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13. ABSTRACT (Maximum 200 Words)

Altered nucleotide excision repair (NER) activity may be a common, initial and detectable event leading to genomic instability in human breast epithelial cells, ductal carcinoma in situ or early invasive breast cancers. Our objective is to study NER activity in primary breast epithelial cells and cancer tissues from women at risk for or diagnosed with breast cancer to determine if NER activity can be reliably and practically quantitated from these tissues and cells.

The three specific aims are:

1) Apply a quantitative local DNA damage immunoflourescent assay to measure NER activity in single cells derived from women at risk for or with breast cancer, from ductal lavage or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.

2) Develop a flow cytometry based immunoassay to measure NER activity in small populations of cells derived from women at risk for or with breast cancer, from ductal lavage or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.

3) Analyze cell based NER activity from patients samples with clinical, pathological and genetic information, including BRCA1 and BRCA2 carrier status, pathologic grade and stage, familial risk, and molecular correlates.

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DNA repair, ductal breast epithelial cells, genomic instability

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Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer

Introduction:

Genomic instability is a hallmark of carcinogenesis in human tumors, including sporadic and familial breast cancer. A major source for genomic instability are defects in mechanisms for the repair of DNA damage or errors in replication. We recently reported that the breast cancer susceptibility gene BRCA1 is directly involved in the regulation of global genomic nucleotide excision repair (NER) through its ability to transcriptionally regulate DNA damage recognition genes. Several lines of evidence suggest that an altered ability to repair DNA adducts due to cellular defects in NER may be a common source for genomic instability in preneoplastic breast tissue, resulting in enhanced mutagenesis of other cancer genes and breast cancer progression. Therefore, we are studying NER activity in primary breast epithelial cells and breast cancer tissues from women at risk for or diagnosed with breast cancer, as well as in mammary epithelial cells from mice as model system. We will take advantage of our high-risk breast cancer clinic, where we currently have ongoing clinical protocols using ductal lavage, random periareolar fine needle aspiration (rpFNA) and MRI directed biopsies to screen women for early breast cancer and to study the genetics of breast epithelial cells. We hypothesize that altered NER activity may be a common, initial and detectable event leading to genomic instability in human breast epithelial cells, ductal carcinoma in situ, or early invasive breast cancers. We will study NER in preneoplastic breast tissue from high and low risk individuals, BRCA1 mutation carriers and others, and invasive breast cancer tissue, using novel cell based functional assays of DNA repair. Our studies may allow, for the first time, a direct assessment of NER activity in primary breast tissue.

Specific Aims and Study Design:

1). Apply a recently developed quantitative local DNA damage immunoflourescent assay to measure NER activity in single cells derived from women at risk for or with breast cancer, from ductal lavage, rpFNA or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.

2). Develop a flow cytometry based immunoassay to measure NER activity in small populations of cells derived from women at risk for or with breast cancer, from ductal lavage, rpFNA or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.

3). Analyze cell based NER activity from 25 patient samples per year over 3 years with clinical, pathological and genetic information, including BRCA1 and BRCA2 carrier status, pathologic grade and stage, familial risk, and molecular correlates.

Identifying altered NER as a central cause of genomic instability relevant to the multistep process of breast cancer carcinogenesis and progression would have major potential impact on both clinical risk assessment and therapeutic approaches to this disease. A sensitive, quantitative and practical method for assessing functional NER activity in breast epithelial cells may allow for predictive phenotypic risk analysis beyond that currently possible using genotyping approaches. Also, assessment of NER activity of invasive breast cancers using standard diagnostic procedures may allow for selection of individualized therapy based on chemosensitivity.

Research Accomplished According the Statement of Work:

Identifying altered NER as a central cause of genomic instability relevant to the multistep process of breast cancer carcinogenesis and progression would have major potential impact on both clinical risk assessment and therapeutic approaches to this disease. For example, a sensitive, quantitative and practical method for assessing functional NER activity in breast epithelial cells may allow for predictive phenotypic risk analysis beyond that currently possible using genotyping approaches. Also, assessment of NER activity of invasive breast cancers using standard diagnostic procedures may allow for selection of individualized therapy based on chemosensitivity.

Task 1: Develop quantitative local DNA damage and repair immunoflourescent assay for use on primary human breast tissues.

During the first year of this project we have developed and optimized a novel technique for the detection of localized DNA damage and damage binding proteins in individual cells, using targeted micro-irradiation techniques and immunofluorescence, based upon recently published work from our laboratory [1, 2 and attached in appendix]. This has provided a powerful *in vivo* method to analyse the function of proteins that regulate DNA repair (Fig. 1).

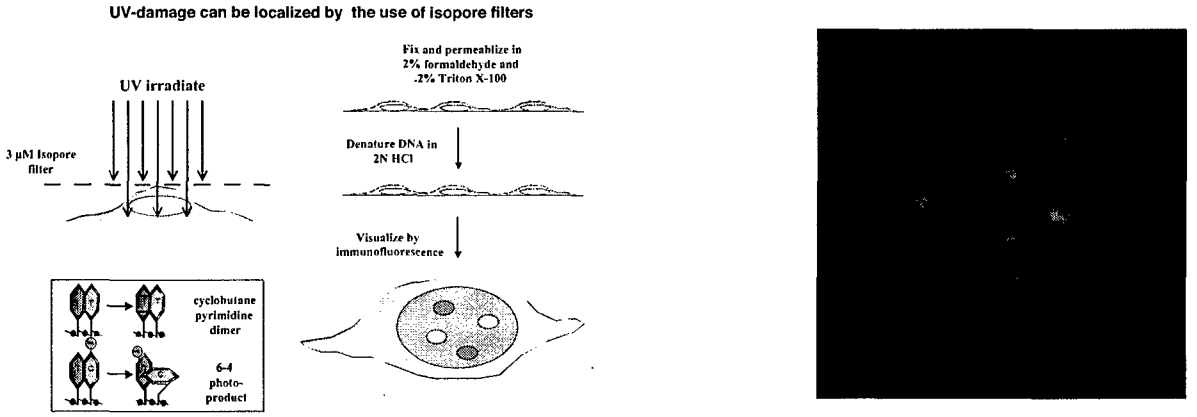

Figure 1. In vivo immunoflourescent detection of localized UV-irradiation induced DNA damage and cellular proteins. Cells are grown on glass coverslips. An isopore filter of 3μm size is presoaked in PBS and placed over the cells, and the cells irradiated through the filter with UVC. The cells are fixed with 2% formaldehyde in 0.2% Triton X-100/PBS, the DNA denatured by 2N HCl for 5 minutes at 37°, incubated in 20% FBS for 30 minutes at RT to block non-specific binding and incubated sequentially with primary and secondary antibodies. The example pictured at right demonstrates a single cell, with 5 discrete irradiated sites within the nucleus, as detected with monoclonal antibodies to CPDs.

We have now adapted this assay to quantitatively measure NER at a single cell level, in vivo. Plated cells are washed with PBS, UV-irradiated through the 3 μm isopore filter, as described above, and either fixed immediately or allowed to repair for 8 or 24 hrs in media and then fixed. Following incubation with monoclonal antibodies to CPDs, as described in Fig. 1, immunoflourescent images of at least 100 cells containing 3 – 7 irradiated sites are captured by a Nikon Eclipse E800 microscope using an RT Slider CCD camera (Spot Diagnostic), and analyzed by Spot RT 3.0 software (Spot Diagnostic) and Quantity One imaging software (Bio-Rad). Spot densitometry is used to analyze average pixel density from all spots detected within at least 100 cells, subtracting background flourescence from adjacent, non-irradiated nuclear areas. Figure 2 demonstrates the quantitative pixelation technique used.

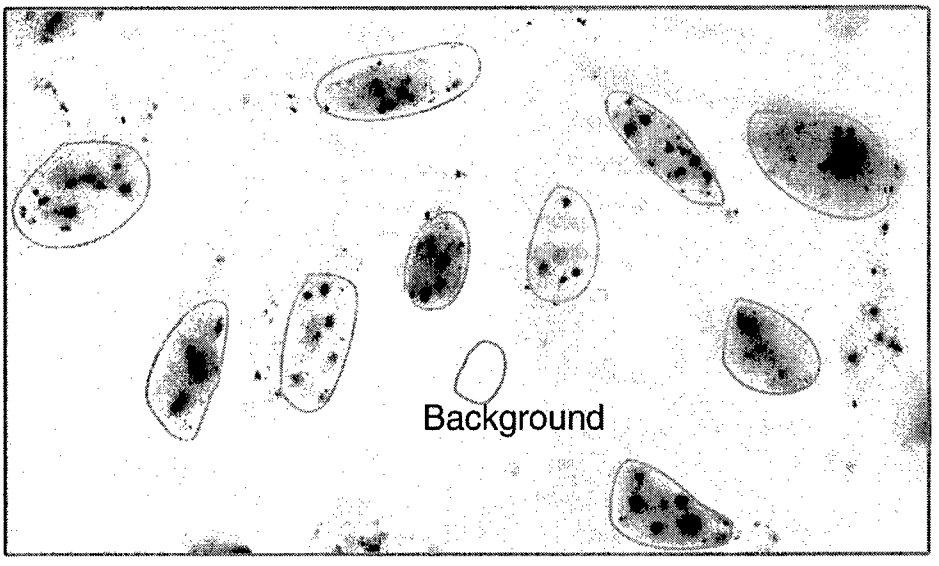

Figure 2. Quantitation of localized UV-irradiation induced DNA damage in primary breast epithelial cells. Cells are processed as described in Fig.1. Computer imaging software used to identify and quantitate spot densitometry using average pixel density.

Task 1a: Optimize technique using cell lines in tissue culture.

We have now successfully used this technique to quantitate NER of cyclobutane pyrimidine dimers (CPDs) from wild-type and several NER deficient fibroblast cell lines (XPA, XPC and p53 mutant) and have obtained similar results compared to traditional methods requiring millions of cells and DNA extraction techniques. Therefore, this approach may prove useful for determining NER activity from clinical samples with limited number of cells nor ability to expand in tissue culture.

Directly relevant to the current project, we have applied this technique to a model cell system. Primary murine mammary epithelial cells were obtained from genetically defined mice allowing for knockout of the mouse Brca1 gene, using a tissue specific conditional cre-lox knockout strategy. We have found that these cells exhibit a significant decrease in NER, as well as increased sensitivity to UV-irradiation and cisplatin, a chemotherapy drug affected by the NER pathway. These results are important in that they confirm the utility of this assay in cells relevant to the current study; being breast epithelial cells containing genetic alterations in the BRCA1 gene. This manuscript has been submitted, and a revision is currently under review [3, and in the appendix].

Task 1b: Optimize technique for use with human breast epithilial cells.

As discussed in detail in Task 3, due to several technical and administrative factors, we were unable to collect as many lavage samples during the first year of this project as planned, and have made appropriate plans to overcome this obstacle going forward. First, the cellular yield from ductal lavage was inconsistent and often too low to provide appropriate numbers of cells for the laboratory studies planned. Neverthess, enough samples were collected to allow us to demonstrate that our quantititative DNA damage and repair assays are applicable to primary changed our clinical protocol to employ random periareolar fine needle aspiration (rpFNA). This technique uses a 21-gauge needle to sample duct cells near the areola in 2 breast quadrants allowing for cytologic assessment and a higher cellular yield for experimental approaches in our

experience than ductal lavage. The rpFNA procedure has been shown Carol Fabian and colleagues to be safe and well-tolerated , with less discomfort and higher cell yield. We have therefore revised our clinical protocol and consent form, and IRB approved versions are attached in the appendix.

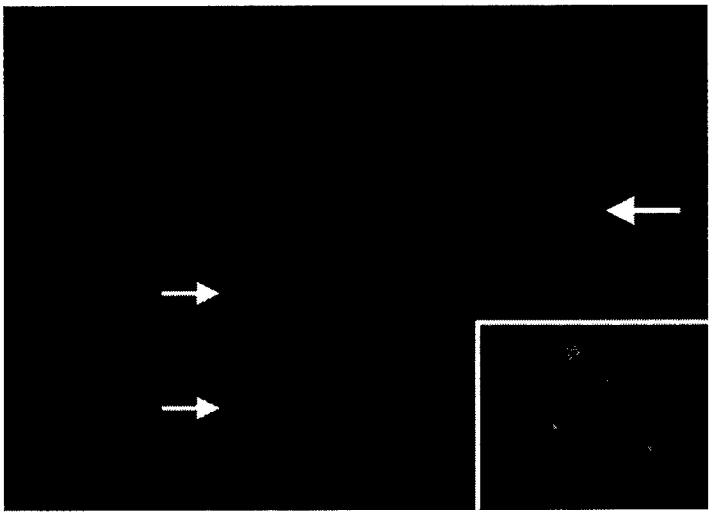

Figure 3. In vivo immunoflourescence and detection of localized DNA damage in primary breast epithelial cells collected by ductal lavage. Breast epithelial cells were collected as detailed in the appended clinical protocol, and processed as described in Fig. 1. The image demonstrates ductal architecture of these samples, and the insert shows detection of localized DNA damage using techniques as described above.

A second obstacle to proceeding with the translational studies of clinical samples was administrative, and is detailed in Task 3. Although our original clinical protocol as submitted with our DOD proposal was approved by the Stanford IRB, the DOD's own local IRB committee took over a year for approval. However, given our concurrent human subjects protocols and trials we were able to collect and freeze a number of samples over the past year, and spent our efforts in optimizing the laboratory techniques necessary, as discussed above. We believe with the change to the rpFNA epithelial cell collection technique, and our expanded clinical patient population and additional complementary clinical trials, that we will rapidly accrue samples going forward.

Task 2. Develop flow cytometry based immunoassay for measurement of NER in primary human breast tissues.

Using the monoclonal antibody to UV-induced CPDs described above, we have also developed a flow cytometry based immunoassay to measure DNA repair in small populations of individual cells. In this approach, cells growing in tissue culture were exposed to UV irradiation at various doses, harvested immediately by trypsinization or allowed various times for repair, and fixed in 2% paraformaldehyde. Following blocking and incubation with anti-CPD antibodies and flourescent secondary antibodies, cells were analyzed using a FACScan flow cytometer, as described in Figure 4. Using this method we have successfully demonstrated a dose-response at physiological levels of UV-irradiation (5 – 10 J/m^2), as well as efficient repair in NER proficient human cells. In addition, we have analyzed repair deficient XP cells, and shown a relative lack of repair. This assay is complementary to the above described assay as it allows analysis of primary human cells of limited quantity, and employs a much lower UV dose.

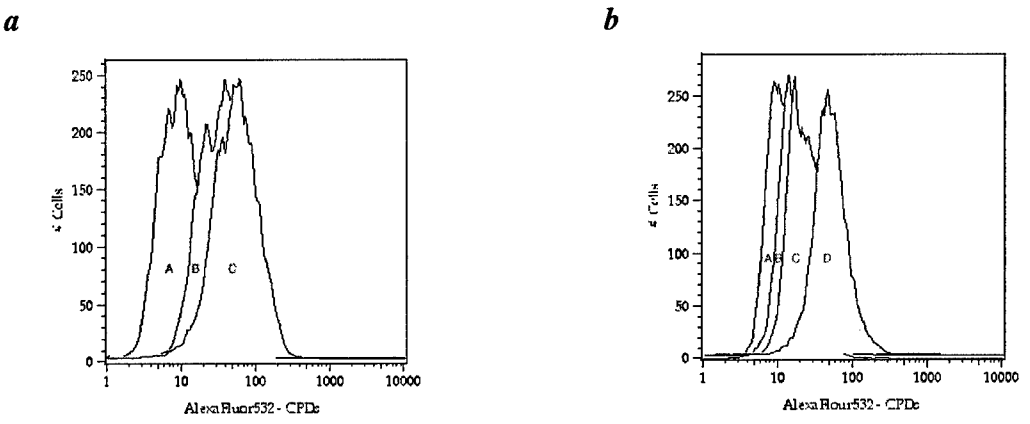

Figure 4. Immunodetection of UV-induced CPDs by flow cytometry.
HCT 116 human colon adenocarcinoma cells were exposed to UV-C irradiation from a germicidal lamp calibrated to deliver a dose of 1 J/m^2/s. For dose studies (***a***), cells were treated with 0 J/m^2 (A), 5 J/m^2 (B), or 10 J/m^2 (C) UV irradiation, harvested immediately by trypsinization, washed twice in ice-cold PBS, and placed on ice for further treatments. For repair time-courses (***b***), cells were treated with 10 J/m^2 UV irradiation and then replaced with fresh media. At the indicated time points, cells were harvested by trypsinization, washed twice in ice-cold PBS, and placed on ice for further treatments (A, unirradiated; B, 20 hrs after UV; C, 4 hrs after UV; D, 0 hr after UV). Approximately 100,000 cells were isolated per sample for further treatment. Cells were resuspended in 800 μl PBS; 200 μl of 10% paraformaldehyde was added dropwise, resulting in a 2% paraformaldehyde solution. Cells were placed on ice for 15 minutes, washed once in ice-cold PBS, then resuspended in 400 μl PBS. 200 μl 6N HCl was added dropwise, yielding a 2N HCl solution. Cells were incubated in a 37°C water bath for 5 minutes, then washed twice in a large volume of PBS to return the suspension to a neutral pH. Cells were incubated in blocking solution (3% FBS, 0.1% Triton-X in PBS) for 30 minutes on ice, then washed once in PBS. Cells were incubated with primary anti-CPD mouse monoclonal antibody for 30 minutes in blocking solution at a concentration of 0.1 μl antibody per 1 million cells. Cells were washed once in PBS, then incubated for 30 minutes on ice with AlexaFlour 532 (Molecular Probes) goat anti-mouse secondary antibody, also at a concentration of 0.1 μl antibody per 1 million cells. After a final PBS wash, cells were analyzed using a FACScan flow cytometer (Becton Dickinson) with an excitation wavelength of 488 nm. Appropriate gates were set to eliminate cell debris. Data from 10,000 events were collected and analyzed using FloJo software.

For similar technical and administrative reasons as cited under Task 1, during the second yeard of this project, we will start collecting appropriate clinical smaples to use in the flow cytometry based immunoassay for measurement of NER in primary human breast tissues.

Task 3 and 4. Collect primary breast epithelial cells from women undergoing comprehensive screening using ductal lavage and MRI directed biopsies.

As discussed in Task 1, due to several technical and administrative factors, we were unable to collect as many lavage samples during the first year of this project as planned, and have made appropriate plans to overcome this obstacle going forward. First, the cellular yield from ductal lavage was inconsistent and often too low to provide appropriate numbers of cells for the laboratory studies planned. Neverthess, enough samples were collected to allow us to demonstrate that our quantititative DNA damage and repair assays are applicable to primary breast epithelial cells (Figure 3). To overcome our yield and consistency issues, we have changed our clinical protocol to employ random periareolar fine needle aspiration (rpFNA). This technique uses a 21-gauge needle to sample duct cells near the areola in 2 breast quadrants allowing for cytologic assessment and a higher cellular yield for experimental approaches in our

experience than ductal lavage. The rpFNA procedure has been shown Carol Fabian and colleagues to be safe and well-tolerated , with less discomfort and higher cell yield. We have therefore revised our clinical protocol and consent form, and IRB approved versions are attached in the appendix.

A second obstacle to proceeding with the translational studies of clinical samples was administrative. Although our original clinical protocol as submitted with our DOD proposal was approved by the Stanford IRB, the DOD's own local IRB committee took over a year for approval. Attached in the appendix are documents pertaining to this, including our final approval notice from 5/30/05, and the revised approved human subjects protocol. As noted in this correspondence, we had several additional Stanford IRB approved human subjects protocols allowing for similar tissue collection by ductal lavage, rpFNA and MRI directed biopsies. The result of this that we have only now started accrual of clinical samples on this human subjects protocol. However, given our concurrent human subjects protocols and trials we were able to collect and freeze a number of samples over the past year, and of course spent our efforts in optimizing the laboratory techniques necessary, as discussed above. Furthermore, we collected and froze breast samples from 3 MRI-directed biopsies, as outlined in the Statement of Work. We believe with the change to the rpFNA epithelial cell collection technique, and our expanded clinical patient population and additional complementary clinical trials, that we will rapidly accrue samples going forward. We are confident that we will be able to obtain sample numbers in line with our proposal, given our expanded clinical trial group, and over 200 women currently participating in comprehensive screening.

Key Research Accomplishments:

- Development of a quantitative immunoassay for measuring DNA repair at a single cell level, and for use with flow cytometry.
- Validation of assay using positive and negative control cell lines in tissue culture with known repair phenotypes.
- Use of assay to demonstrate decreased DNA repair in Brca1-/- murine mammary epithelial cells.
- Demonstration of feasibility of collection and laboratory procedure for introducing and detecting DNA damage in clinical samples.
- Institution of rpFNA as a more reliable and robust method for collection of human breast ductal epithelial cells.
- Approval of human subjects protocol by both Stanford and DOD IRB's.

Reportable Outcomes:

Publications:

Kurian AW, Mills MM, Jaffee M, Sigal BM, Chun NM, Kingham KE, Collins LC, Nowels KW, Plevritis SK, Garber JE, Ford JM and AR Hartman. Ductal lavage of fluid-yielding and non-fluid-yielding ducts in BRCA1 and BRCA2 mutation carriers and other women at high inherited breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **14:** 1082 – 1089 (2005).

Kurian AW, Hartman AR, Mills MA, Ford JM, Daniel BL, and Plevritis SK. Opinions of women with high inherited breast cancer risk about prophylactic mastectomy: an initial evaluation from a screening trial including magnetic resonance imaging and ductal lavage. In Press, *Health Expectations* (2005).

Ghanouni P, Kurian AW, Margolis D, Hartman AR, Mills MA, Plevritis SK, Ford JM and Daniel BL. Ductal pattern enhancement on magnetic resonance imaging of the breast due to ductal lavage. In Press, *American Journal of Roentgenology* (2005).

Hartman AR, Sgasias MK, Sharma V, Turner S, Cowan K and Ford JM. Loss of nucleotide excision repair in brca1-deficient murine mammary epithelial cells. *Submitted* Cancer Research (2005).

Abstracts:

Kurian AW, Daniel BL, Mills MA, Nowels KW, Ford JM, Plevritis SK, Kingham KE, Chun NM, Herfkens RJ, Dirbas FM, Jaffe M, Garber JE, Hartman AR. A pilot breast cancer screening trial for women at high inherited risk using clinical breast exam, mammography, breast MRI and ductal lavage: updated results after median follow-up of fourteen months. San Antonio Breast Cancer Symposium. *Breast Cancer Res. Treatment* **88S1:** 5013 (2004).

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Kumm J, Zhang M, Darcy J, Davis R, Ford J, Ji H. Genomic and proteomic biomarker discovery in cancer: a bioinformatic solution for data validation, quality control analysis and clinical data integration in a multi-institutional setting. *Proc. Am. Assoc. Cancer Res.* **46:** 435 (2005).

Sharma VB, Hartman AR, Cowan K, Ford JM. Enhanced sensitivity to cisplatin and gemcitabine in DNA repair deficient Brca1 null mouse embryonic fibroblasts. *Proc. Am. Assoc. Cancer Res.* **46:** 4390 (2005).

