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**14. ABSTRACT**

Germline mutations in the BRCA1 and BRCA2 tumor suppressor genes are among the strongest predictors of breast cancer known. Yet their true contribution to breast cancer risk has never been characterized at the molecular level. In particular, mutations that lie outside of the exons sequenced during conventional screening have never been sought. Such hypothetical mutations could include those affecting transcription, mRNA splicing/processing, or mRNA processing. We have devised an assay for identifying such potential null mutations based on analysis of retention of heterozygosity of exonic SNPs in the cDNA pool. Thus far, such mutations do not occur at a frequency comparable to conventional mutations. We have further determined that, in roughly one year, we will have recruited enough patient samples with sufficiently high likelihood of carrying mutations (by BRCAPRO scores) to determine whether such mutations occur at a small percent of the frequency of conventional mutations.

**15. SUBJECT TERMS**
BRCA1, BRCA2, breast cancer risk, splicing, nonsense-mediated decay, nonsense-mediated alternate splicing, premature stop codons, functional null alleles, alternate splice variants, null mutations

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## INTRODUCTION:

Germline mutations in the tumor suppressor genes *BRCA1* and *BRCA2* are among the strongest predictors of breast cancer, yet their true contribution to population risk has not been explored at the molecular level. The majority of individuals predicted to carry mutations in *BRCA1* or *BRCA2* because of family history of disease are not found to carry mutations [1]. Additionally, some mutations that map to the *BRCA1* locus are not associated with molecular lesions within the *BRCA1* gene [2]. These observations suggest that some *BRCA1* and /or *BRCA2* mutations may not be detected by conventional direct sequencing of exons. Other types of mutations could include promoter mutations or defects in sites required for correct mRNA splicing/processing [3] (Fig. 1). Such mutations could even include some currently detected and categorized as "variants of uncertain clinical significance." To explore such potential categories of mutation we have developed an assay to identify functional gene defects that result in allele-specific failure in mRNA accumulation. Conversely, the levels of penetrance associated with truncating *BRCA1* and *BRCA2* mutations are highly variable, even though the RNAs transcribed from these genes are predicted to be degraded by nonsense-mediated mRNA decay (NMD). Though NMD is a general phenomenon, it has been shown specifically to regulate *BRCA1* and *BRCA2* RNAs with premature stop codons[4, 5]. One possible explanation may be inefficient NMD for some alleles, or potential use nonsense-associated altered splicing (NAS) [6] resulting in partially functional splice variants. To address these possibilities, we have begun to perform complete *BRCA2* cDNA exon scans from breast cancer and normal cell lines in the presence of inhibitors of NMD as well as endogenous *BRCA2* mutations.

## BODY:

### Task 1. Identify allele-specific *BRCA1*/2 mRNA-reduction phenotypes in *BRCA1* and *BRCA2* mRNA null phenotypes in 324 Cancer Risk Clinic patient samples.

To identify mRNA reduction phenotypes in *BRCA1* or *BRCA2*, we determine whether heterozygous exonic single nucleotide polymorphisms (SNPs) also appear heterozygous in the corresponding cDNA. Heterozygosity will not be seen in the cDNA when one allele carries a mutation affecting mRNA synthesis, processing, or stability. We have selected a panel of exonic SNPs (seven in *BRCA1* and 13 in *BRCA2*, see Table 1) and used these to screen genomic DNA from breast cancer lymphoblastoid cell lines (LCLs). 62/66 (94%) of samples were heterozygous for at least one exonic SNP and could thus be assayed for allele-specific failure of mRNA accumulation. We developed panels of primers for genotyping SNPs by direct sequencing and for amplifying/sequencing corresponding cDNA fragments.

To test the validity of the assay, we grew a commercially available LCL containing a known *BRCA1* truncating mutation and a heterozygous exonic SNP on a separate exon. We have shown the SNP is clearly heterozygous in the genomic DNA but this heterozygosity is not reflected in the cDNA, due to NMD of the mRNA carrying the premature stop codon (Fig. 2). This analysis was applied to 19 patient samples that already tested negative for *BRCA1*/2 mutations by conventional screening and were also

heterozygous for at least one exonic SNP in *BRCA1* or *BRCA2*. In most cases, both *BRCA1* and *BRCA2* were eligible for analysis. In all cases, the heterozygosity of genomic SNPs was reflected in the cDNA. This indicates that none of the patients analyzed carried deleterious *BRCA1*/2 mutations resulting in basal, allele-specific failure of mRNA accumulation. It remains possible that such mutations could exist if the phenotype is expressed in a tissue-specific manner.

