* (Genbank accession number 003986). We found a cumulative incidence of methylation of the three genes in 48 (96%) of 50 surgically excised primary breast tumours and in eight (57%) of 14 of the ductal carcinoma in situ (DCIS) lesions. This analysis highlights the high sensitivity and specificity of a MSP-based

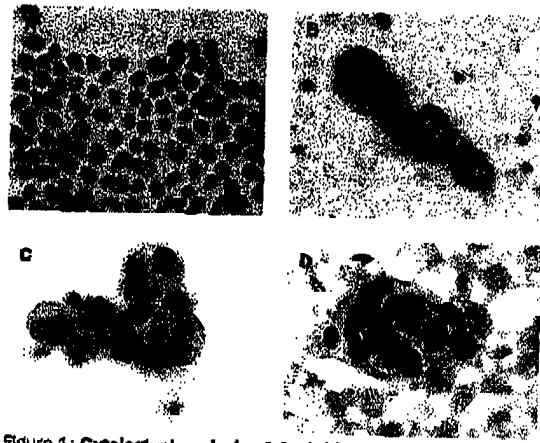


Figure 1: Cytological analysis of ductal lavage fluid  
 A: benign cells. B: atypical with mild changes. C: atypical with substantial changes. D: malignant cells.

test for breast cancer and raises the possibility that it could be applied to the detection of cancer cells in body fluids.

Because most breast cancers arise from the ductal epithelium, atypical and malignant cells can be found in breast ductal fluid. We used two techniques to collect ductal fluid: Routine Operative Breast Endoscopy (ROBE) and ductal lavage. ROBE allowed direct visualisation of macroscopic changes in the ductal epithelium,<sup>1</sup> and recovery of irrigation fluid from the catheter. Ductal lavage through a microcatheter (Pro-Duct Health, CA), enabled collection of breast epithelial cells from the entire ductal tree. We cannulated the individual orifices with a small flexible microcatheter, and up

Diagnosis	<i>Cyclin D2</i>	<i>RAR-β</i>	<i>Twist</i>	Overall methylated*
<b>Tissue</b>	<b>n=140</b>	<b>n=140</b>	<b>n=140</b>	
Invasive breast cancer	25/50	17/50	21/50	48/50 (96%)
Ductal carcinoma in situ	4/14	7/14	4/14	8/14 (57%)
Normal breast tissue	0/20	0/20	0/20	0/20 (0%)
White blood cells	0/56	2/56	0/56	2/56 (4%)
<b>ROBE fluid</b>	<b>n=38</b>	<b>n=37</b>	<b>n=34</b>	
Invasive breast cancer	8/19	12/20	13/18	17/20 (85%)*
Ductal carcinoma in situ	2/6	1/7	0/7	2/7 (29%)
Atypical ductal hyperplasia	1/6	2/6	1/5	2/6 (33%)
No residual tumour	0/4	0/4	0/4	0/4 (0%)
<b>Ductal lavage fluid</b>	<b>n=56</b>	<b>n=56</b>	<b>n=48</b>	
Benign	3/45	2/45	0/35	5/45 (11%)
Atypical with mild changes	0/5	1/5	0/5	1/5 (20%)
Atypical with substantial changes	3/5	2/5	0/5	3/5 (60%)
Malignant	1/1	1/1	0/1	1/1 (100%)

ROBE=routine operative breast endoscopy

### Assessing the use of methylation markers for early detection of breast cancer

\*The number of overall methylated markers was significantly higher in malignant cases (invasive breast cancer and DCIS) than in non-malignant cases (healthy breast tissue, atypical ductal hyperplasias, and in samples from patients with no residual tumour;  $p < 0.01$  by Pearson's  $\chi^2$ ). The number of overall methylated markers was significantly higher in cases classified as "atypical with marked changes" and "malignant", than in cases classified as "benign" and "atypical with mild changes" ( $p < 0.01$  by Pearson's  $\chi^2$ ).

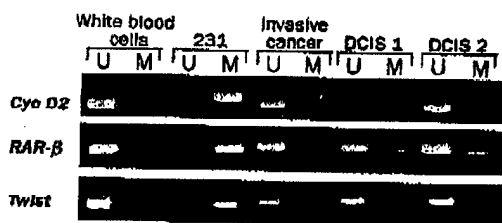


Figure 2: MSP profiles of ductal lavage fluid

U=unmethylated, M=methylated, 231=breast cancer cell line MDAMB 231. From women with invasive cancer and "malignant" cytology, and two women with ductal carcinoma in situ (DCIS1, DCIS2) and a cytology diagnosis of "atypical cells with marked changes". MSP primer sequences were: RAR- $\beta$ : U Forward-5'GGATTGGGATGTTGAGATGT3'; U Reverse-5'CAACC-AATCCAACCAAAACA2'; M Forward 5'GAACGGGAGCGATTGAGT3'; M Reverse-5'GACCAATCCAACCGAAACG3'. Twist: U Forward-5'TTTGGATGGG-GTYGTATTGT3'; U Reverse 5'CCTAACCAACCAACCAACG3' M Forward-5'TTTCGGATGGGATGTTGATG3'; M Reverse-5'AAACGACCTAACCCGAACG2'. Cyclin D2 analysis has been previously described.<sup>1</sup>