Presentations:

Breast Cancer Genetics. Invited Speaker at Inaugural Stanford-Hong Kong Oncology Conference: Update in Breast Cancer Investigation and Treatment. Hong Kong University, April 30, 2004.

Dr. C.P. Manahan Memorial Lecture: Advances in the Genetics of Breast Cancer. Makati Medical Center, Manila. May 2, 2004.

Regulation of Nucleotide Excision Repair in Human Cells. Invited Speaker. 14th International Congress on Photobiology. Jeju, South Korea. June 11, 2004.

Tumor Suppressor Genes and Transcriptional Regulation of Nucleotide Excision Repair. Invited Speaker for Session on "Crossroads of DNA Damage, Cell Cycle, and DNA Repair." 2004 Gordon Research Conference on Mechanisms of Toxicity. Colby College, Maine. July 29, 2004.

Assessing Hereditary Cancer Risk: Appropriate Use of Cancer Genetic Testing and Clinical Management. Invited Speaker. Humboldt-Del Norte Consortium Grand Rounds, Eureka, CA. Sept. 1 – 2, 2004.

Genetic Testing in Breast Cancer: The Who, What, When, Where and Why. Invited Speaker. Community Hospital of the Monterey Peninsula Grand Rounds, Pacific Grove, CA. Sept. 21, 2004.

Role of p53 and BRCA1 in DNA Repair. The Cancer Institute of New Jersey Grand Rounds Speaker. New Brunswick, NJ. April 6. 2005.

Conclusions:

The overall goal of this project is to determine if NER activity can be reliably and practically quantitated from breast epithelial tissues and breast cancer cells derived from women undergoing standard screening and diagnostic procedures. We have developed and validated laboratory assays allowing for this question to be tested in a clinical testing, and have organized a clinical trial to facilitate collection of tissues and outcomes information. Should an NER defect appear to be present in a subset of these women, we will proceed to test our hypothesis in a prospective trial to determine the role of NER in breast cancer risk and clinical outcomes. Therefore, the results of our current proposed study could have a major impact on risk assessment for breast cancer. In addition, since NER is a key pathway affecting the sensitivity of tumor cells to several types of cancer chemotherapeutic drugs, our results could have implications for tailoring drug treatment in invasive breast cancer.

References:

1. Fitch ME, Cross I and Ford JM. p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation induced DNA damage. *Carcinogenesis* **24:** 843 – 850 (2003).

2. Fitch ME, Nakajima S, Yasui A and Ford JM. *In vivo* stimulation of XPC protein binding to UV-induced cyclobutane pyrimidine dimers by the DDB2 gene product. *J. Biol. Chem.*, **278**: 46906 – 46910 (2003).

3. Hartman AR, Sgasias MK, Sharma V, Turner S, Cowan K and Ford JM. Loss of nucleotide excision repair in brca1-deficient murine mammary epithelial cells. *Submitted* (2004).

Appendices:

1. Fitch ME, Cross I and Ford JM. p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation induced DNA damage. *Carcinogenesis* **24:** 843 – 850 (2003).

2. Fitch ME, Nakajima S, Yasui A and Ford JM. *In vivo* stimulation of XPC protein binding to UV-induced cyclobutane pyrimidine dimers by the DDB2 gene product. *J. Biol. Chem.*, **278**: 46906 – 46910 (2003).

3. Kurian AW, Mills MM, Jaffee M, Sigal BM, Chun NM, Kingham KE, Collins LC, Nowels KW, Plevritis SK, Garber JE, Ford JM and AR Hartman. Ductal lavage of fluid-yielding and non-fluid-yielding ducts in BRCA1 and BRCA2 mutation carriers and other women at high inherited breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **14:** 1082 – 1089 (2005).

4. Hartman AR, Sgasias MK, Sharma V, Turner S, Cowan K and Ford JM. Loss of nucleotide excision repair in brca1-deficient murine mammary epithelial cells. *Submitted* (2004).

5. Memo MCMR-ZB-PH (70-1n1) from Jo Collins, M.S.A., Human Subjects Protection Scientist, AMDEX Corporation, regarding review of research project. 3/21/2005.

6. Stanford IRB Approved Research Protocol (Revised 4/07/2005). "Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer."

7. Approval Memo, Award No. W81XWH-04-1-0576, HSRRB Log No. A-12761. Email from Caryn Duchesneau, CIP, USAMRMC Human Subjects Research Review Board (5/30/05).

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p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation-induced DNA damage, *in vivo*

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The p53 tumor suppressor gene is an important mediator of the cellular response to ultraviolet (UV)-irradiation-induced DNA damage and affects the efficiency of the nucleotide excision repair (NER) pathway. The mechanism by which p53 regulates NER may be through its ability to act as a transcription factor, and/or through direct interactions with damaged DNA or the repair machinery. p53 has been shown to regulate the expression of the *DDB2* gene (encoding the p48 protein) and the *XPC* gene, two important components of the NER pathway involved in DNA damage recognition. In this study, a localized UV-irradiation technique was used to examine the localization of p53, p48 and XPC proteins in relation to sites of UV photoproducts, *in vivo*. We did not observe any specific co-localization of p53 with sites of UV-induced DNA damage, but did observe rapid co-localization of both p48 and XPC to these sites. p48 bound to UV photoproducts in cells mutant or deficient for either p53, XPC or XPA, and p48 enhanced XPC binding to lesions, suggesting that p48 is a very early recognition factor of DNA damage. We propose that p53 functions to transcriptionally regulate the *DDB2* and *XPC* NER genes, but does not activate the NER pathway through direct interactions with UV-induced damaged DNA or other repair factors.

Introduction

Preservation of genetic integrity is critical to an organism's survival, and complex repair pathways exist to remove any exogenously or endogenously introduced lesions from the DNA. Nucleotide excision repair (NER) is one highly conserved pathway that serves to remove several types of bulky lesions in DNA, including the major photoproducts induced by ultraviolet (UV) radiation, the cyclobutane pyrimidine dimer (CPD) and the 6–4 photoproduct (6–4PP) (reviewed in ref. 1). NER is the only pathway in humans that removes UV-induced photoproducts. Mutations in NER genes cause the inherited cancer-prone syndrome xeroderma pigmentosum (XP), and result in the developmental and neurological abnormalities seen in Cockaynes syndrome (CS) and trichothiodystrophy (2).

Characterization of the defects in XP cells, which can be grouped into eight complementation groups (XP-A through XP-G and XP-V), has led to a greater understanding of the biochemical events involved in NER. NER proceeds through two distinct, yet overlapping pathways; transcription coupled repair (TCR) that preferentially removes lesions from the transcribed strand of active genes (3), and global genomic repair (GGR) that removes lesions from the overall genome and non-transcribed strands (1). TCR is believed to be activated by the recognition of a stalled RNA polymerase II that acts to recruit the NER machinery (4). The damage recognition steps of GGR are not as well understood, but there are several candidate factors that may be responsible for sensing the damage that is repaired through GGR. The XPC gene product, together with hHR23B, forms a heterodimeric complex that has strong affinity for damaged DNA *in vitro* (5). Mutations or loss of XPC causes complete inhibition of GGR of both CPDs and 6–4PPs *in vitro* and *in vivo*, whereas TCR is unaffected (6,7). The XP-E phenotype is caused by mutations in the *DDB2* gene (8). The protein product of this gene, p48, interacts with the p127 protein (the product of the *DDB1* gene), and together form a UV-damaged DNA binding complex (termed UV-DDB) (9,10). UV-DDB is the most readily detectable UV-damaged DNA binding activity in extracts from human cells, and has a greater affinity for UV-damaged DNA substrates *in vitro* than does the XPC–hHR23B complex or XPA (5). However, the role that p48 and UV-DDB play in NER *in vivo* is not clear because UV-DDB is not required for NER *in vitro* (11), and cells with *DDB2* mutations exhibit only partially diminished GGR rates *in vivo*, with CPD repair affected to a much greater extent than 6–4PP repair (12). UV-DDB does stimulate repair rates *in vivo* when it is microinjected into XP-E cells, and so its function has been proposed to be important for repairing damaged DNA in the context of chromatin (13). XPC–hHR23B also binds UV-damaged DNA with high affinity, and because loss of XPC completely inhibits GGR, it has also been proposed to be a DNA-damage recognition factor (14,15). Key questions remain about how these factors potentially interact with each other during the damage recognition step or activate the remaining enzymes involved in the repair machinery.

We and others have demonstrated that loss of the tumor suppressor protein p53 leads to decreased rates of GGR, but not TCR, following UV-C irradiation and affects CPD repair much more than 6–4PP repair (16–18). This phenotype is reminiscent of p48 deficiency in XP-E cells, and we have in fact demonstrated that p53 regulates both basal and UV-inducible levels of *DDB2* expression (12,19). We have also recently demonstrated that p53 regulates the expression of the *XPC* gene, again affecting both the basal and inducible levels (20). These data suggest that p53 regulates NER through its activities as a transcriptional regulator of genes involved in DNA damage recognition. However, p53 has also been shown to bind to certain repair factors, including XPB and XPD, as well as damaged DNA itself, thus suggesting that it may act as a direct repair factor (21–23).

Abbreviations: CPD, cyclobutane pyrimidine dimer; GGR, global genomic repair; NER, nucleotide excision repair; TCR, transcription coupled repair; Tet, tetracycline; UV, ultraviolet; 6–4PP, pyrimidine (6–4) pyrimidone photoproduct; UV-DDB, UV-damaged DNA binding factor; wt, wild-type; XP, xeroderma pigmentosum.

We have used the newly developed micropore filter local UV-irradiation technique to examine the *in vivo* localization of p53, UV-DDB and XPC after UV-C irradiation to address the question of whether or not these proteins are involved in binding lesions *in vivo* (24,25). This assay is a powerful method to explore protein localization after UV damage because only portions of the nucleus are irradiated through the micropore filter, while the remaining portion of the nucleus that is covered by the filter is blocked from the radiation. We have determined that p53 does not localize to sites of DNA damage at any time following UV irradiation, but that p48 and p127 do so very rapidly. XPC also localizes to lesions, but with apparently slower kinetics than p48. We further demonstrate that the presence of p48 can enhance the binding of XPC to sites of DNA damage and that p53 is not required for the *in vivo* localization of these proteins. We propose that both XPC and p48 are UV-damaged DNA recognition factors *in vivo*, and that p48 particularly functions to seek out CPDs that are located within chromatin structures by its very high affinity for damaged DNA.

Materials and methods

Cell lines

All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics, and were incubated at 37°C and 5% CO_2. Early passage WI38 cells (Repository # GM01604A), which express wild-type (wt) p53, were obtained from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ). WI38 cells were maintained in media as described above, but with 20% FBS, vitamins and non-essential amino acids. 041 TR cells, a subclone of TR9-7 cells obtained from Dr George Stark (Cleveland Clinic Foundation, Cleveland, OH), were constructed from Li–Fraumeni Syndrome 041 human fibroblasts, mutant for p53, into which a tetracycline (Tet)-regulated system for expression of wt p53 was stably transfected (26). 041 TR cells were grown in the presence of 600 μg/ml G418 and 50 μg/ml hygromycin, and maintained in 2 μg/ml of Tet when suppression of wt p53 expression was desired. XP-C cells (XP1MI) have been described (27,28), as have XP-A cells (SV40 XP12RO) (29,30).

Antibodies

Primary antibodies used for immunoblot analysis were mouse monoclonal anti-p53 at a 1:2000 dilution (DO-1, Santa Cruz), mouse anti-p21 at 1:500 (#556430, Pharmingen, CA), mouse anti-tubulin at 1:15,000 (B-5-1-2, Sigma-Aldrich), and mouse anti-XPC at 1:5 (A gift of Eva Lee, University of California, Irvine, CA). Secondary antibody was goat anti-mouse IgG conjugated to HRP diluted 1:5000 (Pierce Biotechnologies, IL). Primary antibodies for immunofluorescence were mouse monoclonal anti-CPD at 1:1500 [TDM2, a gift from Toshio Mori, Nara Medical University, Nara, Japan (31)], rabbit polyclonal anti-p53 at 1:100 (FL393, Santa Cruz, CA), and mouse anti-V5-FITC conjugated at 1:500 (Invitrogen, CA). Secondary antibodies were Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit, both used at 1:500 (Molecular Probes, OR).

Immunoblot

Briefly, cells were grown in a 150 mm dish, washed with PBS, and then some dishes were covered by a 150 mm 3 μm polycarbonate filter (Millipore, MA). The cells were irradiated with the indicated UV dose, the filter was removed, media was replaced, and the cells were returned to the incubator for 16 or 48 h. Cells were harvested in 150 mM NaCl, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 1% Triton X-100, 0.5 mM Pefabloc, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM DTT. Lysates were incubated on ice for 1 h, then sonicated for 20 min at 4°C in a Branson 1510 water bath sonicator. Debris was spun out by 14 000 *g* spin for 10 min. Protein concentration was quantified by the BCA kit (Pierce). Fifty micrograms of freshly boiled lysate was loaded onto a 12% reducing polyacrylamide gel, transferred to Hybond ECL paper (Amersham Biosciences, NJ), probed with the indicated antibodies, visualized by chemiluminescence (Super Signal, Pierce) and exposure to autoradiography film (Eastman Kodak Company).

Immunoslotblot analysis of CPD lesions

Cells were irradiated at the indicated doses in a similar manner as for the immunoblot analysis. Cells were immediately lysed after UV irradiation in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K and 0.1 mg/ml RNase. Genomic DNA was isolated by phenol–chloroform extraction, followed by ethanol precipitation, and the concentration was determined. Twenty-five nanograms of sheared genomic DNA was denatured and slotted onto Hybond N+ nylon membrane (Amersham Biosciences) and CPDs were detected by monoclonal antibody as described previously (16,32). Images were analyzed by Quantity One software (Bio-Rad, CA). The relative amounts of CPDs were normalized to the amount of CPDs in the 20 J/m^2 sample.

UV irradiation

For local UV irradiation, cells were grown overnight on glass coverslips. Prior to irradiation, the media was aspirated, and the cells were washed in PBS. For every experiment using localizing irradiation, an isopore polycarbonate filter of 3 μm size (Millipore) pre-soaked in PBS was placed over the cells, and the cells were irradiated through the filter with varying doses of UV-C (predominantly 254 nm wavelength) from a germicidal lamp calibrated to deliver 10 J/m^2/s. The membrane was removed and the cells were either fixed directly after UV, or medium was replaced and the cells put back in the 37°C incubator for the indicated times.

Immunofluorescence

Cells were grown as indicated on coverslips in a 35 mm dish, washed in PBS, then fixed by 2% formaldehyde in 0.2% Triton X-100/PBS for 10 min on ice. Cells were washed 3× in PBS, then the DNA was denatured by incubation in 2 N HCl for 5 min at 37°C. Cells were incubated in 20% FBS in washing buffer (WB-0.1% Triton X-100 in PBS) for 30 min at room temperature to block non-specific binding. Primary and secondary antibodies were made up in 1% BSA in WB and incubated for 45 min at room temperature. After each antibody step, cells were washed three times for 5 min in WB. When staining for both CPD and the V5 epitope-tagged proteins, a second blocking step of 5 μg/ml mouse IgG (Sigma) was added for 30 min after the CPD and the goat-anti mouse antibodies had been incubated, to block non-specific interactions between them and the V5 antibody. Anti-V5 FITC conjugated antibody was added after the IgG step and incubated for 45 min at room temperature. Coverslips were mounted in VectaShield with DAPI (Vector Laboratories, CA). Images were captured by a Nikon Eclipse E800 microscope using an RT Slider CCD camera (Diagnostic Instruments, MI), analyzed by Spot RT 3.0 software (Diagnostic Instruments) and further adjusted in Adobe Photoshop 6.0.

Transfections

Cells were plated on coverslips in a 35 mm dish 24 h prior to transfection. The *DDB2* full-length cDNA (−20 to 1281 bp not including the final stop TGA) was cloned in frame into the pcDNA3.1 vector (Invitrogen) to pick up the coding sequence of the V5 and His epitope tags at the C-terminus by the Invitrogen TOPO™ method. The cDNA was generated by RT–PCR using RNA isolated from GM38 normal human fibroblasts. XP2RO and XP82TO mutations were introduced into the *DDB2* gene by the use of the QuikChange™ Site Directed mutagenesis kit (Stratagene, CA). To generate the 2RO mutation, codon 273 was changed from CGC to CAC (R>H); for the 82TO mutation, codon 244 was changed from AAA to GAA (K>E). The full-length *p127* (−12 to 3432 bp) and full-length *XPC* (−15 to 2820 bp) cDNAs were cloned into the same vector in a similar manner. *p127* cDNA was a gift from Gilbert Chu (Stanford University). *XPC* cDNA was generated by RT–PCR using RNA from WI38 normal human fibroblasts. All transfections were done using Lipofectamine 2000 (Invitrogen) according to their instructions. When performing double transfections, the p127-V5 or XPC-V5 expressing vectors were transfected with a 5-fold excess of a p48 cDNA (no tag) in a pcDNA3.1 vector. Cells were irradiated 24 h post-transfection.

Results

p53 activation after local UV irradiation

We first established whether p53 is activated by the amount and distribution of DNA damage induced through the micropore filter following UV-C irradiation. The amount of DNA damage induced by varying amounts of UV irradiation delivered through a 3 μm isopore filter was quantified by immunoslotblot analysis of total genomic DNA using a monoclonal antibody against the CPD, and expressed as a percentage of that resulting from 20 J/m^2 UV delivered without a filter (Figure 1A). A UV dose of 100 J/m^2 delivered through the filter induced fewer lesions per cell equivalent than a dose of

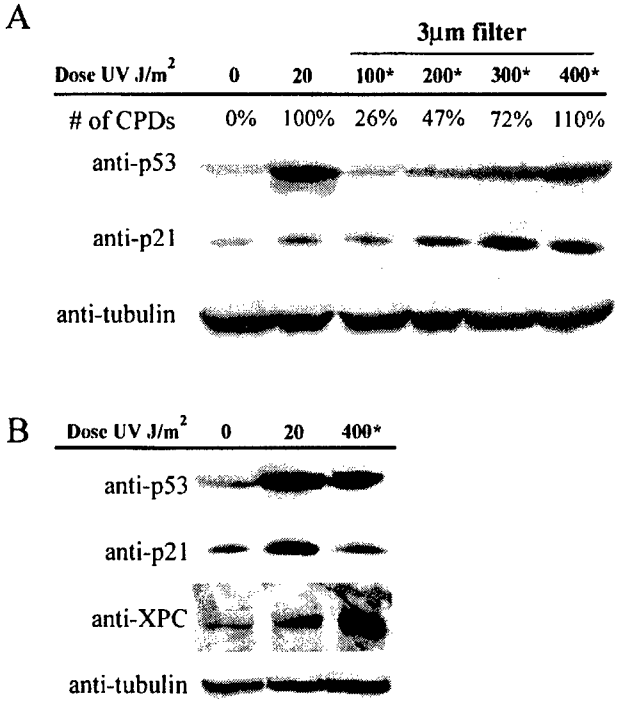

Fig. 1. Response of p53 to local DNA damage in normal human fibroblasts. WI38 cells were irradiated without (lane 2) or with a 3 μm filter at the indicated dose [lanes 3–6 in (**A**) and * in (**B**)]. Cells were harvested (A) 16 h post-UV or (B) 48 h post-UV, and lysates were analyzed by immunoblot to detect p53, p21, XPC and tubulin levels after UV irradiation. Images were compiled using Adobe Photoshop. The relative number of CPDs was quantified by immunoslotblot as described in the Materials and methods.

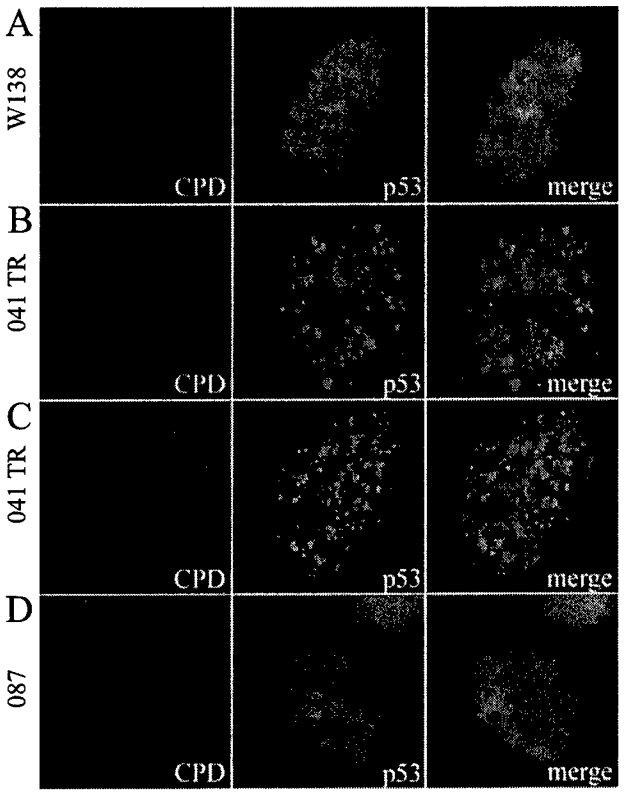

Fig. 2. p53 does not localize to sites of UV damage. (**A**) WI38 normal human fibroblasts were fixed 6 h after 300 J/m^2 UV delivered through a 3 μm filter. (**B** and **C**) 041 TR cells were induced to express p53 20 h prior to UV-irradiation by the withdrawal of Tet. Cells were fixed (B) 15 min or (C) 2 h post 300 J/m^2 UV delivered through a 3 μm filter. (**D**) 087 fibroblasts mutant for p53 were fixed 2 h after 300 J/m^2 UV delivered through a 3 μm filter. UV irradiated sites were visualized by an antibody to the CPD. All fluorescent images were compiled in Adobe Photoshop.