Or results came from a combination of commercially available LCLs and cell lines from the University of Chicago Cancer Risk Clinic (CRC). As a basis of comparison, we determined the likelihood of identifying a conventional *BRCA1* or *BRCA2* mutation in this cohort using BRCAPRO, a scoring mechanism that determines the likelihood of carrying a mutation based on personal and family cancer history. Information required for BRCAPRO scoring was unavailable for the commercial cell lines, but could be determined for the samples from the CRC. The average score per patient was 11.3, suggesting there was an 11.3% likelihood of identifying a *BRCA1* or *BRCA2* mutation per sample by conventional direct sequencing methods. Binomial distribution suggests there was a 90% likelihood of detecting at least one conventional mutation in a similar population that had not already undergone *BRCA1*/2 mutation testing, but only a 35% likelihood of detecting a mutation from a hypothetical category occurring at 20% the frequency of a conventionally detected mutation. Thus, potential functional null mutations with a failed mRNA accumulation phenotype could still exist at a frequency somewhat lower than conventional mutations.

Management of large numbers of LCLs has proven more difficult than anticipated, owing to the limited incubator space in a crowded shared facility. We have therefore decided to minimize the labor by suspending this investigation until the CRC has accrued LCLs from a significant number patients with high BRCAPRO scores. At the rate of accrual of LCLs from patients with known BRCAPRO scores, we anticipate waiting one year would allow us to maximize the likelihood of identifying any example of the hypothetical class of *BRCA1*/2 mutations.

**Task 2. Identify alternate splice variants of *BRCA2*.**

To identify splice variants in *BRCA2* we designed a series of primers to perform exon scanning of *BRCA2* cDNA. These will be used to amplify and sequence cDNA fragments small enough to detect predicted size differences associated with exon-skipping. These primers have been tested on currently available RNAs and conditions have been established for their successful use in both RT-PCR and sequencing reactions. While numerous RT-PCR products from these products could potentially represent *BRCA2* splice variants, the few we have examined thus far by sequencing have been artifacts unrelated to *BRCA2*. We will perform exon scanning on *BRCA2* mRNA prepared from MCF7 (a breast cancer cell line), 184A1 (a non-cancerous breast cell line), and two lymphoblastoid cell lines from unaffected individuals. These cells are growing now.

**Task 3: Identify constitutive patterns of *BRCA1*/2 splice variants in patient samples, identify possible altered patterns in potential germ-line mutation carriers.**

To determine whether NAS may be a general feature of *BRCA2* splice regulation, we will repeat the exon scanning analysis using mRNA from four lymphoblastoid cell lines carrying *BRCA2* mutations 5578delAA, 852delC, 6174delT and S1955X. These cell lines have been made available to us, and we are growing them now.

**Task 4: Identify potential disease-specific alternate splice variants of *BRCA1*/2 in normal breast and breast cancer cell lines.**

See Task 2.

## KEY RESEARCH ACCOMPLISHMENTS:

• We have developed an assay for identifying functional null alleles of any germline mutation that results in allele-specific failure of mRNA accumulation. Such mutations could occur in promoter regions or any sequence required for correct splicing/mRNA processing/stability. Any of these categories of mutation could result in an easily detected functional null mutation, but would not necessarily be detected by conventional mutation screening.

• To clarify the true contribution of germline *BRCA1* and *BRCA2* mutations to breast cancer risk, we have performed the first search for numerous previously unexplored categories of functionally null mutations that would not have been detected by conventional screening methods. So far, these putative mutation categories collectively do not contribute to population risk at a frequency that approaches that of conventional mutations.

## REPORTABLE OUTCOMES:

1. Fackenthal JD, Burrill DR, and Yoshimatsu T. *BRCA1* and *BRCA2* post-transcriptional regulation and cancer risk. (2008) Poster presentation at the Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting, Baltimore, MD.