to 20 mL of saline was introduced in incremental volumes to flush out epithelial cells from the ducts and lobules. The ductal fluid was placed immediately in cytology fixative and prepared with standard millipore filtration devices for cytology assessment and DNA extraction.

We recruited 37 women with biopsy-proven cancer. Women underwent ROBE immediately before definitive surgery and after signing an informed consent form. DNA from both the ductal fluid cells and the matching surgical samples was tested with methylation-specific PCR (MSP) for *Cyclin D2*, *RAR- $\beta$* , and *Twist*.<sup>2,3</sup>

Methylated alleles of at least one of three markers were detected in 17 of 20 irrigation fluid samples from patients with pathology-confirmed invasive carcinoma (table). Healthy breast tissue contained only unmethylated genes (zero samples of 20; table). Methylated alleles for *RAR- $\beta$*  only were noted in two of 56 samples (table).

By contrast, irrigation fluid from four patients who underwent re-excision, but were subsequently found to be tumour-free, contained only unmethylated markers (table). Irrigation fluid from two of seven patients with DCIS (Grade 1-3), and two of six patients with atypical ductal hyperplasias contained hypermethylated markers. DNA samples from 19 of the 20 excised tumour samples were positive by MSP for the presence of methylated markers. Analysis of the irrigation fluid thus missed two MSP-positive samples, presumably because of the low cell yields. Cytology analysis on this fluid was inconclusive in 23 samples due to inadequate cellularity, and no malignant cells were detected in the remaining samples. These results suggest that MSP is sensitive, as the technique detected cancer cells in 85% of ductal fluid samples from patients with breast malignancy, including cases where the material was inadequate for cytology.

We extended our analysis to 56 samples of ductal lavage fluid (obtained after informed consent) from women with non-suspicious mammograms and breast examinations, but at high risk for developing breast cancer (as defined by a Gall index  $\geq 1.7$ , previous history of contralateral breast cancer, or *BRCA1* and *BRCA2* mutations). Using cytopathology, 50 samples were classified as benign or with mild changes, and six samples were classified as atypical with substantial changes or frankly malignant (figure 1). Among the cases with substantially abnormal cells or malignant cells, four of six samples were identified by MSP (67% sensitivity), whereas only five of 45 benign cases were positive (89% specificity; figure 2). Pathologically confirmed breast cancer was subsequently diagnosed in two women with abnormal cytological findings and MSP-positive ductal lavage fluid. A third patient in this category is undergoing further assessment.

These cases indicate the promising potential of the MSP-based method for early detection of breast malignancy, before the appearance of suspicious findings on mammography.

MSP confirmed the cytological finding that led to the diagnosis of breast cancer in two women. In combination with cytology evaluation, MSP of ductal lavage could provide a useful adjunct to mammography in the early diagnosis of breast cancer.

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## MUC 1: a genetic susceptibility to infertility?

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In man and some animals regulation of embryo implantation by endometrial expression of the highly polymorphic MUC 1 mucin has been suggested. We assessed the polymorphism of MUC 1 in women known to be fertile and those with infertility due to suspected failure of embryo implantation. The median of the lower allele size in the infertile group was only 2.5 kb compared with 3.4 kb in the fertile group ( $p=0.0029$ , difference 0.9, [95% CI 0.1-1.3]). Women with unexplained infertility might have a genetic susceptibility to failure of embryo implantation due to small MUC 1 allele size. Despite thorough investigation many cases of infertility remain unexplained. Although morphologically normal embryos are transferred to the uterus in most in-vitro fertilisation (IVF) cycles, successful pregnancy only takes place in about one in five attempts. Since at least 50% of IVF embryos develop to the blastocyst stage in culture, failure of implantation is probably the reason for failure of treatment.<sup>1</sup>

The essential cellular factors in endometrium that contribute to implantation are not fully understood. MUC 1 mucin, an oxygen-glycosylated (O) epithelial glycoprotein, could potentially modulate embryo attachment. It extends beyond the endometrial glycocalyx and is probably the first molecule that the embryo encounters on attachment.<sup>2</sup>