20 J/m^2 without a filter. The average number of CPDs increased proportionally with the dose, such that 400 J/m^2 UV through the filter induced just over 100% of the lesions observed from 20 J/m^2. We examined p53 protein stabilization after local irradiation in p53 wt WI38 normal human fibroblasts using an immunoslotblot analysis (Figure 1). p53 protein levels increased in proportion to the total number of UV-induced lesions per cell, with similar p53 levels observed following 400 J/m^2 under the filter and 20 J/m^2 without a filter at both 16 and 48 h post-irradiation. Levels of the p53-responsive gene product p21 (Cip1/Waf-1) also rose in a dose-dependent manner at both time points examined. Levels of the p53-responsive gene product XPC did not change greatly at the 16 h time point (data not shown). However, by 48 h, we observed a significant increase in XPC protein levels in both the 20 J/m^2 dose and the 400 J/m^2 dose under the filter (Figure 1B). This late induction is similar to our previous results in WI38 primary human fibroblasts (20). Stabilization of p53 and up-regulation of these known p53-response genes are strong indicators that the p53 activation mechanism that responds to DNA damage is activated by local irradiation, and that the mechanism responds proportionally to the total number of lesions in the cell, not their overall distribution.

p53 localization after local UV irradiation

We next examined p53 localization *in vivo* after UV damage in WI38 cells. A dose of 300 J/m^2 was chosen to irradiate the cells through a 3 μm filter because that combination activated p53 sufficiently to detect by immunofluorescence, and the immunoblot analysis confirmed that this dose increased p53 levels and increased levels of known p53 response genes (Figure 1). UV-damaged sites in the nucleus were visualized by a monoclonal antibody to the CPD, and cells typically contained three to 10 discrete irradiated sites that were consistent with the size of the pores in the 3 μm filter. p53 was undetectable by immunofluorescence prior to UV irradiation, presumably because the levels were low in unstimulated cells, and its location was primarily cytoplasmic (data not shown). Once nuclear p53 levels rose to high enough levels to detect by immunofluorescence, between 6 and 20 h post-UV, we were able to assess if p53 localized to areas of damaged DNA to a greater degree than non-damaged areas (Figure 2A). Six hours following UV irradiation, p53 distributed in a punctate pattern in the nucleus, but did not accumulate to a greater degree in areas that had been irradiated, as indicated by the staining pattern of the CPD antibody (Figure 2A). This suggests that p53 does not specifically interact with UV photoproducts or associated NER repair complex proteins or intermediates at this time point. Because p53 was not detected at early times after UV in WI38 cells, and a significant amount of repair occurs soon after UV irradiation, we examined the localization of wt p53 in 041 TR cells, a human cell line derived from a Li–Fraumeni patient that is mutant for p53, but that contains a stably integrated Tet-regulated wt p53 gene (26). We have shown previously that 041 TR cells induce NER and p53-dependent transcriptional regulation of the *DDB2* and *XPC*

genes following removal of Tet (12,16,20). Tet was removed from the cells 20 h prior to UV irradiation, and p53 localization examined immediately after UV, and at several time points thereafter. No localization of p53 to areas of damage occurred either 15 min (Figure 2B) or 2 h (Figure 2C) after irradiation, but a punctate pattern following UV irradiation was observed. The levels of p53 were higher in this cell line due to induced overexpression in comparison with the 6 h time point in WI38 cells, and thus the punctate pattern appeared more pronounced over that observed in WI38 cells. These foci were not distributed evenly throughout the nucleus, but rather appeared diminished and even absent from areas of the nucleus that had been irradiated (Figure 2B and C). To verify that the pattern of distribution observed was characteristic of functional p53, we also examined the localization of mutant p53 after UV irradiation. Li–Fraumeni 087 fibroblasts, that constitutively overexpress a mutant p53 protein containing a point mutation at amino acid position 248 that renders it incapable of specific DNA binding (33), were irradiated with 300 J/m^2 through a filter and again examined for localization of p53 at various times after UV. At several time points examined, the mutant p53 was distributed throughout the nucleus, and there was no specific accumulation seen at UV-irradiated sites, nor did we observe the bright foci appearance (Figure 2D). Taken together, our results with cells expressing wt and transcriptionally deficient p53 suggest that following localized UV irradiation, wt p53 is activated, and may function as a transcription factor at foci, but does not localize to sites of DNA damage where transcription is transiently inhibited.

Co-localization of the p53 target gene product p48 to sites of UV-induced DNA damage

We next examined if several known NER proteins localized to sites of UV damage, and in particular the p53 responsive gene products p48 and XPC. To examine p48 protein localization, we established a human 041 cell line null for p53 that stably expressed a V5 epitope-tagged *DDB2* cDNA (clone 041-p48.6) (33a). We have shown previously that this cell line contains lower levels of endogenous *DDB2* mRNA in comparison with WI38 fibroblasts, and that the mRNA levels do not change following UV-C irradiation (12). Following UV irradiation through a 3 μm filter with 200 J/m^2, p48 was observed to immediately co-localize with UV-induced damage sites, and remained associated with lesions for over 90 min (Figure 3A, and data not shown). By 2 h, the association of p48 with UV lesions was no longer detectable by immunofluorescence. We have observed by western blotting that p48 protein levels decrease significantly in 041-p48.6 cells within 2 h of UV irradiation, and that this loss can be attenuated by the addition of proteasome inhibitors (33a, 34). Therefore, we cannot be certain at times >90 min if p48 is no longer bound to lesions, or is simply undetectable because the majority of the protein has been degraded. We also examined the localization of p48 to sites of DNA damage in NER deficient cell lines using transient transfections with a V5-tagged *DDB2* cDNA in a pcDNA 3.1 expression vector. Transfected p48 co-localized to sites of damage in both XP-C cells and XP-A cells immediately after UV irradiation and remained associated with the lesions for up to 2 h (Figure 3B and C and data not shown). Notably, both of these cell lines are also SV40 T antigen transformed, and so functionally p53 deficient as well, confirming the results from the 041 cells that p48 does not require wt p53 protein to associate with UV lesions. The results in

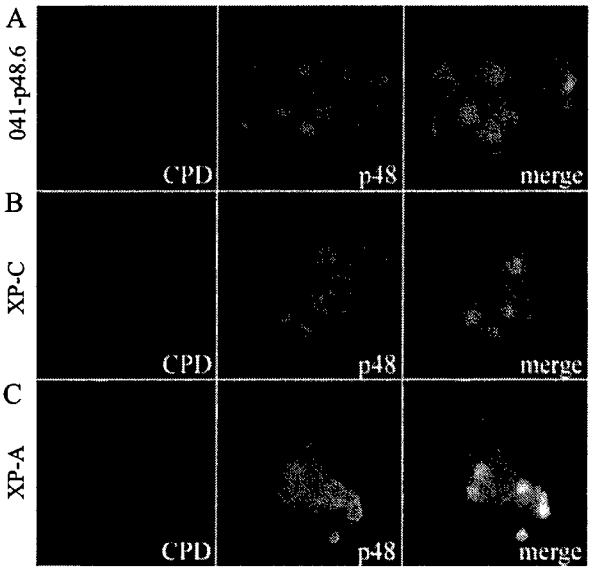

Fig. 3. p48 associates with UV lesions immediately after UV irradiation. (**A**) 041-p48.6 cells stably expressing p48 were fixed immediately after 200 J/m^2 UV delivered through a 3 μm filter. (**B**) XP-C and (**C**) XP-A cells were transfected with a *DDB2*-V5 cDNA 24 h prior to UV treatment and then fixed immediately after UV-irradation. p48 was visualized by an antibody to the V5 epitope.

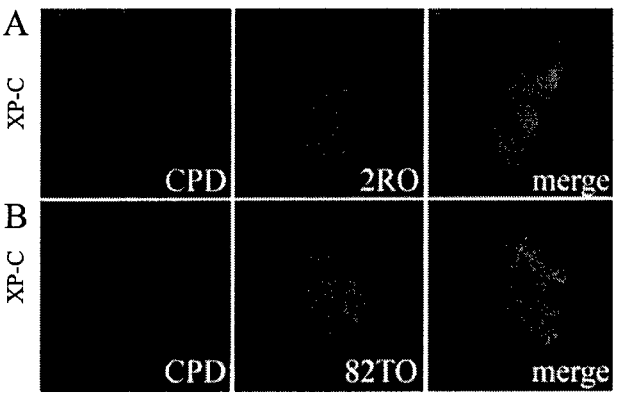

Fig. 4. p48 mutant proteins do not bind to UV lesions. XP-C cells were transfected 24 h prior to 200 J/m^2 UV-irradiation delivered through a 3 μm filter with either a p48 mutant (**A**) 2RO or (**B**) 82TO cDNA expressing plasmid. Cells were fixed 30 min after 200 J/m^2 UV delivered through a 3 μm filter. Both mutant p48 proteins had a C-terminal V5 epitope tag.

these cell lines also demonstrate that p48 does not require XPC or XPA to bind to lesions.

Clinically occurring mutants of DDB2 do not associate with UV lesions

XP2RO and XP82TO are XP-E cell lines that each has a point mutation in the *DDB2* gene (R273H and K244E, respectively) that affects several known p48 functions. For example, both 2RO and 82TO mutant p48 proteins are greatly diminished in their ability to induce the nuclear import of p127 (35,36), and both display no UV-DDB activity *in vitro* (37). We further show that they fail to bind UV-lesions *in vivo* (Figure 4). The localization of each mutant protein was examined at several time points post-UV, and no specific co-localization with lesions was observed, although as described previously

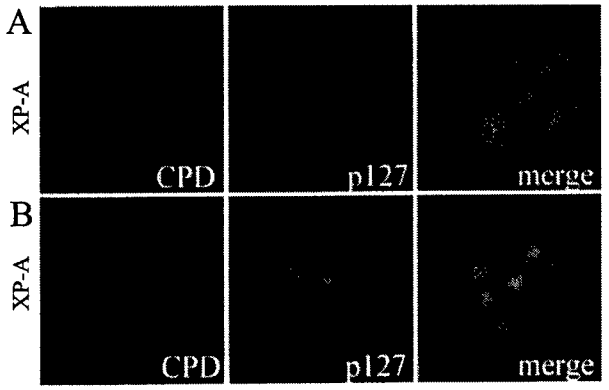

Fig. 5. p127 associates with DNA damage when p48 is present in sufficient levels. XP-A cells were transfected with *p127*-V5 only (**A**) or with *p127*-V5 and *DDB2* (**B**). Cells were UV-irradiated with 200 J/m^2 24 h post-transfection and fixed 10 min post-UV. p127 was visualized by an antibody to the V5 epitope present on the transfected *p127*.

(35,37), these proteins do readily localize to the nucleus (Figure 4A and B).

p127 requires p48 for efficient binding to UV lesions

We next examined the localization of p48's heterodimeric binding partner, p127, following UV-induced DNA damage. XP-A cells were transiently transfected with a V5-tagged *p127* cDNA, irradiated through a 3 μm filter with 200 J/m^2, and examined for localization of p127 at several time points post-UV (Figure 5A). We did not observe any specific co-localization of p127 with CPDs at any time point after UV irradiation. The XP-A cells used in this study were SV40 T-antigen transformed, and thus functionally p53 deficient. Because p53 is known to affect the basal as well as inducible expression levels of *DDB2* (12), we examined the basal *DDB2* mRNA levels in these cells. *DDB2* mRNA levels were lower in these cells in comparison with wt fibroblasts (data not shown), and thus we inferred that endogenous p48 protein levels may also be diminished. p48 has been shown to enhance import of p127 into the nucleus (35), and so p127 may not be present in the nucleus in sufficient enough levels to detect co-localization with UV lesions in these cells. To potentially enhance any binding activity of p127, we performed co-transfection experiments of both *DDB1* and *DDB2* into XP-A cells. When p48 was overexpressed, p127 readily co-localized with lesions within minutes (Figure 5B). As expected, the 2RO and 82TO mutants of p48 were not able to complement p127 binding to lesions when both were transfected into cells (data not shown).

Characterization of XPC binding to UV lesions

Volker *et al.* have reported previously that XPC binding occurs within 15 min in XP-A cells after 30 J/m^2 delivered through a 3 μm filter (14). We used a much higher dose of 200 J/m^2 through a 3 μm filter and observed co-localization of XPC with UV lesions in XP-A cells within 5 min of irradiation (data not shown). However, we did not observe detectable co-localization immediately after UV like that seen for p48 (Figure 6A). To determine if XPC localization, like p127, could be accelerated by increasing the amount of p48 in these cells, we performed co-transfection studies with *XPC* and *DDB2*. When p48 was overexpressed along with XPC in XP-A cells, we observed an enhancement of XPC binding to lesions, so that there was significant XPC binding detectable

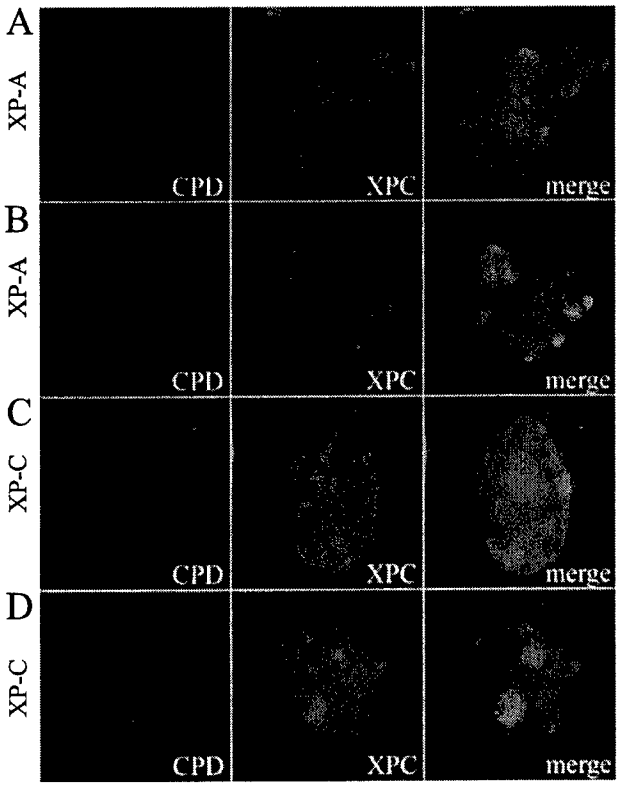

Fig. 6. p48 enhances XPC binding to UV lesions. XP-A cells transfected with either (**A**) *XPC*-V5 only or (**B**) *XPC*-V5 and *DDB2*; XP-C cells transfected with either (**C**) *XPC*-V5 only or (**D**) *XPC*-V5 and *DDB2*. All cells were irradiated with 200 J/m^2 UV 24 h post-transfection and then fixed immediately after UV-irradiation. XPC was visualized by a V5 epitope on the transfected *XPC*.

immediately post-UV in XP-A cells (Figure 6B). We observed the same effects of XPC binding when it was transfected into an XP-C cell line. XPC transfected alone did not co-localize to UV lesions immediately after UV irradiation (Figure 6C). When p48 was co-transfected with XPC into XP-C cells, we observed XPC binding to lesions immediately post-UV like what was observed in XP-A cells (Figure 6D), confirming that p48 can enhance XPC binding to UV-induced lesions.

Discussion

We have used the newly developed technique of micropore filter mediated local UV irradiation to directly examine the localization of several important proteins involved in NER to areas of UV-induced DNA damage *in vivo*. p53 is an important contributor to the cell's response to UV damage, and yet its role in DNA repair is not fully understood. It has been proposed that p53 can directly bind to damaged DNA through its non-specific DNA binding domain, and/or that it can directly interact with other DNA repair factors. There is evidence that p53 activates base excision repair (BER) through such direct interactions (38). Addition of p53 to repair lysates can stimulate BER *in vitro*, and p53 interacts through its N-terminus with the BER enzymes DNA polymerase β, and the 5′ AP endonuclease APE1 (39,40). In contrast, p53 does not stimulate NER *in vitro* when added to lysates (41,42). p53 interacts *in vitro* with XPB, XPD (components of the transcription factor TFIIH), and CSB (21); however, deficiencies in these

proteins lead to defects in TCR. We have shown previously that cells deficient in p53 do not show significant defects in their TCR pathway following UV-C irradiation, and so interactions with these proteins do not readily explain p53's primary role in global genomic NER (17). Several studies have suggested that p53 has an affinity for damaged or altered DNA structures, but these studies used cell extracts on artificial substrates *in vitro* (22,23). A study by Jackson *et al.* (43) examined p53 localization after whole cell UV irradiation using a biotin-dUTP labeling system to indicate sites of DNA repair. Their data were suggestive that p53 does not localize to sites of NER. However, because the whole cell was irradiated, the experiments were difficult to interpret. We show very clearly by the use of local UV irradiation of portions of the cell nucleus that p53 does not localize specifically to sites of UV-induced DNA damage. On the contrary, we see a relative lack of p53 protein in areas of chromatin that have recently been irradiated. A study by Moné *et al.* (25) using this technique showed that transcription is almost completely inhibited in areas that have been irradiated, while chromatin outside of the damaged areas continue to support normal levels of transcription. In fact, our data (Figure 1) suggest that the transcriptional response to UV irradiation is different following local DNA damage than when a similar number of lesions are distributed uniformly throughout the nucleus. For example, Figure 1 demonstrates higher p21 levels in cells 16 h following irradiation with 400 J/m^2 under the filter in comparison to cells irradiated uniformly with 20 J/m^2, even though the p53 levels are lower in the locally irradiated cells. This pattern is also observed for XPC levels 48 h post-irradiation. This may reflect that although the absolute number of lesions is comparable between the uniformly irradiated cells and the locally irradiated cells, the damage incurred through the filter is concentrated in discrete regions of the nucleus, leaving the majority of active chromatin without DNA damage and unhindered to perform transcription. However, XPC levels were not different between local and uniform irradiation at 16 h, and p21 levels were higher in the uniform irradiated cells at 48 h than the locally irradiated. Therefore, whether these observations are due to differential activation of transcription, or effects from differing damage in genomic regions will require further investigation. Taken together, the data strongly suggest that p53 functions primarily as a transcription factor after UV-induced DNA damage, and not as a UV damage recognition factor, and further that, like TFIIH, it is responsive to the mechanisms in the cell that prevent transcription in areas that have been damaged.

We have further characterized the initial cellular activities of several p53-regulated NER proteins, namely the UV-DDB complex made up of p48 and p127, and XPC. We find that p48 binds very rapidly to DNA lesions induced by 200 J/m^2 through a 3 μm filter and can be detected at these sites immediately after irradiation, and up to 90 min later. The immediate binding of p48 to UV lesions occurs regardless of the presence of p53 or of the other repair factors XPC or XPA, consistent with a previous report by Wakasugi *et al.* (44). p127 was also found to localize immediately to sites of DNA damage, but only in the presence of high levels of p48. As there are no known p127 deficient cell lines, we did not determine if p48 equally requires p127 for DNA binding. However, we speculate that either p48 alone binds to UV lesions and then recruits p127, or that together, p48 and p127 bind to lesions. *In vitro* evidence would support the latter hypothesis, as p48 by itself does not have as much UV-DDB activity as p48 with p127 (37). The fact that the p48 2RO and 82TO mutant proteins, which do not efficiently bind to p127, fail to bind to UV lesions also suggests that UV-DDB complex formation is needed for binding to lesions. *p127* is not induced after UV irradiation like *DDB2* (12), and so regulation of p48 levels by p53, and possibly the proteasome [(34) and unpublished data], may suffice for control of this step of DNA repair.

We have further characterized XPC binding to UV lesions. We observed XPC localization to sites of DNA damage in XP-A cells within 5 min of irradiation, yet this clearly occurred with slower kinetics than that observed for p48. Overexpression of p48 accelerated the binding of XPC to lesions by several minutes. This suggests that XPC by itself is fully capable of binding lesions, yet p48/UV-DDB is also a recognition factor that has some activity that can stimulate XPC binding. There is conflicting evidence in the literature about UV-DDB's ability to stimulate NER *in vitro*. One of the first NER reconstitution experiments using purified components by Aboussekhra *et al.* (11) saw little effect with the addition of UV-DDB to the other core NER components on the repair of a UV-damaged substrate. A more recent study has demonstrated that addition of recombinant UV-DDB to the other core components stimulated excision of a CPD by up to 17-fold, but had little or no effect on a 6–4PP containing substrate (44). We now demonstrate that p48 can stimulate XPC binding to lesions *in vivo*. Taken together with the *in vitro* data, our results suggest that UV-DDB may stimulate the rate of GGR by increasing the recognition of UV lesions, and in particular, CPDs. UV-DDB may increase recognition through its actions as a chromatin remodeling factor, for which there is accumulating evidence based on the phenotype of p48 deficient cells, and UV-DDBs ability to stimulate repair of a nucleosome bound damaged substrate (13). 6–4PPs occur with much less frequency in nucleosome bound DNA than CPDs, thus requiring less remodeling for their repair (45). 6–4PPs are more distorting to the overall structure of DNA than CPDs, and so 6–4PPs may be recognized easier and not require a specialized recognition factor that can work in the context of chromatin structure. The enhanced XPC binding we observed could be due to p48 facilitating a more open conformation of the chromatin around UV lesions, CPD's in particular, thus allowing greater access to the lesions.

In this study, an antibody to CPDs was used to identify the areas that have been UV-irradiated. However, 6–4PPs will also be found at these sites depending on the time post-UV and the repair capabilities of the cell type. Therefore, we cannot conclude which lesion is the primary recruiting factor for p48 or XPC. Repair of 6–4PPs is almost complete after several hours in normal cells, while complete CPD removal can take over 24 h. As we indicated, detectable p48 remains associated with the lesions for only a few hours in repair proficient cells, perhaps suggesting that 6–4PPs are the major recruiting lesion. p48 and XPC both bind 6–4PPs with more avidity than CPDs *in vitro* (46). It will be of interest to learn if there is a difference in binding preferences of the proteins to the different photoproducts *in vivo*.

One interesting question that remains to be answered regarding the role of p48 in NER is why *DDB2* basal levels are typically very low and are inducible following UV irradiation, and why p48 is degraded in response to UV irradiation. This degradation potentially occurs due to the ability of p48 to interact with the specific E3 ubiquitin ligase Cul-4A (47,48).

Taken together, one can speculate that the activities of p48 are tightly regulated and potentially harmful to the cell if left unchecked. For example, p48 may have non-specific DNA binding activity leading to gratuitous repair replication and the potential for mutagenesis. If UV-DDB is a chromatin remodeling factor, perhaps it can cause non-specific 'loosening' of chromatin structures that potentially lead to a general deregulation of gene expression because many genes are controlled by their level of chromatin structure. UV-DDB has also been proposed to have transcription activating activities through its ability to interact with E2F1 (35), and these activities may need to be tightly regulated. Another possible explanation for the degradation of p48 is that this process is somehow integral to facilitating NER. Intriguingly, XPC and hHR23B levels have also recently been suggested to be regulated by the proteasome (49), lending further credence to the idea that the proteasome may function in regulating NER, and that replenishment of the levels of these proteins is critical to completion of timely repair.

Another interesting question is how p53 regulated transcription of NER genes is important to the cells response to damage. p53 appears to regulate basal levels of p48 and XPC independent of UV-inducible responses, maintaining higher levels of these gene products in wt cells for immediate use in the early steps of NER (12,19,20). p53's induction of p48 and XPC following DNA damage is somewhat late in the UV response, typically reaching maximal levels for both after 24 h. However, transcriptional induction of p48 and XPC in response to UV may help maintain a critical cellular level of these proteins that have been depleted by proteasomal degradation at sites of DNA damage. In addition, it may also function to ensure recognition of any lingering CPD lesions that have as yet gone unrepaired, and in particular those lesions remaining in less accessible regions of chromatin. Understanding the biochemical functions of UV-DDB and XPC in NER and chromatin remodeling will lead to a greater understanding of the need for transcriptional control of DNA repair genes by factors like p53.