2. Fackenthal JD and Godley LA. (in preparation) Aberrant RNA splicing and its functional consequences in cancer cells.

## CONCLUSION:

Tumor suppressor mutations associated with disease risk can occur anywhere within a gene's structure, including the promoter, intronic and exonic sequences required for correct splicing, and transcribed sequences required for other features of mRNA processing and stability. Mutations in all such sequences have been identified on many genes associated with disease risk [3, 7-24]. Yet commercial screening for germline mutations in genes like *BRCA1* and *BRCA2* focus almost entirely sequence analysis of exons and short intronic sequences at intron/exon boundaries. The recent discovery of *BRCA1* and *BRCA2* gene inactivating large scale genomic rearrangements validates the

notion that previously unexplored categories of mutation contribute to cancer risk [25-29]. These rearrangements, however, do not account for all the previously suspected but undetected. We have developed an assay for identifying several categories of functionally null mutations in *BRCA1* and *BRCA2* that can easily be adapted to any tumor suppressor gene. This is especially important for genes like *BRCA1* and *BRCA2* for which there is no protein-based functional assay and, consequently, no way of clinically testing the significance of unclassified variants. Happily for patients seeking genetic testing for *BRCA1*/*BRCA2* mutations, we have shown that such previously unexplored categories of potential mutation are unlikely to contribute significantly to breast cancer risk. Results were identical in one breast cancer cell line, one non-cancer breast cell line, and two lymphoblastoid cell lines.

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Modulation of soluble CD44 concentrations by hormone and anti-hormone treatment in gynecological tumor cell lines. EMBO Journal. 2001;20(14):3821-30.

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## APPENDICES:

See the attached Era of Hope meeting abstract.

Era of Hope Abstract 2008

June 25-28, Baltimore, MD

*BRCA1* and *BRCA2* post-transcriptional regulation and cancer risk

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Inherited mutations in the tumor suppressor genes *BRCA1* and *BRCA2* are associated with very high lifetime risks of breast cancer. *BRCA1* is especially associated with aggressive, "basal-like" breast tumors that typically test negative for estrogen and progesterone receptors and show no *HER2/neu* amplification ("triple negative" phenotype). Yet the majority of individuals considered likely to carry familial breast cancer susceptibility test negative for *BRCA1/2* mutations. While other risk-associated genes likely account for many of these cases, the true contribution of *BRCA1/2*-associated mutations to inherited breast cancer risk is not known. Numerous functionally unclassified variants are routinely observed during mutation testing, and several potential categories of deleterious mutations might be missed by routine testing methods. In this study we are developing a method for identifying the presence of many potential categories of functional null alleles by phenotype, taking advantage of exonic single nucleotide polymorphisms (SNPs) to track allele-specific defects in mRNA accumulation. We demonstrate that mutations resulting in premature stop codons are subject to nonsense-mediated mRNA decay (NMD) that can be detected with automated sequencing.

One potential category of truncating mutation is cis-acting splicing mutations that could occur at virtually any position within an intron or exon without being recognized as deleterious. To understand better the effects of potential mutations that could affect splicing patterns, it is important to characterize the variety of splice variants produced by the *BRCA1/2* genes. Many *BRCA1* splice variants and alternate protein products have been described, but few *BRCA2* splice variants have been characterized functionally. In this study we systematically scan the *BRCA2* mRNA exons by RT-PCR to identify exon-skipping products characteristic of alternate splice variants. Surprisingly, we find few potential splice variants in lymphoblastoid cell lines, normal breast epithelial cells, and breast tumor cells, even though *BRCA2* contains 27 exons, far more than many genes with numerous alternate splice variants. However, we do see preliminary evidence for a *BRCA2* splice variant that skips exon 11, which is predicted to retain the translational reading frame of the full-length *BRCA2* mRNA. This variant could represent a previously uncharacterized *BRCA2*-encoded protein product.

## SUPPORTING DATA:

See the attached PowerPoint file "Progress Report 08 data.ppt" for figures and table.

**Figure Legends:**

Fig. 1. Hypothetical gene showing positions of mutations that could result in an mRNA-null phenotype. Such mutations might not be recognized or even detected by conventional sequencing of exons and intron/exon boundaries. Mutations could fall within enhancer elements (E) that could lie any distance upstream or downstream of the first exon, promoter regions (P) that might not be recognizable, regions in the 5' untranslated mRNA sequence (5) required for mRNA processing and stability, sequences required for correct splicing (S) that could lie near intron/exon boundaries, deep within introns, or well within exons, or regions in the 3' untranslated sequence (3) required for transcription termination in the primary transcript, and processing/stability in mature messages.