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In Vivo Recruitment of XPC to UV-induced Cyclobutane Pyrimidine Dimers by the *DDB2* Gene Product*

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The initial step in mammalian nucleotide excision repair (NER) of the major UV-induced photoproducts, cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4PPs), requires lesion recognition. It is believed that the heterodimeric proteins XPC/hHR23B and UV-DDB (UV-damaged DNA binding factor, composed of the p48 and p127 subunits) perform this function in genomic DNA, but their requirement and lesion specificity *in vivo* remains unknown. Using repair-deficient xeroderma pigmentosum (XP)-A cells that stably express photoproduct-specific photolyases, we determined the binding characteristics of p48 and XPC to either CPDs or 6–4PPs *in vivo*. p48 localized to UV-irradiated sites that contained either CPDs or 6–4PPs. However, XPC localized only to UV-irradiated sites that contained 6–4PPs, suggesting that XPC does not efficiently recognize CPDs *in vivo*. XPC did localize to CPDs when p48 was overexpressed in the same cell, signifying that p48 activates the recruitment of XPC to CPDs and may be the initial recognition factor in the NER pathway.

The majority of DNA damage induced by ultraviolet light is caused by the transformation of adjacent pyrimidines into either cyclobutane pyrimidine dimers (CPDs),[1] or pyrimidine (6–4) pyrimidone photoproducts (6–4PPs). In human cells, these photoproducts are repaired exclusively through the nucleotide excision repair (NER) pathway, and loss of NER leads to the skin cancer-prone syndrome xeroderma pigmentosum (XP). NER can be further subdivided into two pathways, global genomic repair (GGR), which repairs lesions found throughout the genome, and transcription-coupled repair (TCR), which removes lesions selectively from the transcribed strand of active genes (reviewed in Refs. 1 and 2). TCR is believed to be activated by the recognition of a stalled RNA polymerase at the lesion. Recognition of lesions by the GGR pathway is less well understood. Mutations in the *XPC* and *DDB2* genes, resulting in the XP-C and XP-E complementation groups, respectively, lead to a loss in GGR but not TCR, suggesting that these genes encode for the UV-damage recognition factors initiating the GGR pathway (3, 4). Further evidence that XPC and p48 (the protein product of the *DDB2* gene) are DNA damage recognition factors comes from *in vitro* and *in vivo* experiments of the interaction of these proteins with UV-induced DNA lesions. *In vitro* binding studies have shown that XPC complexed with the hHR23B protein has a greater affinity for naked UV-damaged DNA than undamaged DNA and has a greater affinity for the 6–4PP than the CPD (5–8). p48 and the p127 product of the *DDB1* gene make up the UV-DDB complex, which has also been shown to have a much greater affinity for UV-damaged DNA than undamaged and, like XPC, shows a stronger *in vitro* binding affinity for the 6–4PP over the CPD (6, 9, 10). The precise role of UV-DDB in GGR is not well understood because the repair reaction can be recapitulated *in vitro* in its absence (11), although inclusion of UV-DDB can stimulate repair 2–17-fold (12, 13).

One caveat of these *in vitro* binding studies is that the DNA substrates were not bound by nucleosomes or folded into higher order chromatin structures as would be found in an intact nucleus that had sustained DNA damage. Regulation of many DNA processes, such as transcription and replication, involves chromatin remodeling; it is now clear that DNA repair also requires chromatin remodeling to access damaged nucleotides (14, 15). Binding studies performed *in vivo* are necessary to fully understand how these structures may be affecting damage recognition. Use of a localized UV irradiation technique whereby only parts of the nucleus are irradiated through a micropore polycarbonate filter is a powerful tool for analyzing protein localization to sites of UV irradiation *in vivo*. We and others have used this technique to demonstrate that p48 and XPC bind rapidly to areas that have been irradiated and that the binding of both of these factors can occur in the absence of other important repair factors, such as XPA and p53 (13, 16, 17).

Although UV photoproducts are repaired solely by the NER pathway in human cells, many other organisms possess an alternative pathway for the repair of individual UV photoproducts by the use of single enzymes called photolyases (18). Once bound specifically to the lesion, these enzymes use energy from the visible spectrum of light to reverse the UV photoproduct without any incision or religation event, a process termed photoreactivation. Photolyases specific for either the CPD or the 6–4PP have been described from bacteria, lower eukaryotes, plants, and even marsupials (19). UV irradiation induces substantially more CPDs than 6–4PPs, yet the repair, or consequence of having unrepaired lesions, of either of the individual photoproducts in DNA is unclear. Therefore, generation of an

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[1] The abbreviations used are: CPD, cyclobutane pyrimidine dimer; NER, nucleotide excision repair; 6–4PP, pyrimidine (6–4) pyrimidone photoproduct; PBS, phosphate-buffered saline; XP, xeroderma pigmentosum; UV-DDB, UV-damaged DNA binding factor; GGR, global genomic repair; TCR, transcription-coupled repair.

experimental system with defined UV photoproducts is of great interest for understanding the physiologic response to UV irradiation. Photolyases have recently been used in mammalian systems to address the contribution of the individual photoproducts to the mutagenic effects of UV-B (20), the apoptotic response of HeLa cells to UV-C (21), and also UV resistance in whole animals (22). These studies have all suggested that the majority of the cellular response to UV is attributable to CPDs. Understanding the mechanism of recognition of CPDs *versus* 6–4PPs in the repair process is therefore of great importance. We have used NER-deficient XP-A cells that stably express either a heterologous CPD-specific photolyase or a 6–4PP-specific photolyase to address the specific roles *in vivo* of p48 and XPC in recognition of the individual photoproducts after UV irradiation.

EXPERIMENTAL PROCEDURES

Antibodies—For immunoblots, rabbit polyclonal anti-photolyase antibodies were used at 1:2000 and horseradish peroxidase-conjugated donkey anti-rabbit at 1:5000 (Pierce) for chemiluminescent detection (anti-photolyase antibodies were gifts from Dr. Andre P. M. Eker, Erasmus University, Rotterdam, The Netherlands). 50 μg of protein was loaded on a 12% SDS-PAGE for analysis of the photolyases. Primary antibodies for immunofluorescence were mouse monoclonal anti-CPD at 1:1500 (TDM2), mouse monoclonal anti-6–4PP at 1:400 (64M-2) (gifts from Toshio Mori, Nara Medical University, Nara, Japan) (23), rabbit polyclonal anti-photolyase antibodies at 1:500, and mouse anti-V5 fluorescein isothiocyanate conjugated at 1:500 (Invitrogen). Secondary antibodies were Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit, both used at 1:500 (Molecular Probes). Immunoslotblot detection of photoproducts was performed as previously described (24).

Cell Lines—All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, antibiotics, and 500 μg/ml G418 (for maintenance of transgenic photolyase genes) and were incubated at 37 °C and 5% CO_2. XP12ROSV, an SV40-transformed cell line derived from an XP-A patient, is mutated in the *XPA* gene and possesses no NER activity (25). For expression of photolyase genes in the XP12ROSV cell line, a cDNA of the CPD photolyase gene (CPDphr) derived from rat kangaroo *Potorous tridactylis* (19) and a cDNA of the 6–4PP photolyase gene (6–4phr) derived from plant *Arabidopsis thaliana* (26) were used.[2] The cDNA of each gene was introduced into the vector pCY4B, which contains the cytomegalovirus enhancer, chicken β-actin promoter, and rabbit poly(A) signal (27). CPD-3 cells were further subcloned by single cell dilution.

UV Irradiation—For photoreactivation, cells were irradiated at the indicated dose of UV-C using a germicidal lamp (predominantly 254 nm). After UV-C exposure, cells were incubated in Dulbecco's modified Eagle's medium without phenol red and exposed to photoreactivating 360-nm UV-A light from two bulbs (Sankyo Denki, F15T8BLB 15W; PGC Scientific) from above. To block shorter wavelengths, the black lights were filtered through one 5-mm borosilicate glass plate. The distance from the bulbs to the cells was ~15 cm. For local UV irradiation, cells were grown overnight on glass coverslips. Prior to irradiation, the media were aspirated, and the cells were washed in PBS. For every experiment using localized irradiation, a 3-μm isopore polycarbonate filter (Millipore) presoaked in PBS was placed over the cells, and the cells were irradiated through the filter with 200 J/m^2 of UV-C from a germicidal lamp calibrated to deliver 10 J/m^2/s. The membrane was removed, and the cells were photoreactivated for 2 h under UV-A blacklight.

Immunofluorescence—Cells were grown as indicated on coverslips in a 35-mm dish, washed in PBS, then fixed by 2% formaldehyde in 0.2% Triton X-100/PBS for 10 min on ice. Cells were washed three times in PBS, and then the DNA was denatured by incubation in 2 N HCl for 5 min at 37 °C. Cells were incubated in 20% fetal bovine serum in washing buffer (0.1% Triton X-100 in PBS) for 30 min at room temperature to block nonspecific binding. Primary and secondary antibodies were made up in 1% bovine serum albumin in washing buffer and incubated for 45 min at room temperature. After each antibody step, cells were washed three times for 5 min in washing buffer. When staining for both CPDs or 6–4PPs and the V5 epitope-tagged proteins, a second blocking step of 5 μg/ml mouse IgG (Sigma) was added for 30 min after the CPD and the goat-anti mouse antibodies had been incubated to block nonspecific interactions between them and the V5 antibody. Anti-V5 fluorescein isothiocyanate-conjugated antibody was added after the IgG step and incubated for 45 min at room temperature. Coverslips were mounted in VectaShield with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were captured by a Nikon Eclipse E800 microscope (with ×60 oil objective) using an RT Slider CCD camera (Spot Diagnostic), analyzed by Spot RT 3.0 software (Spot Diagnostic), and further adjusted in Adobe PhotoShop 6.0.

[2] S. Nakajima and A. Yasui, manuscript in preparation.

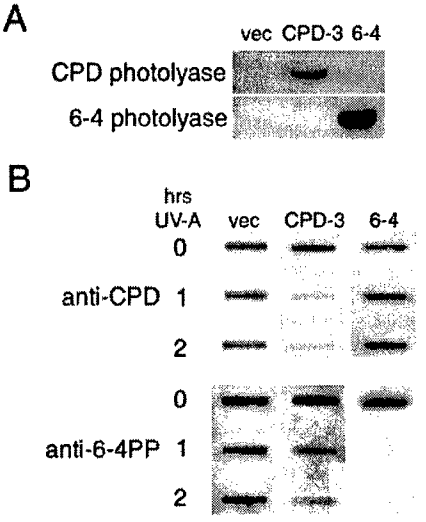

FIG. 1. **Expression of photoproduct-specific photolyases and photoreversal.** *A*, Western blot analysis showing the specific expression of CPD photolyase in CPD-3 cells, 6–4PP photolyase in 6–4 cells, and the negative vector-only control cell line. *B*, immunoslotblot analysis of photoproduct removal by the specific photolyases. Photolyase-expressing XP-A cells were irradiated with 20 J/m^2 UV-C and then exposed to UV-A photoreactivating light for the indicated time.

RESULTS

Characterization of Photolyase-expressing Cell Lines—XP-A cell lines were established that stably express either the CPD photolyase from the rat kangaroo *Potorous tridactylis* or the 6–4PP photolyase from *Arabidopsis thaliana*. The advantage of using XP-A cells completely deficient in repair of both CPDs and 6–4PPs (28, 29) is that any removal of a photoproduct occurs solely through the action of the photolyase and not through the NER pathway. Fig. 1*A* is an immunoblot demonstrating the specific expression of each photolyase in XP-A cells; CPD-3 cells express the CPD photolyase, and 6–4 cells express the 6–4PP photolyase. We examined the kinetics of photoreactivation by first irradiating the photolyase-expressing cells with 20 J/m^2 of UV-C and then exposing the cells for varying times to the reactivating energy found in UV-A light. Substantial photoreactivation was observed with one hour of UV-A treatment as measured using monoclonal antibodies to the specific photoproducts (Fig. 1*B*). By two hours, ~83% of CPDs had been photoreversed from CPD-3 cells. Increasing lengths of UV-A treatment did not cause significantly more reversal of CPDs (data not shown). Repair of 6–4PPs was much more efficient in the 6–4 cells, with 98% of the 6–4PPs reversed following 2 h of UV-A treatment (Fig. 1*B*). It is not clear why 6–4PPs were repaired so much more efficiently, but one factor may be that 6–4PPs typically occur outside of chromatinized regions and so may be more accessible to the enzyme (30). Previous work (31, 32) has demonstrated that nucleosomes inhibit the activity of photolyase on CPDs, suggesting that the residual CPDs seen in the UV-A-treated CPD-3 cells may reside within nucleosomal structures. Exposure of the cells to UV-A light did not induce more CPDs or 6–4PPs, as indicated by the lack of change in the band intensities of the

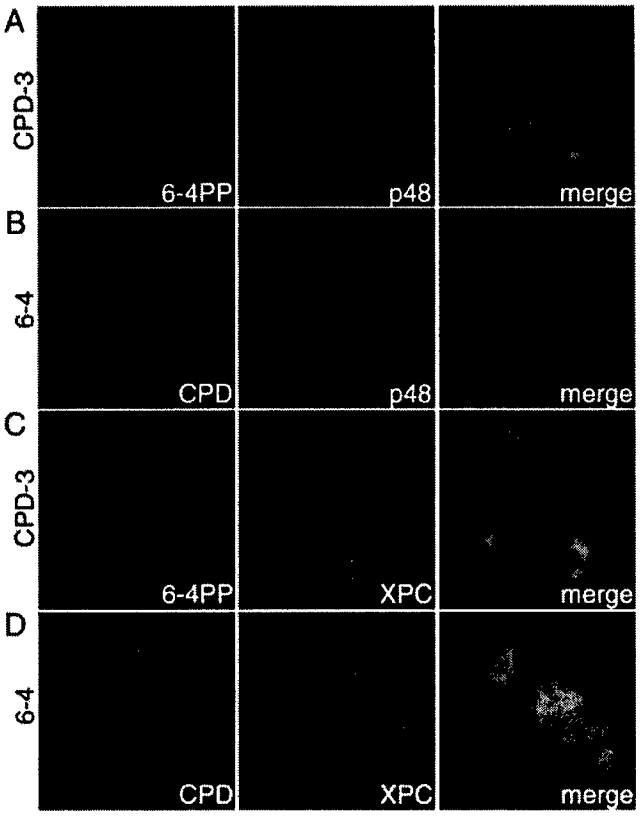

FIG. 2. ***In vivo* localization of p48 and XPC to either the CPD or the 6–4PP.** CPD-3 cells (*A* and *C*) or 6–4 cells (*B* and *D*) were irradiated with 200 J/m^2 UV-C through a 3-μm isopore filter and photoreactivated under UV-A blacklight for 2 h. 6–4PP (*A* and *C*) or CPD (*B* and *D*) photoproducts were visualized by monoclonal antibody specific to each photoproduct. p48 or XPC was transfected 24 h prior to UV irradiation and detected by an antibody to the V5 epitope present on the transgene.

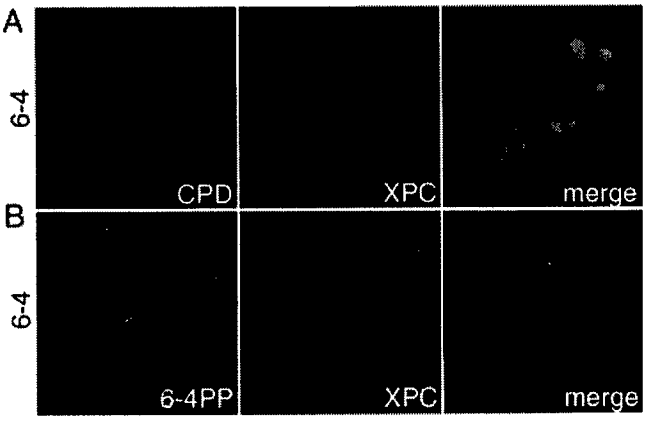

FIG. 3. **p48 stimulates the binding of XPC to CPDs *in vivo*.** 6–4 cells were irradiated with 200 J/m^2 UV-C through a 3-μm isopore filter and photoreactivated under UV-A blacklight for 2 h. CPDs (*A*) or 6–4PPs (*B*) were visualized by a monoclonal antibody specific to the photoproduct. XPC and p48 were cotransfected 24 h prior to UV irradiation, and XPC was detected by an antibody to the V5 epitope present on the XPC transgene. p48 cDNA was transfected in 10-fold excess of the XPC-V5 cDNA. The *dashed lines* in *panel B* outline the nuclei of two representative cells (one of which expresses the transgenes and one which does not), as determined by 4,6-diamidino-2-phenylindole (DAPI) staining.

vector-only expressing cells at the indicated time points. Notably, 6–4PPs also diminished in the CPD-3 cells after two hours of UV-A treatment to ~30% of original levels, possibly because of some cross-reactivity between the CPD photolyase and the 6–4PP.

Localization of p48 and XPC to DNA Damage after Photoreactivation of CPDs or 6–4PPs—Having established the characteristics of each photolyase clone, we utilized these cells to determine the binding properties of p48 or XPC to the individual photoproducts *in vivo*. For the study of each protein, we transiently transfected the photolyase-expressing XP-A cells with either a *DDB2* or *XPC* cDNA that had an additional V5 epitope tag to aid in detection and then used indirect immunofluorescence to visualize both the protein and photoproduct of interest. Irradiation was performed using the localized introduction of lesions through a micropore polycarbonate filter and colocalization of nuclear proteins to sites of DNA damage determined using specific monoclonal antibodies to CPDs and 6–4PPs (23). Photoreactivation under UV-A light was carried out for 2 h because this was sufficient to remove nearly all of the 6–4PPs in the 6–4 photolyase-expressing cells and the majority of CPDs in the CPD-3 photolyase-expressing cells (Fig. 1*B*).

Fig. 2 shows representative images of p48 and XPC binding to lesions induced by 200 J/m^2 UV-C through a 3-μm polycarbonate filter and then subjected to 2 h of UV-A reactivating light. We have previously used the local UV irradiation assay to demonstrate that a dose of 200 J/m^2 through a 3-μm filter was able to activate the tumor suppressor protein p53 and that this dose induced irradiated sites that efficiently bound both p48 and XPC (16). In CPD-3 cells, where the majority of CPDs have been removed by the CPD-specific photolyase, p48 clearly localized to sites that contain 6–4PPs (Fig. 2*A*). This localization pattern was observed in 100 of 105 cells examined visually (95.2%). One caveat to this finding is that because of the small number of photolyase-resistant CPDs remaining in these irradiated sites, we have not definitively determined if p48 bound only to 6–4PPs or if the residual CPDs are the cause of the binding pattern seen. In 6–4 cells that express the 6–4PP-specific photolyase, p48 localized to sites that contained CPDs, as shown in Fig. 2*B*. This colocalization pattern was observed in 88 of 112 cells examined visually (78.5%). The binding of p48 to the irradiated sites in the 6–4 cells after 2 h of reactivation appears to be specific to CPDs because the vast majority of 6–4PPs had been removed by the photolyase at this time point (Fig. 1*B*). There were not as many cells with the specific colocalization pattern of p48 and CPDs in 6–4 cells as there were for p48 and 6–4PPs in the CPD-3 cells (78.5 *versus* 95.2%), suggesting that CPDs may not be as strong a recognition signal as the 6–4PP for p48 *in vivo*. This would be consistent with the *in vitro* findings that UV-DDB has a higher affinity for 6–4PPs over CPDs (6, 9). The lower percentage of localization observed in the 6–4 photolyase-expressing cells also suggests that the high percentage of colocalization of p48 to irradiated sites observed in the CPD-3 cells was indeed due to p48 recognizing the 6–4PPs and not only the residual CPDs.

The XPC binding pattern was also determined in the photolyase-expressing XP-A cells. Fig. 2*C* is a representative image of XPC binding to irradiated sites that contained 6–4PPs in CPD-3 cells 2 h after UV-C irradiation and reactivation with UV-A. The colocalization of XPC to 6–4PP sites was observed in 99 of 108 cells (91.7%), suggesting that XPC is proficient at recognizing the 6–4PP *in vivo*. In contrast, 2 h after reactivation XPC did not colocalize to irradiated sites that contained only CPDs in 6–4 cells, as seen in Fig. 2*D*. Only 9 of 110 cells examined (8.2%) demonstrated an appreciable amount of colocalization of XPC to CPD-containing sites. The majority of 6–4PPs have been removed from these sites. Thus it can be inferred that the remaining CPDs were not able to efficiently recruit enough XPC to these sites to be seen by indirect immu-

TABLE I
Colocalization data of p48 and XPC in photolyase-expressing cells

Cell line	Transfected cDNA	Percent remaining CPDs	Percent remaining 6–4PPs	No. of transfected cells counted	No. of cells with colocalization	Percent
CPD-3	DDB2	17	30	105	100[a]	95.2
CPD-3	XPC	17	30	108	99[b]	91.7
6–4	DDB2	98	2	112	88[a]	78.6
6–4	XPC	98	2	110	9[b]	8.2
6–4	XPC + DDB2	98	2	115	100[b]	87.0

[a] p48 colocalization.
[b] XPC colocalization.

nofluorescence. The lack of binding to CPDs in the 6–4 cells further demonstrated that it was the 6–4PPs that were recognized by XPC in the CPD-3 cells and not the residual CPDs that were not removed by the CPD photolyase. This *in vivo* data is again consistent with *in vitro* binding studies that demonstrated that XPC has a much greater affinity for the 6–4PP than the CPD (6–8). This is the first *in vivo* demonstration that p48 is a more efficient recognition factor for CPDs than XPC (78.5% positive colocalization verses 8.2%).

p48 Activates XPC Binding to CPD-containing Sites—The different binding patterns observed between p48 and XPC led to the hypothesis that p48 may stimulate XPC binding to CPDs *in vivo*, thereby explaining why XP-E cells mutant for *DDB2* are predominantly deficient in the GGR of CPDs, more than 6–4PPs (4). We have previously observed that p48 can stimulate XPC binding to UV-irradiated sites that contained both CPDs and 6–4PPs (16). Fig. 3 is a representative image of 6–4 cells that were transfected with both XPC and p48, irradiated with 200 J/m^2 UV-C, and then photoreactivated with 2 h of UV-A to remove the 6–4PPs. In comparison to Fig. 2*D* where no detectable XPC binding in 6–4 cells was seen after photoreactivation, XPC strongly colocalized to irradiated sites that contain only CPDs in cells that overexpressed p48 (Fig. 3*A*). This pattern was observed in 100 of 115 cells examined (87.0%). This clearly demonstrates that p48 stimulates XPC binding to CPDs *in vivo* and provides the mechanism for the previously observed enhancement of XPC binding by p48. One caveat to our transfection studies is that these conditions are not the same as the photoreversal conditions used to determine the efficiency of the 6–4 photolyase in Fig. 1 because we have overexpressed ectopic p48 and XPC, which potentially could interfere with the action of the 6–4 photolyase. We therefore also stained for 6–4PPs in the transfected 6–4 cells after 2 h of photoreactivating UV-A in the presence of overexpressed p48 and XPC. Fig. 3*B* shows two representative cells from a transfection experiment with both XPC and p48 transgenes as in Fig. 3*A*, only now stained for 6–4PPs and XPC. XPC can be seen localizing to areas that are presumably irradiated sites that contain CPDs, but there are no discernable 6–4PPs and the XPC-expressing cell has the same background staining pattern as the cell below it, which is not expressing the transgenic XPC and p48. These results were verified in whole cell irradiation experiments (20 J/m^2, 2-h photoreactivation); again we did not observe any residual 6–4PPs in cells that had overexpressed p48 or XPC alone or in combination over cells that did not express the transgenes. In conclusion, we do not believe that p48 or XPC interferes with the action of the 6–4 photolyase and that the results we observe are due to p48 activating the binding of XPC to CPDs. Table I summarizes the colocalization data of p48 and XPC in the photolyase-expressing cells.

DISCUSSION

We have used XP-A cells that express specific DNA photolyases to define the *in vivo* binding properties of p48 and XPC. p48 bound to both lesion types, with a slight preference for the 6–4PP. XPC, in contrast, showed a very strong binding preference for the 6–4PP over the CPD. Our data corroborate *in vitro* studies of XPC and p48 binding preferences (6–10). Overexpression of p48 was able to dramatically alter the binding properties of XPC so that significant colocalization of XPC was observed to CPD-only-containing sites. Loss of p48 in XP-E cells causes a decrease in repair of CPDs but does not greatly affect the repair of 6–4PPs (4). The ability of XPC to readily recognize 6–4PPs but not CPDs *in vivo* correlates with the phenotype of XP-E cells and demonstrates *in vivo* that XPC can function to recognize 6–4PPs. When p48 is present at higher levels, as with overexpression in our study or through upregulation by p53 both at basal levels and after DNA damage (4), p48 is able to recruit XPC to CPDs and therefore stimulate repair of these lesions. This also explains recent observations by Wang *et al.* (33) describing a p53-dependent effect on binding of XPC and TFIIH to UV-irradiated sites, where they speculated that regulation of a p53-dependent gene may affect the recruitment of XPC to irradiated sites, and specifically to CPDs.

This is the first demonstration *in vivo* of the mechanism by which p48 stimulates DNA repair through the NER pathway. There is some repair of CPDs in XP-E cells, and this may occur through the recognition of CPDs by XPC when other chromatin remodeling processes occur, such as those related to transcription or DNA replication. The *in vitro* binding studies of XPC to CPDs would suggest that there is some affinity between the two, yet the fact that we did not observe significant binding of XPC to CPDs when p48 was not overexpressed suggests that the amount of XPC binding at any one time was not enough to allow detection by our technique. Although p48 clearly enhances the binding of XPC to CPDs, we do not know the mechanism for this stimulation. p48 has recently been shown to interact with the COP9 signalosome (34), a complex that has ubiquitin ligase activity. This would suggest a mechanism whereby p48 mediates ubiquitin ligation of substrates around CPDs, possibly including histones, which may lead to nucleosome unfolding and thereby allow access of XPC and the remaining components of the NER machinery to CPDs. p48 is itself degraded rapidly after UV irradiation via the ubiquitin-mediated proteasome (24, 35); XPC stability is also regulated through the proteasome (36). Clearly, regulation of ubiquitin pathways is an important feature of NER and an exciting new avenue to explore in the understanding of NER.

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Ductal Lavage of Fluid-Yielding and Non–Fluid-Yielding Ducts in BRCA1 and BRCA2 Mutation Carriers and Other Women at High Inherited Breast Cancer Risk

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Abstract

Objective: **Nipple fluid production and atypical breast duct cells in women at high risk of breast cancer have been associated with further increased risk. Most publications on ductal lavage for cell collection report cannulating fluid-yielding ducts only. We report lavage of fluid-yielding and non–fluid-yielding ducts in women at high inherited breast cancer risk.**

Methods: **A pilot breast cancer screening study including ductal lavage was conducted in 75 women at high inherited risk, 56 (74.7%) of whom had BRCA1/2 mutations. Ductal lavage was attempted in any duct identifiable with a catheter.**

Results: **Ducts were successfully catheterized in 60 of 75 patients (80%). Successfully catheterized patients were younger (median age 41 versus 53 years, $P = 0.0003$) and more often premenopausal (51.7% versus 20%, $P = 0.041$). Thirty-one successfully catheterized patients [51.6%, 95% confidence interval (39.4-63.9%)] had non–fluid-yielding ducts only. Seventeen patients [28.3% (18.5-40.9%)] had atypical cells. Twelve of seventeen [70.6% (46.8-87.2%)] samples with atypia were from non–fluid-yielding ducts. Patients with non–fluid-yielding ducts (versus fluid-yielding ducts) were more likely to have had prior cancer (48.4% versus 17.2%, $P = 0.014$) or chemotherapy (45.2% versus 17.2%, $P = 0.027$); this was also true in patients with atypia from non–fluid-yielding ducts.**

Conclusion: **Successfully lavaged women were younger and more often premenopausal. Atypical cells can be found in non–fluid-yielding ducts in patients at high inherited breast cancer risk. Non–fluid-yielding ducts, and atypia from non–fluid-yielding ducts, are more common in patients with prior cancer and chemotherapy. Larger studies are needed to identify risk factors and prognostic significance associated with atypia and non–fluid-yielding ducts in high-risk populations, and define their role as biomarkers.** (Cancer Epidemiol Biomarkers Prev 2005;14(5):1082–9)

Introduction

Women with an inherited predisposition to develop breast cancer are a group at very high risk of the disease, one which has not been well-served by standard screening techniques. It is estimated that 9,000 to 18,000 cases of breast cancer in the U.S. per year are attributable to inherited risk. A large percentage of such cases are related to deleterious mutations in the breast cancer susceptibility genes BRCA1 and BRCA2, of which some 1 in 500 to 800 American women are estimated to be carriers (1, 2). Lifetime risks of breast cancer in women with BRCA mutations have been reported in the 45% to 82% range (3, 4). A minority of such women choose to undergo prophylactic mastectomy, which is the most effective available preventive method (5-10); bilateral salpingo-oophorectomy (generally done to decrease the high risk of ovarian cancer in BRCA mutation carriers) and tamoxifen are also used as breast cancer–risk reducing strategies in this population (11-15).

For the majority of women with an inherited predisposition to breast cancer who do not choose prophylactic mastectomy, intensive screening is an emerging alternative. Standard mammographic screening has been shown to be of inadequate sensitivity in this group of generally young women (16). A high incidence of interval cancers has been reported with mammographic screening in this population (17). Increasingly, breast magnetic resonance imaging (MRI) is being incorporated, both within and outside of research protocols, as a screening technique in these high-risk women, with encouraging reports of high tumor detection rates at early stages (18-23). We have recently reported on the use of mammography, high-quality breast MRI, clinical breast examination, and ductal lavage as a comprehensive screening protocol for women at high inherited risk of developing breast cancer (24). In this population, we have identified high-risk lesions by MRI screen detection and by cytologic assessment of ductal cells (24). Because of the high cancer risk in these patients, there is great need to develop and validate novel breast screening techniques, with the ultimate goal of improving their breast cancer outcomes through early detection.

Most breast cancers start in the breast ducts, and early, potentially premalignant alterations in the ductal epithelium are beginning to be defined (25-27). For these reasons, evaluation of breast duct cells is an emerging technique for breast cancer risk assessment, and for the discovery of potential biomarkers which may serve as intermediate end points in trials of cancer prevention agents (28-34). Various minimally invasive methods exist for collecting breast duct

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cells, including nipple suction aspiration and random periareolar fine-needle aspiration; the finding of atypia in cells collected by each of these techniques has been associated with increased subsequent breast cancer risk (35, 36). A more recently developed cell collection method, ductal lavage, uses a small catheter inserted into the nipple to collect cells lining the breast ducts (37). Advantages of ductal lavage include higher average cell yield than nipple suction aspiration, and relative anatomic specificity, with the ability to resample a specific abnormal duct over time (24, 37, 38). Potential disadvantages include reports of low cancer detection rate in patients with known malignancy, possibly because of duct occlusion by tumor (39, 40); this finding has led to speculation that ductal lavage may be a more appropriate technique for risk assessment than for cancer diagnosis. In a large, multicenter study evaluating ductal lavage for tolerability and cell yield, ductal lavage was attempted only in those ducts which yielded fluid on nipple suction aspiration (fluid-yielding ducts; ref. 37), a strategy based on previous reports that women with fluid-yielding ducts were at higher risk of breast cancer than those with non–fluid-yielding ducts (35, 41, 42).

Our breast screening protocol for women with high inherited risk of breast cancer, combining annual ductal lavage, breast MRI, mammography, and biannual clinical breast exam, was initiated with a goal of improving early detection of cancer and high-risk breast lesions (24, 38). Early in the course of this study, we observed that a higher proportion of our patients than the 16% reported in previous series (37) did not yield fluid on nipple suction aspiration, and we commenced lavage of non–fluid-yielding ducts, as well as fluid-yielding ducts. We now report results of ductal lavage of fluid-yielding ducts and non–fluid-yielding ducts, with associated reproductive and life-style characteristics, in women enrolled in this breast screening protocol.

Materials and Methods

Patient Population. After study approval by Institutional Review Boards at both centers, in accordance with assurances filed and approved by the Department of Health and Human Services, participants were recruited from cancer genetics clinics at Stanford University School of Medicine and the Dana-Farber Cancer Institute. Inclusion criteria and patient enrollment procedures at both centers were similar. Eligibility criteria at both centers included a documented BRCA1 or BRCA2 mutation; at Stanford University School of Medicine, patients were also eligible if they had no BRCA mutation, but had a >10% risk of developing breast cancer at 10 years based on the Claus model, which incorporates only family history of breast cancer (43-45). If patients had a personal history of breast cancer and no mutation in BRCA1 or BRCA2, the Claus model was used to calculate predicted risk for an unaffected sister; if this risk was >10%, the patient was eligible for participation. Only the unaffected breast was eligible for lavage in patients with prior breast cancer history. Participants had to be at least 25 years of age, or 5 years younger than the earliest age at which a relative was diagnosed with breast cancer. Patients with a history of breast cancer or ovarian cancer had to have completed adjuvant therapy at least 1 year previously. Patients who had had prior breast surgery which seemed to distort the duct system, including incisions near or involving the nipple, were not eligible for ductal lavage of that breast, given our concern for potential increase in infection risk under those circumstances. Informed consent was obtained from all patients, and all study procedures were compliant with regulations of the Health Insurance Portability and Accountability Act of 1996. Alternatives to study participation were offered to all patients.

Screening Protocol. Participants were enrolled in a pilot breast screening study incorporating mammography, MRI, and ductal lavage, with the goal of evaluating these combined techniques for their ability to detect high-risk and malignant breast lesions. The breast screening protocol and its preliminary results have been described in detail previously (24, 38, 46). The protocol included twice yearly clinical breast exam, yearly mammogram, MRI, and ductal lavage. Abnormality detected on clinical breast exam required 3 to 4 months follow-up clinical breast exam or biopsy, as determined by clinical features; further imaging, including ultrasound and additional mammographic views, was done as prompted by clinical findings. Abnormal MRI or mammogram required 6 months of follow-up or biopsy, as determined by radiographic features. Atypical cells on ductal lavage required 6-month interval follow-up ductal lavage and 6-month follow-up MRI of the affected breast. Enrollment began in September of 2001, and accrual continues.

Ductal Lavage Protocol. Participants were anesthetized topically with 4% lidocaine cream applied to the nipple 20 to 30 minutes prior to the procedure. Nipple suction aspiration was done to identify any fluid-yielding ducts. Attempts were made to cannulate any duct, regardless of fluid status, which could be identified using a dilator coated in 1% xylocaine gel, and subsequently a catheter (Cytyc Health Corporation, Boxborough MA; Acueity, Palo Alto, CA). If resistance was met on attempt to catheterize a duct, gentle pressure was applied; if further resistance was encountered, or if the patient experienced discomfort, no further attempt was made to catheterize that duct. Once the catheter was inserted into the duct, 3 to 5 mL of 1% lidocaine was injected, followed by approximately 15 mL of normal saline, in aliquots of 5 mL per injection. Following each injected aliquot of normal saline, breast massage was done and fluid collected via the lavage catheter. The location of each lavaged duct was marked in all cases by assigning a location on a two-dimensional grid, and in most cases by inserting a metal clip provided for this purpose (Acueity) and recording its location via photograph. A cytologic diagnosis of normal cells, insufficient cellular material for diagnosis, mild atypia, marked atypia, or malignant cells was made for each specimen. A representation of a benign and an atypical cytologic reading is presented in Figs. 1 and 2. Time constraints limited attempted cannulation to approximately two to three ducts per breast. Both medical oncologists performing the ductal lavage procedure (A.W. Kurian and A.R. Hartman) and both pathologists interpreting the cytologic specimens (L.C. Collins and K.W. Nowels) were trained by the same methods, as published by Dooley et al. (37).

Statistical Analysis. Univariate analysis of patient characteristics associated with the results of ductal lavage was done using Fisher's exact test for categorical data, and the Mann-Whitney U test for continuous data. All P values are two-sided. Logistic regression was used to identify those variables which are most significant independent predictors of fluid-yielding versus non–fluid-yielding duct status.

Results

Patient Characteristics. Patient characteristics are presented in Table 1. A total of 75 patients underwent attempted lavage; 24 patients were enrolled from the Dana-Farber Cancer Institute, and 51 from Stanford University Medical Center. Comparison of baseline clinical characteristics between patients from the two participating centers revealed no statistically significant differences in median age, BRCA1 or BRCA2 mutation status, prior breast or ovarian cancer, prior chemotherapy or radiation therapy, prior breast biopsy, prior

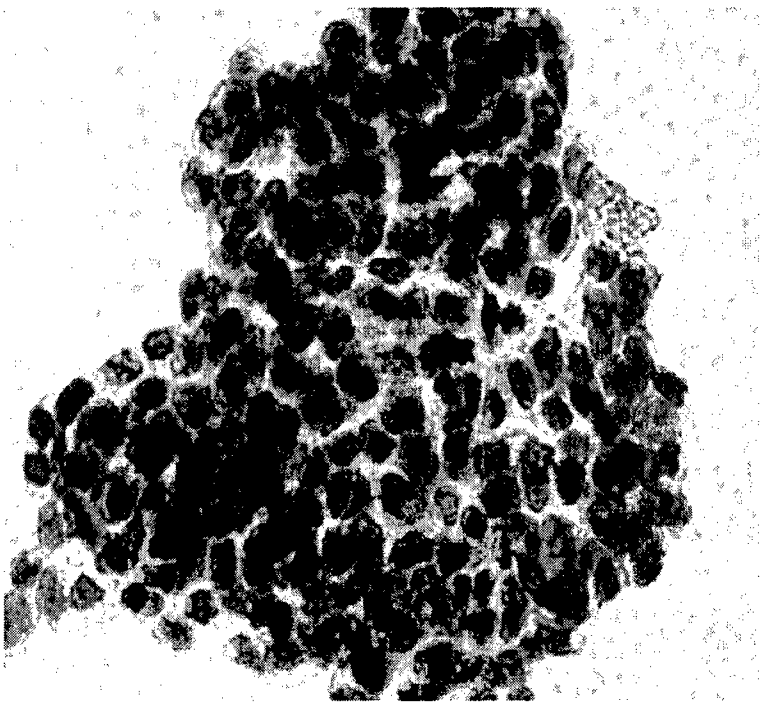

Figure 1. Benign cells from ductal lavage.

use of tamoxifen or other selective estrogen response modulator, hormone replacement therapy, or oral contraceptive pill use, premenopausal status, or fluid yield on nipple suction aspiration. Compared with patients at Stanford University Medical Center, patients at the Dana-Farber Cancer Institute were significantly more likely to be parous (87.5% versus 62.7%, $P = 0.03$), to have breastfed (79.2% versus 43.1%, $P = 0.006$), and to have had a prior bilateral salpingo-oophorectomy (70.8% versus 39.2%, $P = 0.01$, data not shown). A catheter could be inserted into one or more ducts in 60 patients [80%, 95% confidence interval (69.5-87.7%)]. Four patients were African-American, one was Asian-American, and 70 were Caucasian. Given the very small number of patients who were not Caucasian, analyses by race were not done. The median age of all patients in whom ductal lavage was attempted was 43 years. Ductal lavage was considered successful if a catheter could be inserted into a duct, and saline instilled. In all patients who underwent successful

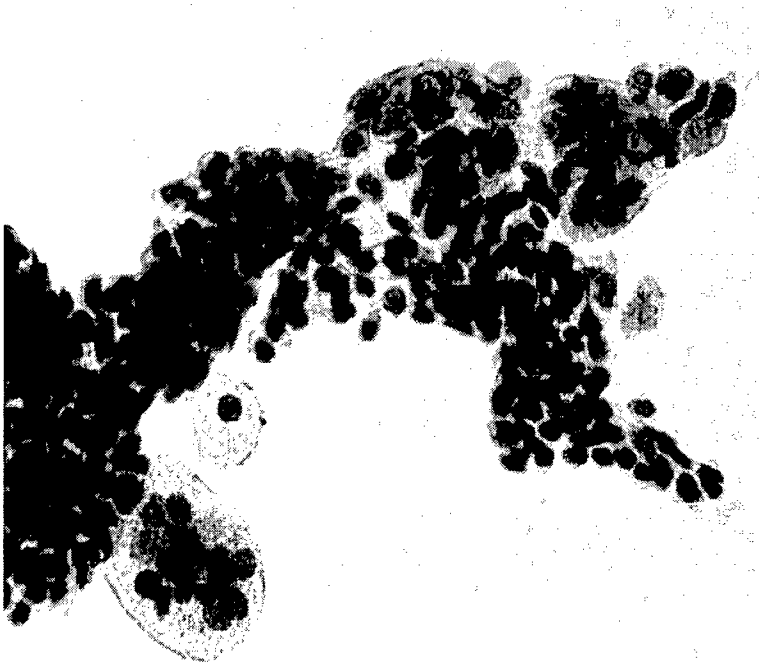

Figure 2. Atypical cells from ductal lavage.

Table 1. Patient characteristics and ductal lavage success

Patient characteristics	Ductal lavage attempted (n = 75)	Ductal lavage successful* (n = 60)	Ductal lavage unsuccessful† (n = 15)	Two-sided P value‡,§
Median age (years)	43	41	53	0.0003
BRCA1	43 (57.3%)	34 (56.7%)	9 (60%)	1.0
BRCA2	13 (17.3%)	11 (18.3%)	2 (13.3%)	1.0
Prior breast cancer	19 (25.3%)	14 (23.3%)	5 (33.3%)	0.51
Prior ovarian cancer	8 (10.7%)	6 (10%)	2 (13.3%)	0.66
Prior chemotherapy	24 (32%)	19 (31.7%)	5 (33.3%)	1.0
Prior breast radiation	12 (16%)	9 (15%)	3 (20%)	0.70
Prior or current selective estrogen response modulator use	15 (20%)	10 (16.7%)	5 (33.3%)	0.16
Prior or current oral contraceptive pill use	62 (82.7%)	50 (83.3%)	12 (80%)	0.72
Prior or current hormone replacement therapy use	20 (26.7%)	15 (25%)	5 (33.3%)	0.53
Parous	53 (70.7%)	43 (71.7%)	10 (66.7%)	0.76
Breastfed	41 (54.7%)	34 (56.7%)	7 (46.7%)	0.57
Premenopausal	31 (41.3%)	31 (51.7%)	3 (20%)	0.041
Bilateral salpingo-oophorectomy before ductal lavage	37 (49.3)	29 (48.3%)	8 (53.3%)	0.78
Prior breast biopsy	40 (53.3%)	32 (53.3%)	8 (53.3%)	1.0
Ever fluid-yielding on suction aspiration	30 (40%)	29 (48.3%)	1 (6.7%)	0.0029

*Ductal lavage considered successful if a catheter could be inserted into one or more ducts, and saline instilled.
† Ductal lavage considered unsuccessful if a catheter could not be inserted into any duct, or saline could not be instilled.
‡ Two-sided P values from Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables.
§For comparison of successful versus unsuccessfully lavaged patients.

catheterization, median age was 41 years, whereas in all patients who could not be catheterized, median age was 53 years (P = 0.0003). Patients who could be successfully catheterized were more likely to be premenopausal than patients who could not (51.7% versus 20%, P = 0.041). Only 1 of the 15 patients who could not be catheterized yielded fluid on nipple aspiration, compared with 29 of the 60 successfully catheterized patients (6.7% versus 48.3%, P = 0.0029). No significant differences in BRCA mutation status, prior breast or ovarian cancer, prior chemotherapy or radiation therapy, prior breast biopsy, prior selective estrogen response modulator use, hormone replacement therapy or oral contraceptive pill use, parity, breastfeeding, or prior bilateral salpingo-oophorectomy were noted between patients who could and could not be successfully catheterized.

Ductal Lavage Cytology. Patient characteristics according to ductal lavage cytology are summarized in Table 2. Eight patients had insufficient cellular material for diagnosis [13.3% (6.7-24.5%)]; compared with the 35 patients with benign cytology [58.3% (45.8-70.0%)], patients with insufficient cellular material for diagnosis were more likely to have had prior breast cancer (62.5% versus 14.3%, P = 0.01), to have had prior chemotherapy (62.5% versus 20%, P = 0.028) or to have taken tamoxifen or another selective estrogen response modulator (50% versus 11.4%, P = 0.028). The median age of patients with insufficient cellular material for diagnosis was 44.5 years, and the median age of patients with benign cytology was 42 years (P = 0.072). Of 8 patients with insufficient cellular material for diagnosis, 1 yielded fluid on nipple suction aspiration, compared with 19 of 35 patients with benign cytology (12.5% versus 54.3%, P = 0.05). Seventeen patients were found to have mildly atypical cytology [28.3% (18.5-40.9%)]. Of 17 patients with atypia, 9 yielded fluid from any duct on nipple suction aspiration [52.9% (31.1-74.0%)]; in 12 of these 17 patients, the ducts which produced atypia were non–fluid-yielding. No significant differences in mean age, BRCA mutation status, prior breast or ovarian cancer, prior chemotherapy or radiation therapy, prior breast biopsy, prior selective estrogen response modulator, hormone replacement therapy or oral contraceptive pill use, parity, breastfeeding, menopausal status, or prior bilateral salpingo-oophorectomy were noted between patients with atypical and benign cytology.