Fig. 2. A *BRCA1* variant with a premature stop codon results in NMD, seen as allele-specific failure in mRNA accumulation. A. Genomic *BRCA1* sequence surrounding the heterozygous exonic polymorphism 2430 T>C. The T allele is in phase with the E1250X mutation. B. cDNA sequence of the same region from the same patient, showing clear failure of accumulation of mRNA from mutant allele.

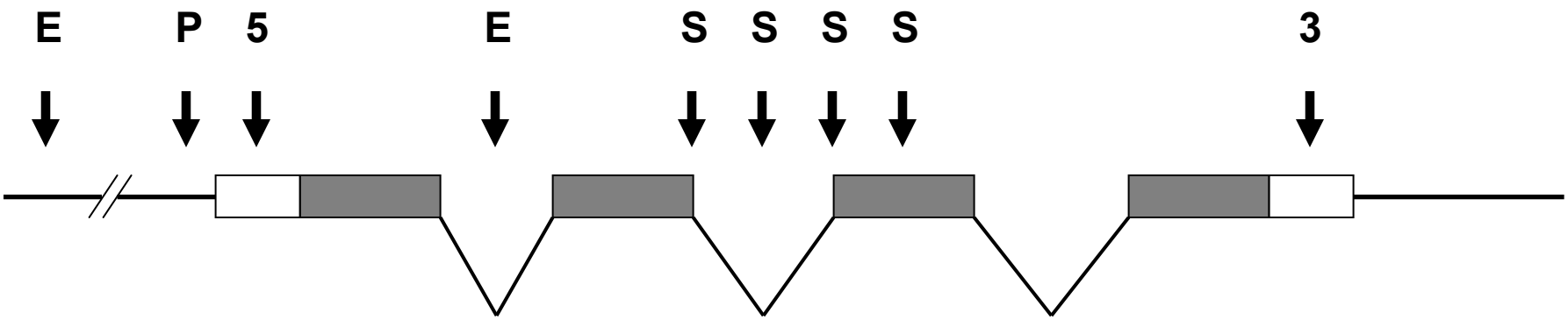


Figure 1.

A


QuickTime™ and a
decompressor
are needed to see this picture.

B

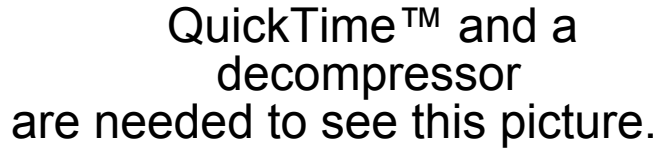
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Figure 2.

| Exonic Location | SNP name from dbSNP | cDNA location | Expected Het. Frequencies | Experimental Het. Frequencies |
|---|---|---|---|---|
| BRCA1 | | | | |
| exon 11e | rs1799949 | 2201 | 0.39 | 0.289 |
| exon 11f | rs16940 | 2430 | 0.38 | 0.339 |
| exon 11g | rs799917 | 2731 | 0.412 | 0.452 |
| exon 11i | rs16941 | 3232 | 0.38 | 0.339 |
| exon 11j | rs16942 | 3667 | 0.39 | 0.317 |
| exon 13 | rs1060915 | 4427 | 0.374 | 0.34 |
| exon 16 | rs1799966 | 4956 | 0.392 | 0.327 |
| BRCA2 | | | | |
| exon 2 | rs1799943 | 203 | 0.412 | 0.175 |
| exon 10a | rs766173 | 1093 | 0.069 | 0.125 |
| exon 10b | rs144848 | 1342 | 0.406 | 0.333 |
| exon 10c | rs1801439 | 1593 | 0.083 | 0.1 |
| exon 10e | rs1046984 | 2024 | 0.241 | 0.1 |
| exon 11.1 | rs1801499 | 2457 | 0.111 | 0.061 |
| exon 11.3 | rs11799944 | 3199 | 0.078 | 0.1 |
| exon 11.4 | rs1801406 | 3624 | 0.4 | 0.352 |
| exon 11.5 | rs543304 | 4035 | 0.273 | 0.298 |
| exon 11.11 | rs4987117 | 5972 | 0.079 | 0 |
| exon 14 | rs199955 | 7470 | 0.33 | 0.262 |
| exon 27b | rs1801426 | 10462 | 0.088 | 0.089 |
| exon 27b | rs115869 | 10590 | 0.235 | ND |

Table 1. Exonic SNPs used to monitor allele-specific failures in mRNA accumulation. ND, not determined.