Fluid-Yielding Status. Patient characteristics according to fluid-yielding status are summarized in Table 3. Twenty-nine patients had one or more fluid-yielding ducts on at least one occasion [48.3% (36.2-60.8%)] and 31 patients [51.7% (39.3-63.9%)] had only non–fluid-yielding ducts on all occasions. Patients with non–fluid-yielding ducts were significantly more likely than patients with fluid-yielding ducts to have had prior breast or ovarian cancer (48.4% versus 17.2%, P = 0.014) or prior chemotherapy (45.2% versus 17.2%, P = 0.027). No significant differences in mean age, BRCA mutation status, prior radiation therapy, prior breast biopsy, prior selective estrogen response modulator, hormone replacement therapy or

Table 2. Patient characteristics and ductal lavage cytology results

Patient characteristics*	Insufficient cellular material for diagnosis (n = 8)	Benign cells (n = 35)	Two-sided P value†,‡	Atypical cells (n = 17)	Two-sided P value†,§
Median age (years)	44.5	42	0.072	38	0.63
Prior breast cancer	5 (62.5%)	5 (14.3%)	0.01	4 (23.5%)	0.45
Prior ovarian cancer	0 (0%)	3 (8.6%)	1.0	3 (17.7%)	0.38
Prior breast or ovarian cancer	5 (62.5%)	8 (22.9%)	0.042	7 (41.2%)	0.20
Prior chemotherapy	5 (62.5%)	7 (20.0%)	0.028	7 (41.2%)	0.18
Prior breast radiation	3 (37.5%)	3 (8.6%)	0.067	3 (17.7%)	0.38
Ever fluid-yielding on suction aspiration	1 (12.5%)	19 (54.3%)	0.05	9 (52.9%)	1.0

*There were no significant differences between groups in BRCA mutation status, prior or current selective estrogen response modulator, oral contraceptive, and hormone replacement therapy use, parity, prior breast-feeding, menopausal status, bilateral salpingo-oophorectomy before ductal lavage, and prior breast biopsy.
† Two-sided P values from Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables.
‡ For comparison of patients with insufficient cellular material for diagnosis versus patients with benign cells.
§For comparison of patients with benign cells versus patients with atypical cells.

Table 3. Patient characteristics and fluid-yielding duct status

Patient characteristics	Any fluid-yielding duct (n = 29)	All non–fluid-yielding ducts (n = 31)	Two-sided P value*
Median age (years)	42	40	1.0
BRCA mutation	20 (70%)	25 (80.7%)	0.38
Prior breast cancer	4 (13.8%)	10 (32.3%)	0.129
Prior ovarian cancer	1 (3.5%)	5 (16.1%)	0.196
Prior breast or ovarian cancer	5 (17.2%)	15 (48.4%)	0.014
Prior chemotherapy	5 (17.2%)	14 (45.2%)	0.027
Prior breast radiation	3 (10.3%)	6 (19.4%)	0.47
Prior or current selective estrogen response modulator use	3 (10.3%)	7 (22.6%)	0.30
Prior or current oral contraceptive pill use	25 (86.2%)	25 (80.7%)	0.73
Prior or current hormone replacement therapy use	7 (24.1%)	8 (25.8%)	1.0
Parous	21 (72.4%)	22 (71%)	1.0
Breastfed	15 (51.7%)	19 (61.3%)	0.60
Premenopausal	16 (55.2%)	15 (48.4%)	0.62
Bilateral salpingo-oophorectomy before ductal lavage	13 (44.8%)	16 (51.6%)	0.62
Prior breast biopsy	15 (51.7%)	17 (54.8%)	1.0

*Two-sided P values from Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables.

oral contraceptive pill use, parity, breastfeeding, menopausal status or prior bilateral salpingo-oophorectomy were noted between patients with fluid-yielding ducts and with non–fluid-yielding ducts on univariate analysis. Logistic regression was used to identify independently predictive variables. Forward stepwise selection procedure, starting with the model with no predictors, was used for model-building purposes. Analysis was done using the statistical software S-PLUS version 6.1 for Windows (Insightful Corporation, Seattle, WA). All variables in Table 3 except for premenopausal status were allowed to enter the model; the premenopausal variable was removed because it was perfectly correlated with the bilateral salpingo-oophorectomy variable. As a result of this stepwise procedure, a model with a single predictor, prior breast or ovarian cancer, was selected. This variable had the smallest P value using Fisher's exact test. The same model was selected using backward stepwise selection, starting with a model containing 11 variables (all the variables in Table 3 excluding premenopausal status, prior breast and prior ovarian cancer variables). The variables of prior breast or ovarian cancer and prior chemotherapy were highly correlated: only one woman with prior history of cancer was not treated with chemotherapy, but for all others, a prior history of cancer implied having received chemotherapy. Given this fact, the effects of these two variables were difficult to separate, although both model selection procedures preferred the prior cancer variable.

Atypia and Fluid-Yielding Status. Characteristics of patients with atypia, by fluid-yielding status, are described in Table 4. Of 17 patients with atypical cells, 12 had atypia from non–fluid-yielding ducts only [70.6% (46.8-87.2%)]. Of the remaining five patients, three had atypia from fluid-yielding ducts only [17.7% (5.5-42.1%)] and two had atypia from both fluid-yielding ducts and non–fluid-yielding ducts [11.8% (2.1-35.9%)]. Given the small numbers, patients with atypia from any fluid-yielding ducts [n = 5; 29.4% (13.1-53.7%)] were analyzed as a group. Patients with atypia from non–fluid-yielding ducts only were significantly more likely than patients with atypia from any fluid-yielding ducts to have had prior breast or ovarian cancer (58.3% versus 0%, P = 0.044) or to have had prior chemotherapy (58.3% versus 0%, P = 0.044). No significant differences in mean age, BRCA mutation status, prior radiation therapy, prior breast biopsy, prior selective estrogen response modulator, hormone replacement therapy or oral contraceptive pill use, parity, breastfeeding, menopausal status or prior bilateral salpingo-oophorectomy were noted between patients with atypia from non–fluid-yielding ducts only and patients with atypia from any fluid-yielding ducts.

Discussion

To our knowledge, this is the first characterization of ductal lavage of non–fluid-yielding ducts in high-risk women, with a report on associated atypical cells. The present finding of

Table 4. Patient characteristics and atypia by fluid-yielding duct status

Patient characteristics	Atypia from all non–fluid-yielding ducts (n = 12)	Atypia from any fluid-yielding duct (n = 5)	Two-sided P value*
Median age (years)	39	38	0.96
BRCA mutation†	9 (75%)	2 (40.0%)	0.28
Prior breast cancer	4 (33.3%)	0 (0%)	0.26
Prior ovarian cancer	3 (25%)	0 (0%)	0.51
Prior breast or ovarian cancer	7 (58.3%)	0 (0%)	0.044
Prior chemotherapy	7 (58.3%)	0 (0%)	0.044
Prior breast radiation	3 (25%)	0 (0%)	0.51
Prior or current selective estrogen response modulator use	2 (16.7%)	0 (0%)	1.0
Prior or current oral contraceptive pill use	10 (83.3%)	3 (60%)	0.54
Prior or current hormone replacement therapy use	5 (41.7%)	1 (20%)	0.60
Parous	8 (66.7%)	3 (60%)	1.0
Breastfed	10 (83.3%)	2 (40%)	0.12
Premenopausal	4 (33.3%)	4 (80%)	0.13
Bilateral salpingo-oophorectomy before ductal lavage	8 (66.7%)	1 (20%)	0.13
Prior breast biopsy	7 (58.3%)	1 (20%)	0.29

*Two-sided P values from Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables.
†Of the nine BRCA mutation carriers with atypia from non–fluid-yielding ducts, seven had BRCA1 mutations, and two had BRCA2 mutations; of the two BRCA mutation carriers with atypia from fluid yielding ducts, one had a BRCA1 mutation, and one had a BRCA2 mutation.

atypical cells associated with non–fluid-yielding ducts, at least as frequently as in fluid-yielding ducts, suggests that non–fluid-yielding ducts in women with an inherited predisposition to breast cancer might be associated with higher risk than previously supposed.

Prospective evaluation of outcomes associated with breast duct cytology was first done using nipple suction aspiration (35, 41, 42). Collection of nipple aspirate fluid has been reported in a clinic and population-based sample of women at varying levels of breast cancer risk; women who did not yield nipple aspirate fluid (15% of the studied population) were chosen as the reference group, based on previous observations suggesting that such women would have the lowest risk of breast cancer (47). At a mean 12.7 years of follow-up, relative breast cancer risk of 1.8 was reported in women with normal nipple aspirate fluid cytology, and relative risk of 10.3 was reported in women with atypical nipple aspirate fluid cytology, versus those without nipple aspirate fluid (35). With increased patient numbers and years of follow-up, the authors reported relative breast cancer risk of 1.2 to 1.6 with normal nipple aspirate fluid cytology, and 2.0 to 2.8 with abnormal nipple aspirate fluid cytology, compared with a relative risk of 1.0 in women without nipple aspirate fluid (42). Most subsequent studies of ductal lavage have reported cannulating fluid-yielding ducts only (30, 31, 34), and thus little is known about the prevalence of abnormal cytology from non–fluid-yielding ducts. A recent study reported atypical cells in non–fluid-yielding ducts of lower-risk women (48). Our finding that 12 of 17 patients [70.6% (46.8-87.2%)] with atypical cells produced them from non–fluid-yielding ducts provides evidence that fluid yield is not a prerequisite for cytologic abnormality in high-risk women. It is consistent with a recent report that atypical cells have been collected by random periareolar fine-needle aspiration in patients with non–fluid-yielding ducts on suction (49). It may also provide some explanation for reports of ductal lavage's poor performance as a diagnostic tool in patients with known breast cancer: several of the breast cancer cases considered to have been missed by ductal lavage in a prior publication occurred in patients who had non–fluid-yielding ducts only, in whom ductal lavage was not attempted (40). Given the 2- to 5-fold increase in subsequent breast cancer risk observed in women with atypia in nipple aspirate fluid or on random periareolar fine-needle aspiration (36, 42), our results suggest that non–fluid-yielding ducts should be evaluated when ductal lavage is done in high-risk women. Furthermore, they suggest that reassessment of ductal lavage's performance as a diagnostic tool in women with known breast cancer is warranted, including lavage of non–fluid-yielding ducts as well as fluid-yielding ducts.

One potential explanation for our finding of atypia from non–fluid-yielding ducts might relate to the study population: patients in our breast screening protocol were selected because of their strong inherited predisposition to develop breast cancer. Notably, 7 of the 12 patients with atypia from non–fluid-yielding ducts had mutations in BRCA1, a finding which seems consistent with the high incidence of atypical hyperplasia reported in prophylactic mastectomy specimens of BRCA mutation carriers (50). Patients who carry BRCA1 mutations have a high incidence of estrogen and progesterone receptor–negative tumors; it could be that breast cancer risk in these patients, for which ductal atypia may be a biomarker, is less related to the hormonal factors thought to associate with fluid-yielding ducts than it is in other patient populations. However, one caveat to this hypothesis is the reduction in breast cancer risk seen in BRCA1 mutation carriers after oophorectomy (11-13, 51). It is important to note that the presence of mildly atypical cells collected via ductal lavage has not yet been prospectively associated with increased breast cancer risk, in BRCA mutation carriers or in other patient groups, and that studies with longer follow-up, in larger numbers of women, will be necessary to determine whether such an association exists. It will also be important to determine whether women with atypia from non–fluid-yielding ducts are at risk for different kinds of cancer (for example, a higher incidence of estrogen receptor–negative tumors) than women with atypia from fluid-yielding ducts. If so, then the combination of atypia and fluid-yielding status could have value as a prognostic biomarker, and as a surrogate end point for trials of targeted chemopreventive agents.

On analysis of successfully catheterized patients by fluid-yielding status, factors which differed significantly were: having a history of prior breast or ovarian cancer and having received chemotherapy. History of breast or ovarian cancer remained a significant predictor in multivariate analysis (given the very close correlation between cancer history and chemotherapy, the ability of chemotherapy to add to a model incorporating prior cancer was limited). Consideration of our results and of those previously reported suggests that nipple fluid production may be associated with reproductive and hormonal factors such as ovarian function; our finding of higher breast and ovarian cancer incidence among patients with non–fluid-yielding ducts may reflect the antihormonal maneuvers (selective estrogen response modulator use, bilateral salpingo-oophorectomy, and potential for chemotherapy-induced amenorrhea) used to treat these cancers. The potential relation between fluid-yielding ducts and ovarian function may partially explain the previously observed association of nipple aspirate fluid with increased breast cancer risk (35, 41, 42), given that longer exposure to higher levels of hormones produced by the ovary is likely a mechanism of this observed effect.

Previous authors have reported that age is related to fluid yield (47); a recent Australian study has confirmed the finding of higher fluid-yield and cell count on ductal lavage in premenopausal women (52). Our results show similar trends. The median age of patients who could not be successfully catheterized was 53 years; the median age of patients who could be successfully catheterized, but had only insufficient cellular material for diagnosis, was 44.5 years. Both numbers were larger (in the former case, significantly so) than the median ages of patients who could be successfully catheterized (41 years) or had benign cytology (42 years), respectively. Patients who could not be catheterized were significantly less likely to yield fluid on nipple suction aspiration or to be premenopausal than patients who could. Patients who yielded only insufficient cellular material for diagnosis on catheterization had a higher likelihood of prior breast or ovarian cancer, chemotherapy, selective estrogen response modulator use, and had a nonsignificant trend toward a lower rate of fluid on nipple suction aspiration than patients with benign cytology. Our findings and those of others suggest a decline in patency and fluid production of the ductal system, initially manifested by decreased cellularity of lavage specimens, and associated with falling levels of estrogen and progesterone (which would likely decline after treatment with chemotherapy or selective estrogen response modulators, and with rising age). They are consistent with known proliferative effects of estrogen on the mammary epithelium at various stages in development, as observed in murine models (53). Future studies of high-risk women who are postmenopausal or aged 50 or older should evaluate other methods of assessing ductal cytology, such as random periareolar fine-needle aspiration, which do not rely upon fluid production on aspiration or duct patency. Exploration of methods to increase duct patency, including use of topical nitroglycerin as has been previously reported, might also be effective in cytologic evaluation of this population (54).

Strengths of our study include its prospective, multi-institutional nature. Patients enrolled at the two institutions

had generally similar clinical characteristics; the finding of a higher rate of bilateral salpingo-oophorectomy in the Dana-Farber Cancer Institute group may represent a higher percentage of BRCA1 (70.8% versus 50.9%, $P = 0.14$) and BRCA2 (29.1% versus 11.8%, $P = 0.10$) mutation carriers, although this did not reach statistical significance. The higher rate of parity and breastfeeding among the Dana-Farber Cancer Institute patients might reflect their slightly older median age (45 versus 43 years, $P = 0.22$), a difference which also did not reach statistical significance. Given the overall similarity of populations at both institutions, and the equivalent procedural and diagnostic methods used, these differences seem unlikely to have significantly affected the combined results. One limitation is the absence of a normal control group; future larger studies should include a group of average-risk women who will similarly be evaluated for atypia on ductal lavage, and for subsequent breast cancer incidence. As previously noted, the diagnosis of mild atypia by ductal lavage has not yet been prospectively associated with increased breast cancer risk (unlike atypical findings on nipple aspirate fluid or random periareolar fine-needle aspiration), and artifacts related to the lavage technique cannot be excluded as a contributor to the present findings (35, 36, 42). However, it is notable that a similar atypia rate was found in our population (28.3%) as has been found in other studies of ductal lavage (21%), and random periareolar fine-needle aspiration (24%) among cohorts of high-risk women, which suggests some consistency between these techniques (36, 37). Another limitation is the small number of women of races other than Caucasian, particularly given previous reports of racial differences in nipple aspirate fluid yield (47). Finally, time and technical limitations permitted cannulation of only 2 to 3 ducts per breast, from an estimated total of 6 to 12 (24); uncertainty remains as to whether ductal atypia represents a field effect, involving multiple breast ducts in an affected woman, or is specific to one or a few affected ducts only. With eventual improvements in ductal lavage technology, it is anticipated that the majority of breast ducts in one woman might be lavaged and their cytology compared, which would help to address this question.

The present results show that non–fluid-yielding ducts produce atypical cells in women with an inherited predisposition to develop breast cancer. They suggest that fluid-yielding status is inversely associated with prior cancer and its treatment by chemotherapy (perhaps consistent with an antihormonal mechanism), and that other strategies than ductal lavage may be preferable for cytologic evaluation of postmenopausal women, in whom successful catheterization was less often possible. Future studies of ductal lavage should include evaluation of non–fluid-yielding ducts, and alternate methods, such as random periareolar fine-needle aspiration, for the evaluation of women whose ducts cannot be cannulated, or yield only insufficient cellular material for diagnosis. Longer follow-up of a larger number of patients will be necessary to establish the clinical significance of ductal atypia in women at high inherited risk; we are currently embarked on a prospective, multi-institutional breast cancer screening trial which will address this question.

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Loss of Nucleotide Excision Repair in Brca1-Deficient Murine Mammary Epithelial Cells

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ABSTRACT:

The BRCA1 breast cancer susceptibility gene has been implicated in many cellular processes, yet its specific mechanism of tumor suppression remains unclear. BRCA1 plays a role in several DNA repair pathways including nucleotide excision repair (NER). Here we report enhanced cellular sensitivity to UV irradiation and decreased NER activity in mouse mammary epithelial cells that are genetically deficient for brca1. Loss of brca1 expression also leads to a blunted induction of DNA damage inducible expression of the xeroderma complementation group C gene, but not ddb2, the damaged-DNA binding protein that is mutated in xeroderma complementation group E. These results confirm our earlier findings that BRCA1 is involved in global genomic NER, and affects this process in part through regulation of DNA damage recognition gene expression.

INTRODUCTION:

Inheritance of a mutation in the BRCA1 gene confers a 45-65% average lifetime risk for women of developing breast cancer and an 11-39% average lifetime risk of developing ovarian cancer (1). BRCA1-associated breast cancers have a characteristic phenotype; in general these tumors have a high mitotic index, contain p53 mutations, and do not express estrogen or progesterone receptors (2). Mutations in the p53 tumor suppressor gene are found in 70-80% of breast cancers that occur in women who carry a BRCA1 mutation but only 30% of BRCA1 wildtype breast tumors, implying that loss of p53 function is an important event in the pathogenesis of BRCA1-mutated tumors (2-4). Experiments in mice have shown that homozygous inactivation of brca1$^{\Delta 11/\Delta 11}$ results in embryonic lethality that is partially rescued by inactivation of p53, suggesting that loss of p53 in brca1-deficient cells is necessary for cell viability and may be a required event in the pathogenesis of BRCA1-associated human breast cancers (5, 6).

We have recently shown in human tumor cells that BRCA1 directly affects the nucleotide excision repair (NER) pathway (7, 8). NER is the DNA repair pathway that removes DNA adducts formed by ultraviolet (UV)-irradiation such as cyclobutane pyrimidine dimers (CPDs), and carcinogens including polyaromatic hydrocarbons, tobacco (benzo[a]pyrene-7,8-diol-9,10-epoxide) and endogenous carcinogens (9, 10). NER can be subdivided into two genetically distinct pathways; global genomic repair (GGR) that targets and removes lesions from the whole genome and transcription-coupled repair (TCR) that preferentially removes lesions from the transcribed strand of expressed genes

(11). We have previously shown that loss of expression of the p53 tumor suppressor gene specifically results in deficient GGR of UV-C induced CPDs and several carcinogens, but does not affect TCR of these lesions (9, 12-14). We have found that both p53 and BRCA1 may affect GGR through transcriptional regulation of NER genes involved in the recognition of adducts in genomic DNA including XPC, DDB2 (the genes mutated in xeroderma pigmentosum complementation groups C and E, respectively) and GADD45, a growth arrest and DNA damage inducible gene that may facilitate chromatin unwinding in regions of damaged DNA (12, 15-19). Furthermore, overexpression of BRCA1 can compensate for loss of p53 in maintaining GGR in human cells through upregulation of XPC, DDB2, and GADD45 (7).

In this study, we provide direct genetic support for our earlier results showing that BRCA1 is involved in NER by demonstrating that mouse mammary epithelial cells (MMECs) deficient for p53 undergo a significant reduction of GGR of CPDs when brca1 expression is lost. In addition, these cells are more sensitive to UV-radiation compared to brca1+/+ cells and have a blunted induction of xpc expression in response to DNA damage.

METHODS:

Generation of Brca1$^{+/+}$ and Brca1$^{-/-}$ MMECs.

MMECs were isolated from brca $^{fl/fl}$ mice (20). These mice carry loxP sites flanking exon 11 of the *brca1* gene and develop normally.[5] Brca1$^{fl/fl}$ MMECs were infected with an HPV-16E6 (Neo^{+}) retrovirus to inhibit p53 function and immortalize the cells. Brca1$^{-/-}$ MMECs were generated by deleting exon 11 of *brca1* following transfection with pBabe-Cre (Puro^{+}) retrovirus.

mRNA Expression of Brca1

Brca1 mRNA was measured from total RNA obtained from MMECs using reverse transcription with oligo dT, followed by PCR amplification of brca1 exon 11 sequences (forward primer: 5'-TTCCCTGCTTCCAACACTTCATG, reverse primer: 5'-TCCTCATTCCCACACTGGTGACTC). The transcribed exon 11 of brca1 generated a 322-bp product.

Northern Blotting:

E6 and E6Cre cells were exposed to 10 J/m^2 of UV-C and either harvested immediately or incubated in media and harvested 6 and 24 h later. RNA was isolated using GenElute^{TM} Total RNA mammalian kit (Sigma). For Northern hybridizations, 15 ug of total RNA was separated on a 1% glyoxal gel (Ambion). *Xpc* and *gapdh* mRNA probes

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were generated using a plasmid containing a T7 promoter (GeneStorm^{TM} and Ambion respectively). Radioactivity was detected with a phosphorimager (Bio-Rad). Band density was calculated using Quantity One Software (Bio-Rad).

RT-PCR:

Real-time quantitative PCR reactions was performed on an ABI Prism 7900HT Sequence Detector System (Applied Biosystems, Foster City, CA). For each PCR run, a master mixture was prepared at 4 °C with 1X TaqMan buffer: 5.5 mM $MgCl_2$; 200mM dATP, dCTP and dGTP and 400mM dUTP; 0.01U/ul of AmpErase UNG; 0.025 U/ul of AmpiTaq Gold DNA polymerase (PE Applied Biosystem, Foster City, CA). The *ddb2* and *gapdh* genes were amplified in the presence of 200 nM of *ddb2* or *gapdh* specific primers.

Global Genomic NER Immunoassay:

Repair of CPDs and 6-4 photoproducts from total genomic DNA in the various cell lines at different times following UV irradiation was measured using an immunoslotblot assay, as previously described (7, 19). Monoclonal antibodies specific for either CPDs (1:1000) or 6-4 photoproducts (1:500) were kindly supplied by Toshio Mori (Nara Medical University, Japan). Data from triplicate DNA samples from three different biological experiments were averaged. Statistical analysis of differences in DNA repair curves due to expression of brca1 were performed using the unpaired T-test.

Analysis of Strand-specific DNA Repair:

To determine the rate of removal of CPDs from the transcribed strand of a specific gene

fragment, strand-specific RNA probes were used to evaluate the frequency of CPDs in a 14-kb *Bam*H1 restriction fragment spanning the central region of the mouse *dhfr* gene, as previously described (13, 18). Cells were irradiated with 10 J/m^2 of UV-C, lysed immediately for an initial sample, or incubated for up to 24 hrs to allow photoproduct repair. The frequency of induction and rate of removal of CPDs from transcribed strand and non-transcribed strand of the *dhfr* gene was measured by treating purified *Bam*HI-digested DNA with bacteriophage T4 endonuclease V (generously supplied by R. Stephen Lloyd, University of Texas-Galveston), and then quantifying the reappearance of the full-length restriction fragments in DNA from cells allowed various times to remove the lesions using denaturing electrophoresis and Southern blotting.

Cell Sensitivity Assay:

For assessment of UV sensitivity, E6 and E6Cre cells were plated in 96 well plates at a density of 10^3 cells/well in triplicate, and allowed to attach overnight. The cells were then washed with PBS, and 2 columns each of the plate (16 wells) exposed to UV-C irradiation at doses of 0, 5, 10, 20, and 30 J/m^2. The cells were allowed to incubate at 37°C for 48 hours at which time the media was removed and the cells were fed with fresh media and MTT solution (5 mg/ml in PBS). The MTT formazan crystals were dissolved in 200 ul/well of DMSO and 25 ul/well glycine buffer. Absorbance was recorded at 570 nm and a logarithmic plot of absorbance versus UV-dose was recorded. Cell viability as determined by the MTT assay was expressed as the ratio of the treated cells to that of the untreated controls at each dose. The IC_{50} for each cell line was determined and significance values calculated using an exact Wilcoxon rank sum test for UV-C.

RESULTS:

Studies of the effect of loss of brca1 expression on NER were performed in MMECs allowing for the disruption of the *brca1* gene following transfection with the Cre recombinase in tissue culture. Brca1$^{fl/fl}$ MMECs were infected with HPV-16E6 to inhibit p53 function and the cell lines thus termed E6 (p53 null; brca1+/+) and E6Cre (p53 null; brca1-/-) (Figure 1). We have previously shown that the overall level and rate of GGR of CPDs in murine tissues are decreased compared to humans (18, 21). Therefore, we anticipated that levels of repair would be even lower than what we usually observe in human p53-deficient cells.

Loss of brca1 expression resulted in a decrease in GGR of CPDs 24 hrs after UV-irradiation from 22% in the E6 cells to 12% in the E6Cre cells ($p=0.025$, Figure 2). Both E6 and E6Cre cells repaired nearly 100% of UV-induced 6-4 photoproducts by 24 hrs (data not shown). Analysis of CPD repair within the transcribed strand of the expressed mouse *dhfr* gene did not show any difference between the E6 and E6Cre cells (>70% repair at 24 hours, data not shown), confirming our earlier results showing that brca1 has no effect on transcription-coupled repair of UV-photoproducts (7).

We have previously shown that *DDB2* and *XPC* are p53-regulated genes required for efficient GGR of CPDs in human cells (12, 15, 17). We and others have shown that BRCA1 regulates expression of human *DDB2*, providing a potential mechanism for the effect of murine brca1 on NER (7, 22, 23). Therefore, we evaluated the effect of brca1

on *ddb2* and *xpc* gene expression in MMECs. Loss of brca1 expression caused a transient decrease in *xpc* mRNA induction after exposure to UV-C irradiation in both cell lines. Minimal induction was observed in E6Cre cells, however a 2.6-fold induction of *xpc* was observed at 24 hrs in E6 cells (Figure 3). While we have previously shown in human cells that *XPC* induction after DNA damage is p53-regulated and is involved in the earliest steps of DNA damage recognition (15, 16), the regulation of *xpc* expression in mouse cells has not been previously studied.

Loss of brca1 expression had no effect on *ddb2* gene expression as measured by RT-PCR (data not shown). Expression of *ddb2* was measured from total RNA from E6Cre and E6 cells before and after UV-C irradiation. The mouse *ddb2* promoter lacks the p53 response element identified in human *DDB2*, thereby contributing to its low levels of basal expression and GGR (24). Therefore, this result was not unexpected and confirmed the specificity of the effect of brca1 on *xpc* induction.

To explore the biological consequence of brca1 deficiency in MMECs, we examined cell survival after treatment with UV-C irradiation. As shown in Figure 4, there is a dose-dependent decrease in cellular viability in both brca1$^{+/+}$ and brca1$^{-/-}$ MMECs following exposure to increasing doses of UV-C irradiation. However, loss of brca1 was associated with a 3-fold increase in sensitivity to UV-C relative to that in brca1$^{+/+}$ MMECs ($p=0.029$). Brca1-/- MMECs were not significantly more sensitive to doxorubicin or docetaxel, cytotoxic agents not repaired by the NER pathway (data not shown).

DISCUSSION:

We have previously shown that overexpression of human BRCA1 enhances GGR through transcriptional regulation of NER genes, including *XPC, DDB2*, and *GADD45* (7). Here we confirm our earlier findings that BRCA1 is involved in NER by demonstrating that loss of murine brca1 expression reduces the rate of GGR of CPDs, blunts *xpc* transcriptional induction after UV-C irradiation in MMECs, and significantly increases sensitivity to UV-irradiation. This is the first reported demonstration of the direct effect of genetic loss of brca1 expression on GGR of CPDs.

Loss of brca1 expression resulted in loss of *xpc* induction after UV-irradiation, consistent with our previous results showing that overexpression of BRCA1 protein results in XPC induction in p53-deficient cells (7). Several lines of evidence suggest that BRCA1 may act as a transcriptional activator; BRCA1 has a C-terminal transactivating domain (25-28) that has been found to stimulate transcription from the p21, p53, bax, and GADD45 promoters (29, 30). BRCA1 has been found to complex with RNA polymerase II and has been shown to regulate transcription of GADD45 through interactions with the transcriptional co-activator CtIP and co-repressor ZBRK1 (26, 29, 31, 32). More recently, Cable et al. found that BRCA1 binds to specific DNA sequences in target genes including GADD45, XPD, and XPG via physical association between the US2F family proteins of transcription factors (33). We and others have shown that XPC is required for efficient 6-4 photoproducts and CPD removal, *in vivo* (15). Transcriptional regulation of XPC is partially p53 dependent. However, loss of p53-dependent induction of XPC after DNA damage may be compensated for by BRCA1-transcriptional up-regulation of XPC

and the maintenance of GGR of CPDs (8). Therefore, p53 and BRCA1 appear to orchestrate the regulation of the initial DNA damage recognition steps in GGR.

We also found in human cells that BRCA1 overexpression could induce DDB2 (7). Recent data from our laboratory strongly suggests that DDB2 is the initial DNA damage recognition factor for CPDs and its presence is required for XPC binding to this type of damaged DNA (34). However, loss of brca1 expression had no effect on murine ddb2 gene expression in MMECs. Rodent cells have low basal expression of ddb2 in many, but not all tissues. Mouse ddb2 does not contain a functional p53 response element and consequently transcription of ddb2 is not induced by p53 (24). Consequently, most rodent cells are deficient in GGR of CPDs (18), and show increased GGR when exogenous DDB2 is overexpressed (35).

Our current results in MMECs, and previous findings in human tumor cells (7, 8), suggest that p53 and BRCA1 act independently to regulate GGR, though potentially through similar downstream NER target genes. However, in both these studies, p53 was inactivated through HPVE6 expression, resulting in degradation and loss of detectable p53 protein. It remains formally possible, however, that BRCA1 affects NER through p53 regulation, as suggested by others (22, 23), and further experiments with additional genetic models are necessary to distinguish between these possibilities.

Many lesions in DNA are targeted by NER and the inability to repair these lesions may lead to mutagenesis and breast cancer. Polymorphisms in the XPD and XPG genes are

associated with higher levels of polycyclic aromatic hydrocarbons and CPDs in DNA from breast cancer samples compared to normal controls (35-37). BRCA1 has recently been shown to transcriptionally regulate these genes (38). Loss of heterozygosity of XP genes have also been seen in breast cancer and other solid tumors (38). Therefore, the inability to repair DNA adducts due to a defect in NER may play a role in the development of breast cancer.

There is a growing body of data suggesting that loss of BRCA1 function may play a role in the development of a substantial number of breast cancers. Thirty percent of breast cancers demonstrate promoter hypermethylation of BRCA1 (39). There is data that BRCA1-associated ovarian cancers have a better five year disease-free and overall survival than non-BRCA1-associated ovarian cancers (40). One explanation maybe that the standard treatment of ovarian cancer includes platinum based chemotherapy regimens. Previous studies in human MCF-7 breast cancer cells and mouse ES cells have indicated a role for BRCA1 in determining the cellular sensitivity to cisplatin (41-43), and we have observed a similar increase in cisplatin sensitivity in brca1-/- compared to brca1+/+ MMECs.[5] It is thought that the primary mechanism for the repair of cisplatin DNA lesions is NER (44-45), suggesting that this cisplatin sensitivity is due to BRCA1-associated NER.

To date, cisplatin has not been widely used in the treatment of breast cancer. Our results suggest that breast cancer cells deficient in BRCA1 and p53-dependent NER pathways may be particularly sensitive to the cytotoxic effects of the platinum class of agents, and

that these should be tested as therapies for BRCA1-related cancers. The exploitation of drugs that are targeted by DNA repair pathways in breast cancer may improve patients' treatment response and survival and needs to be expeditiously evaluated.

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FIGURE LEGENDS

Figure 1. mRNA expression of brca1 in brca1+/+ and brca1-/- MMECs.

Loss of exon 11 sequences from brca1 mRNA. Total RNA from MMECs were examined by RT-PCR. An exon 11-specific 392-bp product is formed in $Brca1^{+/+}$ MMECs but not in the $brca1^{-/-}$ MMECs. Actin gene expression was used as control.

Figure 2: Effect of lack of brca1 on GGR in MMECs. GGR of CPDs in p53 null; brca1+/+ E6 and p53 null; brca1-/- E6Cre cells were measured using an immunoslot blot assay. ^{3}H-thymidine labeled cells were exposed to 10 J/m^2 UV-irradiation and collected at the indicated times. Genomic DNA from unirradiated cells was loaded as a control for nonspecific antibody binding. Data from triplicate DNA samples from three different biological experiments were averaged. The solid lines represent repair in the E6 cells with brca1 expression; the hatched lines are from the E6Cre cells with lack of brca1 expression.

Figure 3: Effect of lack of brca1 expression on xpc induction in MMECs. Xpc expression in E6 and E6Cre cells were measured using an RNA probe specific for mouse xpc. E6 and E6Cre cells were exposed to 10 J/m^2 of UV and either harvested immediately or incubated in media and harvested 6 and 24 h later.

Figure 4. Sensitivity of $brca^{-/-}$ MMECs to UV-irradiation. The fraction of viable cells was assessed using the colorimetric MTT assay, as described in the Methods section.

Figure 1

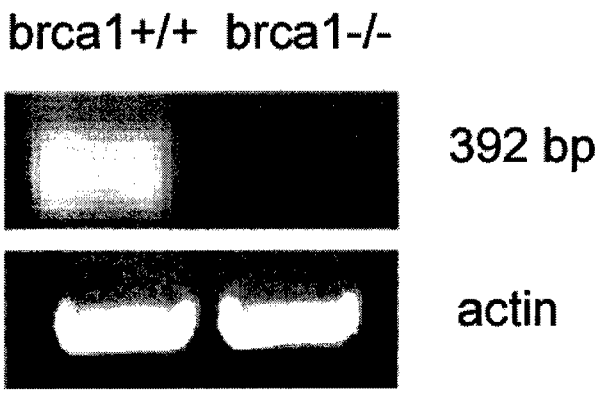


Figure 2

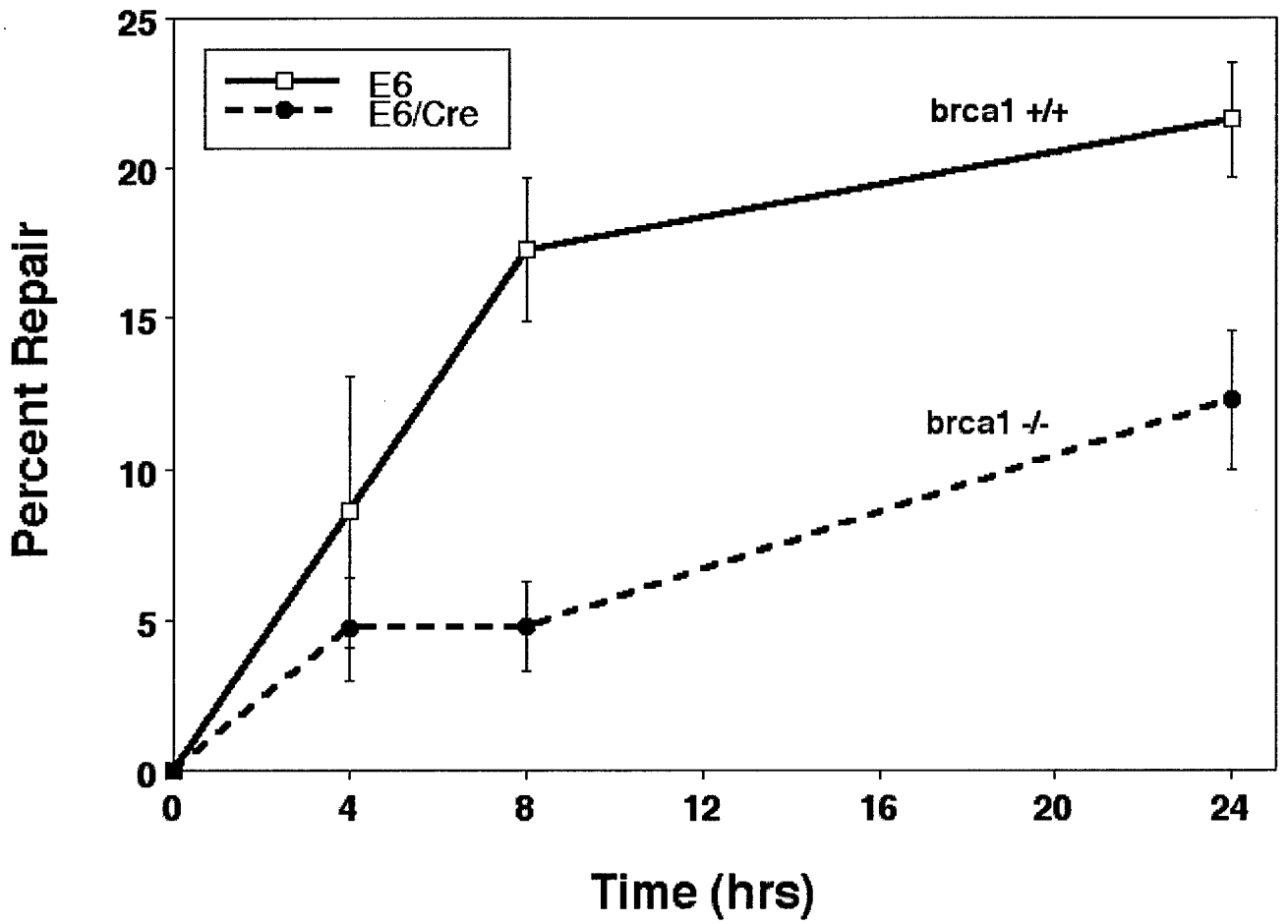
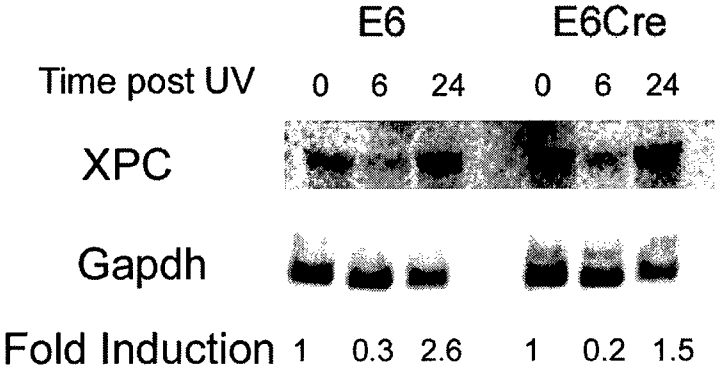

25
20
15
10
5
0
Percent Repair
E6
E6/Cre
brca1 +/+
brca1 -/-
0
4
8
12
16
20
24
Time (hrs)

Figure 3


E6
E6Cre
Time post UV
0 6 24 0 6 24
XPC
Gapdh
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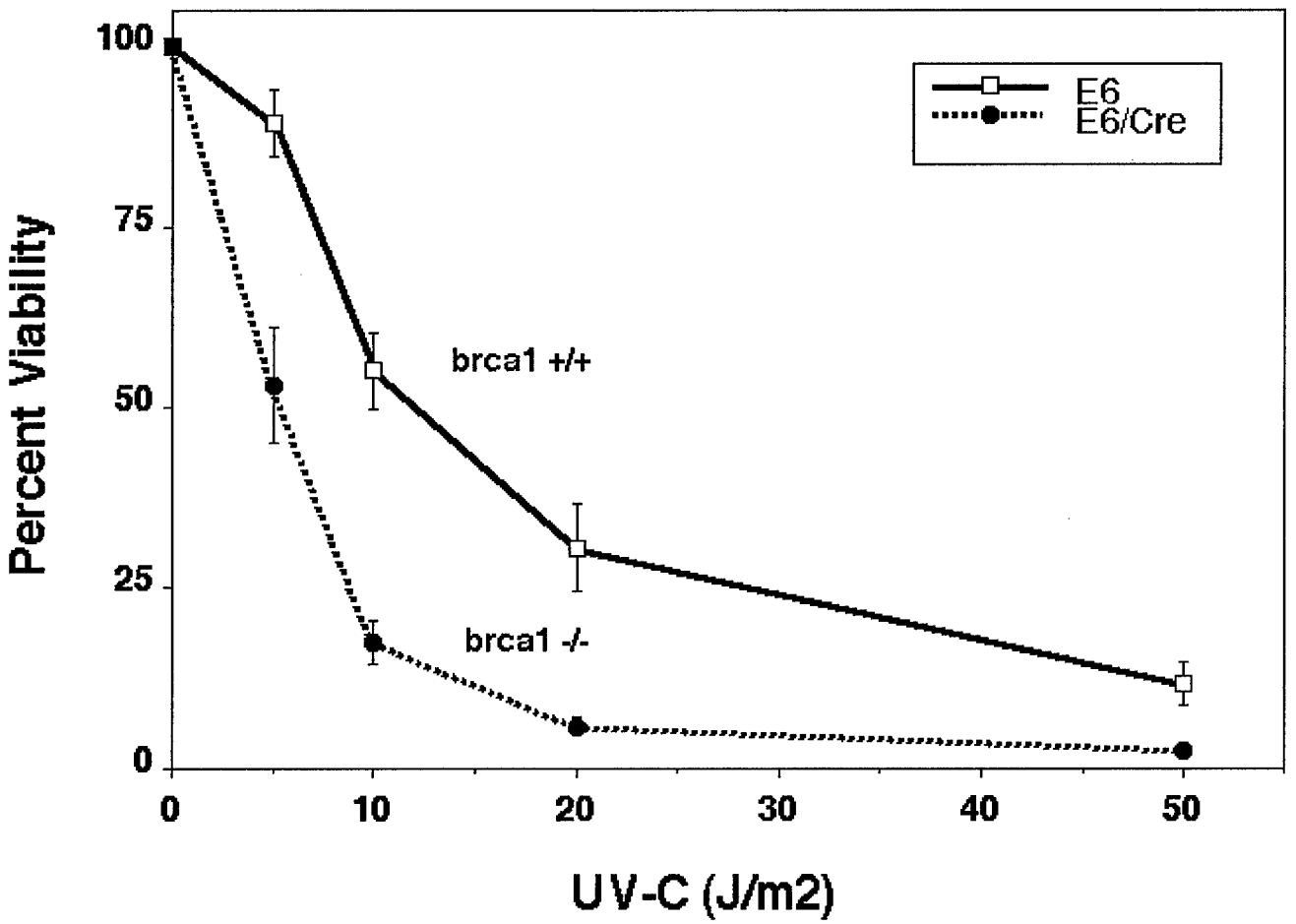
1 0.3 2.6 1 0.2 1.5

Figure 4


100
75
50
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0
Percent Viability
0
10
20
30
40
50
UV-C (J/m2)
E6
E6/Cre
brca1 +/+
brca1 -/-

Research Protocol
(Revised 4/07/2005)

Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer
BC031865

1. Protocol Title. Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer

2. Phase. Not applicable

3. Principal Investigator: James M. Ford, M.D.
Bldg. CCSR Room 1115
269 Campus Dr
Stanford CA 94305-5151
e-mail address: jmf@stanford.edu

4. Location of Study: Stanford University and Stanford Medical Center
Stanford, CA 94305

5. Time required to Complete
Research will be accomplished in three years.

6. Objectives.

Altered nucleotide excision repair (NER) activity may be a common, initial and detectable event leading to genomic instability in human breast epithelial cells, ductal carcinoma in situ or early invasive breast cancers. Our objective is to study NER activity in primary breast epithelial cells and cancer tissues from women at risk for or diagnosed with breast cancer to determine if NER activity can be reliably and practically quantitated from these tissues and cells.

The three specific aims are:

1) Apply a recently developed quantitative local DNA damage immunoflourescent assay to measure NER activity in single cells derived from women at risk for or with breast cancer, from ductal lavage or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.

2) Apply a newly developed flow cytometry based immunoassay to measure NER activity in small populations of cells derived from women at risk for or with breast cancer, from ductal lavage or MRI directed biopsy samples of breast epithelial cells or tumor cells, and from appropriate controls.

3) Analyze cell based NER activity from patients samples with clinical, pathological and genetic information, including BRCA1 and BRCA2 carrier status, pathologic grade and stage, familial risk, and molecular correlates.

7. Study Population

a. Target Populaton
Subjects will be patients who are already participating in either the comprehensive screening protocol (Stanford Protocol # 77716) or the genetic and pathological study (Stanford Protocol #78102). We plan to recruit a total of 100 subjects.

b. Inclusion/Exclusion criteria
Children are excluded from this protocol because they have a low risk of developing breast cancer even if they carry a BRCA1/2 mutation. Samples will be obtained from eligible female subjects between the ages of 21 and 65, who are enrolled in one or both of the two Stanford (non

DOD funded) protocols. Subjects who are enrolled in Protocol 77716, Comprehensive Screening for Women at at High Risk for Breast Cancer, are women between the ages of 21 and 65 who are either a BRCA1 or 2 carrier or have at least a 10% risk of developing breast cancer in the next 10 years. Subjects enrolled in Protocol 78102, Genetic and Pathologfcal Studies of BRCA1/BRCA2: Associated Tumors and Blood Samples, may or may not be at high risk for breast cancer, who are having a breast biopsy prompted by a concerning finding on either MRI, mammogram or clinical breast exam. Women who are not enrolled in either protocol are excluded from this study.

Men are excluded from this protocol because their risk for getting breast cancer is very low.

c. Pregnant subjects
Pregnant subjects will be excluded from this protocol. The effects of ductal lavage is unknown on pregnant subjects.

8. Protocol Design

a. Subject Identification

Patients' identities will be kept as confidential as possible as required by law. Except as required by law, patients will not be identified by name, social security number, address, telephone number, or any other direct personal identifier. Research records may be disclosed outside of Stanford, but in this case, patients will be identified only by a unique code number. Information about the code will be kept in a secure location and access limited to research study personnel.

Tissues and samples will be stored under a unique identifier. All patient information will be stripped from the sample. Subjects' names or other public identifiers will not be included with any data shared with other investigators.

b. Description of the recruitment process. Dr. Ford and Dr. Sharma will enroll patients through the Breast Cancer Genetics Clinic and patients will be enrolled in either one or both of their ongoing protocols (78102 and 77716) and will be consented. Informed consent includes permission to use their samples for research purposes. We are requesting a waiver of consent since subjects have already consented to use of their specimens for research purposes.

c. Description of the Informed Consent process
Specific consent forms for collecting ductal fluids and breast ductal cells and enrollment in the comprehensive screening protocol have been generated and are already in use. Patients sign a consent form at an earlier visit consenting to allow us to use a small portion of their ductal lavage sample for research/banking and complete a separate consent form to obtain tissue samples from breast biopsies. A waiver of informed consent for this protocol is requested because consent is obtained as part of the two Stanford protocols (#77716 and #76102). This protocol meets the criteria for a waiver of consent because 1) the research involves no more than minimal risk to the subjects; and (2) the waiver or alteration will not adversely affect the rights and welfare of the subjects.

d. Subject assignment. Not applicable.

e. Subject Screening Procedures. Not applicable

f. Data Collection Procedures.
This protocol is a lab-based study intended to analyze and evaluate biological specimens, previously collected from other studies, for NER activity. Results of this analysis will be stored in a secure database at the Stanford Cancer Genetics office. Tissues and lavage samples will be stored using a unique identifier. Subjects' names or other public identifiers will not be included

with any data. Lab analysis will be correlated with patient's BRCA 1 or 2 mutation status, pathologic grade and stage of breast cancer is diagnosed, familial risk and molecular correlates.

g. Clinical assessments

As this protocol is lab-based. There will be no clinical assessments.

h. Research Interventions

i. Data Analysis

Lab analysis will be correlated with patient's BRCA 1 or 2 mutation status, pathologic grade and stage if breast cancer if diagnosed, familial risk and molecular correlates.

Statistical Analysis will be conducted in conjunction with Sylvia Plevritis, Ph.D., Assistant Professor of Radiology and Bronislava Sigal, Ph.D. in the Stanford Department of Radiology.

9. Risks/Benefits Assessment

a. Risks

Although every possible effort will be made to maintain confidentiality regarding the handling. sorting, labeling, sharing and securing of the research data, there is always a risk of breach of this confidentiality. Because genetic research raises certain questions, possible risks if there is a breach could include anxiety, other psychological distress, and the possibility of insurance and job discrimination. These risks can change depending on the results of the research and whether investigators might retain the identified samples, e.g. as part of routine care, but not for additional research.

b. Benefits

Data generated by analyzing these specimens for NER activity may very helpful in understanding the multi-step pathway of carcinogenesis of breast cancer in these patients. In addition, the research proposed here may identify more effective chemotherapy for women with BRCA1 and 2 mutations who develop breast cancer. There could be no direct benefits to the subject.

c. Payment There will be no compensation for participation in the study.

10. Reporting of serious or unexpected adverse events.

Unanticipated problems involving risk to subjects or others, serious adverse events related to participating in the study and all subject deaths should be promptly reported by phone (301-619-2165), by email (hsrrb@det.amedd.mil) , or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board (HSRRB). A complete written report should follow the initial notification. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-ZB-QH, 504 Scott Street, Fort Detrick, Maryland 21701-5012.

Meredith Mills who has 9 years of experience in clinical trials will complete adverse event forms. We anticipate very few if any adverse events since this protocol is lab-based. If adverse events occur, they will be reported to the Stanford IRB and our data and safety monitoring board immediately.

A formal data and safety monitoring committee has been created consisting of 2 medical oncology breast cancer specialists, Drs. Frank Stockdale and Bob Carlson and one surgical oncologist, Dr. Stephanie Jeffries. All three physician's are not directly involved in the study and are experts in the area of breast cancer treatment and prevention. All adverse events will be immediately reported to the board. In addition, they will meet on a biannual basis to discuss data and safety aspects of the screening trial. The potential adverse effects of DL are documented extensively in the consent form for the screening protocol. In addition, we have included further potential adverse effect including psychological harm in the form of anxiety to the consent form. All patients will be offered follow-up appointments with our genetic counselors after one round of screening to discuss these issues. If further counseling is needed, patients will be referred out for professional psychological counseling.

11. Description of Protocol Drugs or Devices. Not applicable

12. Disposition of Data. Lab analysis of ductal lavage samples and breast biopsies will be stored in a separate database in Dr. Ford's lab with a unique identifier with no personal identifiers. Lab analysis will be correlated with patient's BRCA 1 or 2 mutation status, pathologic grade and stage if breast cancer is diagnosed, familial risk and molecular correlates. Data will be stored for a minimum of five years after the completion of the project.

13. Modification of Protocol. Any modification to the protocol will be sent to the Stanford IRB as well as the HSRRB. All continuing review reports and final study reports, with associated local IRB documentation will be submitted to the HSRRB for review.

14. Departure from the Protocol. Any departure from the protocol will be sent to the Stanford IRB as well as the HSRRB.

15. Roles and Responsibilities of Study Personnel.

Principal Investigator

James M. Ford, M.D., Principal Investigator (20% effort), is an Assistant Professor of Medicine and Genetics, in the Division of Oncology, at Stanford University School of Medicine, and is the Director of the Program for Applied Cancer Genetics and the Cancer Genetics Clinic. Dr. Ford is an internationally recognized expert in the fields of DNA repair and the molecular genetics of cancer, and has developed both laboratory and clinical research programs focused on these areas of cancer research. He will be responsible for the supervision and direction of all research activities related to the project. He will meet regularly with individual personnel to plan experiments and to review data. He will continue to be active in the laboratory, particularly to oversee the specialized molecular biological assays of DNA repair that he has developed, and to teach these techniques to new students and fellows. Dr. Ford also developed and directs the Stanford Cancer Genetics Clinic, that provides consultation, counseling and genetic testing for individuals with a high-risk for breast cancer due to familial inheritance. It is from this clinic that patients will be recruited for the currently proposed study.

Vandana Sharma, M.D., Ph.D., (75% effort), is currently an oncology fellow trainee in the Division of Oncology at Stanford, and joined Dr. Ford's laboratory in July 2003 to begin research on the molecular genetics of breast cancer. Dr. Sharma received her medical and graduate school training of the University of Chicago, where she studied the transcriptional regulation of the estrogen receptor in breast cancer, and is devoted to pursuing a career in translational breast cancer research. Dr. Sharma will devote all of her research efforts toward this project, and will have less than 20% effort directed at non-research clinical activities during the funding period.

16. Volunteer Registry Database. Not applicable.

17. Medical care for research-related injuries.

All forms of medical diagnosis and treatment -- whether routine or experimental -- involve some risk of injury. In spite of all precautions, a patient might develop medical complications from participating in this study. If such complications arise, the researchers will assist the patient in obtaining appropriate medical treatment but this study does not provide financial assistance for additional medical or other costs.

A-12761
Ford

MCMR-ZB-PH (70-1n1) 21 March 2005

MEMORANDUM FOR RECORD

SUBJECT: Protocol Entitled, "Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer", Submitted by James M. Ford, M.D., Stanford University, Stanford, California, Proposal Log Number BC031865, Award Number W81XWH-04-1-0576, HSRRB Log Number A-12761

1. History and Purpose of Review. This is the first review of this research project submitted by Dr. James M. Ford at Stanford University in California. This project was awarded under the Breast Cancer Research Program COREFAC Award. The project will be conducted at the Stanford University and Stanford Medical Center sites. The Department of Defense (DOD) research project is attached to two larger, in-house studies (non-DOD funded) conducted at Stanford University. To succinctly reflect the research activity funded by the DOD, a stand alone protocol was developed. This protocol is pending local IRB approval. On 18 February 2005, the following documents were submitted by the principal investigator for second level review:

a. A "revised" unapproved DOD stand alone protocol (dated 10 February 2005),

b. An informed consent for protocol #77716 (Comprehensive Screening for Women at High Risk for Breast Cancer),

c. An informed consent for protocol #76102 (Genetic and Pathological Studies of BRCA1/BRCA2: Associated Tumors and Blood Samples),

d. IRB approval letters for two protocols (#77716 and #76102),

e. A continuing review report for protocol #76102 (submitted to the local IRB),

f. A notice from the local IRB to the PI to renew protocol #77716 (dated 08 September 2004),

g. An IRB application from the PI to the local IRB to renew protocol #77716, and

h. A certificate of education training in Human Subjects Protection for Dr. James M. Ford.

2. Background. The overall purpose of this research project is to study Nucleotide Excision Repair (NER) activity in primary breast epithelial cells and cancer tissues from women at risk for or diagnosed with breast cancer to determine if NER activity can be

reliably and practically quantitated from these tissues and cells. In order to reach this conclusion, the investigators plan to do the following:

a. Analyze samples for loss of nucleotide excision repair, obtained from performing ductal lavage on patients participating in an in-house study (protocol #77716), and

b. Obtain breast biopsy samples from patients who undergo breast biopsy in another in-house study (protocol #76102).

3. Status of Regulatory Reviews.

a. Scientific Review. During 20 July to 22 July 2003, the U.S. Army Medical Research and Materiel Command Congressionally Directed Medical Research Programs 2003 (BCRP) reviewed this project proposal. The committee found that the proposed research could have a major impact on risk assessment for breast cancer.

b. Institutional Assurance. Stanford University has a Federal wide assurance number (FWA#00000935), which expires on 10 August 2007.

c. IRB of Record and Level of Risk Assessment. As previously stated, the DOD funded stand alone protocol is pending local IRB review. The level of risk assessment will be provided in the IRB approval letter.

d. Additional Reviews Required. With the exception of the HSRRB review no other reviews are necessary.

4. DoD Unique Requirements.

a. 10 USC 980 – Advanced Informed Consent Requirement. As previously stated, the collection of research samples are obtained through two in-house studies conducted at Stanford University (non-DOD funded). Consent for participating in these clinical trials will be obtained prior to collecting research samples for the DOD protocol.

b. DoD Medical Monitor Requirement. Not applicable.

c. Ombudsman Requirement. Not applicable.

5. Human Subjects Protection Scientist's Assessment. Although, the DOD funded protocol is pending IRB approval, the current research design (lab-based only), appears to be no greater than minimal risk. This assessment will be confirmed after receipt and review of the IRB approved protocol.

6. Requirements.

a. Information & Documents.

(1) Provide an IRB approval letter for the DOD protocol once all revisions have been made and determined adequate. The letter must contain the following information:

- The type of review conducted (i.e., full board, expedited),
- The approval date and approval period (i.e., start date and end date), and
- The continuing review due date (i.e., approval period end date)

The DOD protocol has been submitted to Stanford's IRB and is attached. It takes approximately 6 weeks for approval. We will submit approval letter when we receive notification from the IRB.

(2) Request a waiver of informed consent from your IRB of Record for the DOD protocol because this research appears to be no more than minimal risk and it is not practicable to obtain informed consent from each donor. Whereas, the consents obtained for the in-house protocols (#76102 and #77716) adequately cover the use of and banking biological samples for future research. The IRB approval letter should indicate whether or not a waiver of informed consent has been approved.

See response to #1 above and attached protocol. Protocol was submitted requesting a waiver of consent.

(3) Provide an IRB approval letter extending protocol #76102 since study approval expires on 08 March 2005. (Refer to your IRB approval letter for this study dated 09 March 2004)

Done.

(4) Provide a current consent for protocol #76102, which expires on 08 March 2005.

Done.

(5) Provide a certificate of education for Human Subjects Protection training for Dr. Vandana Sharma.

Done.

(6) Confirm whether Drs. Ford and Sharma are the only investigators approved to conduct this research activity. If other investigators will assist in this research activity for the DOD protocol, please provide for each a current Biosketch and certificate of education for Human Subjects Protection training.

Dr. Ford and Dr. Sharma will be doing all the lab-based research,

b. Revisions to be made to the Protocol.

(1) Confirm the sample size (e.g., the "exact" number of tissue samples to be collected to conduct this research activity).

Done.

(2) Provide the rationale for collecting blood to do genetic testing. For example, will the blood samples be sent to a laboratory to check for alternations in the BRCA1 and BRCA 2 genes?

No genetic testing is done for this protocol. Genetic testing is offered to patients <u>prior</u> to their enrollment in any of the Stanford protocols. We will not be requesting blood from subjects for this protocol.

(3) In section 8(i), provide the rationale for collecting data for analysis of genetic alternations. For example, to determine are specific alternations in BRCA1 and BRCA2 more common in certain patient populations?

We have chosen a high-risk population because these patients are already undergoing ductal lavage and breast biopsy as part of their screening and/or medical care and if significant differences are detected in NER activity in these patients, this data will be important for risk assessment and chemosensitivity prediction on women at risk for or diagnosed with breast cancer. Please refer to the original proposal and the technical abstract for further clarification.

(4) In section 8(i), explain the type of procedure used to conduct the cytologic evaluation. For example, does the procedure entail using a random fine needle aspiration as stated on pages 1, 2, and 6 in the consent form for protocol #77716?

At this point we do not plan to use random fine needle aspiration to obtain samples, although rpFNA is soon going to be offered to women participating in Stanford's protocol 77716. Samples will be obtained via ductal lavage or breast biopsy. When either of these samples are obtained a portion of the sample will be sent to the pathology lab at Stanford Hospital for diagnostic purpose (cytologic evaluation.) The cytologic evaluation is NOT part of the DOD protocol.

(5) Outline how information collected from samples relates to the data analysis plan. Refer to page 3 of the protocol which states, "Half of the sample collected will be for cytologic evaluation and the other half will be collected for analysis of genetic alterations".

When samples are obtained a portion of the sample will be sent the pathology lab at Stanford Hospital for diagnostic purposes. This is NOT part of the DOD protocol.

(6) Explain the process for labeling and storing biological specimens for this research.

When specimens are obtained they will be assigned a unique identifier and samples will be labeled with this unique identifier. Samples will be stripped of any identifiers (i.e. Medical record number, name, address, social security number). Specimens will be stored in Dr. Ford's lab, CCSR Building, 269 Campus Drive, Stanford, CA 94305.

(7) Add language regarding the submission of continuing review reports and final study reports, with associated local IRB documentation, to the HSRRB for review.

Done.

(8) Include a statement that the DOD protocol is a lab based study intended to analyze and evaluate biological specimens previously collected from other studies. Consider incorporating this statement in the section "Protocol Design".

Done.

(9) Rewrite the inclusion and exclusion criteria and the recruitment process in section 7 "Study Population" and in section 8 "Protocol Design" respectively to reflect the research work funded for the DOD protocol. *For example*, state that samples will be obtained from eligible female volunteers, 21 years of age and over (*specify exact age range*), who enrolled in one or both of the two in-house (non DOD funded) protocols. Since recruitment of volunteers is not applicable for this lab based study, please state that samples (i.e., blood and tissue) will be collected from volunteers recruited to participate in two (non DOD funded) in-house studies conducted at Stanford University.

Done.

(10) Add a new sentence in section 8(f) to give the data collection procedures more clarity. For example, data for this research will be obtained from analyzing and evaluating previously collected samples from two in-house studies at Stanford University. In addition, further describe the data collection process for lab results (e.g., genetic testing).

Please refer to the attached the original proposal and technical abstract. There will be no genetic testing done on these samples. The purpose of this study is to attempt to identify altered NER as a central cause of genomic instability relevant to the multi-step process of breast cancer carcinogenesis and progression. Some previously collected data about the subject will be correlated with these lab results, including BRCA 1 or 2 status, family history, breast cancer histology, stage and grade. All this data will be deidentified as well.

(11) Include a statement that breach of confidentiality is a risk in section 8(a).

Done.

(12) Add information about how volunteers could be affected in the event of breach of confidentiality regarding the handling, storing, labeling, sharing, and securing

research data. In addition, describe what security procedures are in place to minimize or manage risks to ensure human subjects protection.

Done.

(13) All protocols should contain language of the HSRRB clause, "Reporting responsibilities for unanticipated problems and serious adverse events" because unanticipated problems can occur in a study that does not require a research and/or clinical intervention. The clause reads , *"Unanticipated problems involving risk to subjects or others, serious adverse events related to participation in the study and all subject deaths should be promptly reported by phone (301-619-2165), by email (hsrrb@det.amedd.mil), or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board (HSRRB). A complete written report should follow the initial notification. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-ZB-QH, 504 Scott Street, Fort Detrick, Maryland 21702-5012".*

Done.

(14) Incorporate a statement in section "Study Population" that samples to be used in this lab based research will be obtained from volunteers enrolled in two in-house protocols (#77716 and #76102).

Done.

(15) Incorporate a statement in sections 8(b) and 8(c) "Description of the Recruitment Process" and "Description of the Informed Consent Process" respectively, that a waiver of informed consent for the DOD funded protocol is requested because consent is obtained as part of two in-house (non-DOD funded) protocols (#77716 and #76102). In addition, explain why the study meets the criteria for a waiver of informed consent.

Done.

(16) Remove any information in the revised DOD funded protocol (dated 10 February 2005) regarding risks to volunteers that are relative to the two in-house (non-DOD funded) protocols (#77716 and #76102).

Done.

(17) Explain how the risks of confidentiality and privacy will be minimized or managed to protect volunteers.

Done.

(18) Remove all statements irrelevant to the lab based research work funded for the DOD protocol. For example, it is appropriate to remove information about performing the ductual lavage procedure, photographing breasts, and stating the effects of MRI on the fetus.

Done.

7. Recommendations.

a. If there is a need to include photographing breasts in the DOD funded protocol, consider modifying the protocol to be more succinct about the procedure. According to my conversation with Ms. Meredith Mills of 04 February 2005 and your letter addressed to me (received on 18 February 2005 through Dr. Katherine Moore via e-mail), photographing the nipple after the ductal lavage was discontinued. The revised DOD protocol now states that the investigators " *may*" photograph the breast nipple after the ductal lavage procedure. In contrast, on page 2 of the consent for protocol #77716, it states that "the investigators *will* photograph your breast nipple after your ductal lavage procedure". If your final decision is to take pictures of the volunteers' nipples/breasts, the consent form for protocol #77716 will suffice. If you decide to not take pictures of the volunteers' nipples/breasts or if this procedure is not applicable to this research activity, please remove this information from the DOD funded protocol.

Removed.

b. Please confirm whether Dr. Vandana Sharma or Dr. Allison Kurian will perform the ductal lavage procedure. The DOD funded protocol states the former (page 5). In contrast, the consent form (page 1) for one of the two in-house (non-DOD funded) protocols (#77716) appears to indicate that Dr. Allison Kurian and/or her associates will do so. If Dr. Sharma (an oncology fellow trainee) is a member of Dr. Kurian's team, please state so in order to resolve this discrepancy.

Dr. Kurian and Dr. Sharma are part of the same clinical oncology team. Both will be present during the ductal lavage procedure, but only Dr. Sharma will be analyzing the samples. Attached is Dr. Kurian's Biosketch and Human Subjects Certification. No part of Dr. Kurian's salary is funded by the DOD.

c. In section 7(b) on page 1 of the protocol, consider rewriting the last sentence, "This excludes children under the age of 21." Legally, individuals 18 - 21 years of age are considered adults instead of "children".

Done.

d. Consider including background information regarding similar research conducted in the protocol.

Please see technical abstract (attached) and original proposal.

e. Consider defining scientific terms (i.e., nucleotide excision repair, genomic instability, BRCA1, and BRCA2) in the protocol.

Please see original proposal.

8. Observations.

a. Please refer to the U.S. Army Medical Research and Material Command website: https://mrmc.detrick.army.mil/crprcqhspd.asp under Medical Research and Development, Regulatory Compliance and Quality, Human Subjects protection for additional information that may be helpful in preparing your documents. This website provides protocol development guidelines, a sample consent form document, and other reference materials.

Jo A. Collins, M.S.A.
Human Subjects Protection Scientist
AMDEX Corporation

X-Sieve: CMU Sieve 2.2
Subject: A-12761 Approval Memo, Award No. W81XWH-04-1-0576
Date: Mon, 30 May 2005 22:18:58 -0400
Thread-Topic: A-12761 Approval Memo, Award No. W81XWH-04-1-0576
Thread-Index: AcVlhxpN9vTGcEEPQxqgVZ5Ci+j04w==
From: "Duchesneau, Caryn L Ms USAMRMC" <caryn.duchesneau@us.army.mil>
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Cc: "Moore, Katherine H Dr USAMRMC" <katherine.moore@us.army.mil>,
"McAllister, Pat M Ms USAMRAA" <pat.mcallister@us.army.mil>,
"Brosch, Laura R COL USAMRMC" <laura.brosch@us.army.mil>,
"Duchesneau, Caryn L Ms USAMRMC" <caryn.duchesneau@us.army.mil>,
"Bennett, Jodi H Ms USAMRMC" <jodi.bennett@us.army.mil>,
"Pascal, Louise M Ms AMDEX" <louise.pascal1@us.army.mil>,
"Collins, Jo A Ms AMDEX" <Jo.Collins@DET.AMEDD.ARMY.MIL>

Protocol, "Loss of Nucleotide Excision Repair as a Source of Genomic Instability in Breast Cancer", Submitted by James M. Ford, M.D., Stanford University, Stanford, California, Local IRB Protocol No. 96042, Proposal Log No. BC031865, Award No. W81XWH-04-1-0576, HSRRB Log No. A-12761

1. The subject protocol and supportive materials received 18 February 2005 through 26 May 2005 have been reviewed and found to comply with applicable human subjects protection regulations. There are no outstanding human subjects protection issues to be resolved.

2. The subject protocol is no greater than minimal risk and is approved for the use of human anatomical substances and associated data.

3. A waiver of consent has been granted for this study in accordance with 32 CFR 219.116(d): (1) the research involves no greater than minimal risk to subjects, (2) the waiver or alternation will not adversely affect the rights and welfare of the subjects, (3) the research could not practicably be carried out without the waiver of alteration, and (4) there is no need to provide the subjects with additional pertinent information from this study.

4. In accordance with 32 CFR 219, a continuing review report must be submitted to the local IRB at least annually. A copy of the continuing review report, approved by the IRB of Record should be submitted to the U.S. Army Medical Research and Materiel Command, Office of Research Protections, 504 Scott Street, Fort Detrick, Frederick, Maryland 21702-5012 upon receipt of approval by the IRB of Record. It appears that the continuing review report is due on 16 May 2006.

5. Any protocol modifications (including but not limited to changes in the principal investigator, inclusion/exclusion criteria, number of specimens to be used, study site, or procedures) must be submitted as a written amendment for the HSRRB review and approval before implementing the changes.

6. Submission of the Volunteer Registry Data sheet is not required.

7. When available, provide a copy of the final study report to the HSRRB.

8. The point of contact for this action is Ms. Jo A. Collins, Human Subjects Protection Scientist, at (301) 619-2380.

Caryn L. Duchesneau, CIP

Vice Chair, Human Subjects

Research Review Board

Note: The official signed copy of this approval is housed with the protocol file at the Office of Research Protections, 504 Scott Street, Fort Detrick, MD 21702. Signed copies will be provided upon request.