Acknowledgments

I heartily acknowledge Dr. Jeff Griffith, my advisor and dissertation chair, for the continual encouragement, training and support during the years I spent in his lab. I could not have done this without his guidance and direction.

Special thanks to my committee members, Dr. Steve Belinsky, Dr. Frank Gilliland, Dr. Robert Moyzis, Dr. John Omdahl and Dr. Tom Williams, for their time and valuable input to this study.

I wish to acknowledge the Biomedical Sciences program and the Department of Biochemistry and Molecular Biology of the University of New Mexico and Los Alamos National Laboratory, Life Sciences Division for funding my research. Also a special thanks to the New Mexico Women's Health Study and the New Mexico Tumor Registry for providing the samples used in this investigation.

For all of their assistance during this project, I thank Chris Stidely for her help with data analysis and interpretation of the results; Nancy Joste for her help with the tumor block pathology; Marilyn Fore for all her work on the DNA extraction procedure; and Myrna Jones for the instruction on automated sequencing.

Thank you to my fellow graduate students and lab members, for all of their support, assistance, encouragement and general comic relief.

A special thanks to my wife, for her unfailing love and superb proofreading skills, as well as my family, for their love, inspiration, encouragement and support.

Sequence Variations in the Breast Cancer Gene BRCA1 in a New Mexico Hispanic Population

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B.S., Microbiology with Chemistry minor, University of Oklahoma, 1993
Ph.D., Biomedical Sciences, University of New Mexico, 2000

ABSTRACT

Breast cancer is the most common malignancy in women. Breast cancer incidence and mortality rates vary between social and ethnic groups. This variation is also apparent within the three main racial groups of New Mexico, with the non-Hispanic whites having the highest rate of breast cancer, followed by Hispanics and then Native Americans. Recently, *BRCA1* was identified as a tumor suppressor gene involved in familial and early onset breast/ovarian cancer. BRCA1 appears to be involved in DNA repair, transcription and replication, as well as cell cycle control and apoptosis. With hundreds of reports on *BRCA1* sequence variants involving many different ethnic groups, it is clear that *BRCA1* mutations differ between ethnic groups in type and frequency. The three main populations of New Mexico offer a unique opportunity to study *BRCA1* sequence variations in different racial groups that live in the same geographic area. Reports from other locations indicate that Hispanics in general have a lower frequency of familial breast cancer than Caucasians. A reduction of *BRCA1* mutation frequencies in New Mexico Hispanics could contribute to the observed breast cancer rates. To test this hypothesis, blood samples obtained by the New Mexico Women's Health Study were analyzed using various methods to search *BRCA1* exons 2, 5, 11, 13, 16, 18, 20, 21 and 24 for sequence variations. No mutations were found in any of these exons, which comprise 93% of the reported mutations. Of the nine polymorphisms identified, a novel association was observed with the two polymorphisms 4427T/C and S1613G and women who were diagnosed with breast cancer before age 45. Women with the T/C-A/G genotype are diagnosed 7.8 years earlier, and the C/C-G/G genotype 10.5 years earlier, than those with the T/T-A/A genotype (p= 0.036). The absence of *BRCA1* mutations indicates that the difference in breast cancer rates between the New Mexico Caucasians and Hispanics is most likely influenced by both genetic and environmental factors. While this particular population is not likely to benefit from routine screening of this gene, it is clear that a woman's racial background needs to be considered when genetic testing for *BRCA1* is undertaken.

Introduction

Breast cancer is an ancient disease. Egyptians described breast tumors in 3,000 B.C., followed by the accounts of Greek and Roman physicians including Hippocrates and Cornelius Celsus. Various reports of breast cancer are found throughout writings from the Middle Ages as well, indicating that this cancer has been a continuous affliction of women in western civilizations (Donegan, 1995). Now, almost 5,000 years after the Egyptians described it, some of the causes of breast cancer are beginning to be understood, though any practical means of total prevention or cure are not in sight. The incidence rates of new breast cancer cases continue without significant decrease, though extensive screening and early detection programs have had some impact on the mortality rate. New insights and approaches are needed to address this enduring disease.

Breast cancer incidence and mortality

Breast cancer is the most common malignancy in women. In 1998 the American Cancer Society (ACS) estimated that approximately 178,700 new cases of invasive breast cancer would be diagnosed that year and that 43,500 women would die as a result. The ACS also estimated that approximately one out of every eight women in the United States that lived beyond 85 years would develop breast cancer. This corresponds to a cumulative risk of 12.6% by age 85 for Americans, as reported by Szabo and King (1995). The incidence and mortality of breast cancer increases as women age, with 76.8% of newly diagnosed cases being over the age of 50. Breast cancer is rare in women under 30 (one case per 100,000), however the rate climbs to 25 cases for women ages 30-34, 121.7 for women 40-44 and 244.8 for women 50-54 (ACS, 1998).

The incidence of breast cancer in the United States has been increasing steadily until recently. Between 1940 and 1982 the annual rate of increase was 1% per year, which increased to 4% during 1982-1987, then leveled off to essentially zero increase from 1990 to 1994. The ACS speculates that the steady increase in breast cancer incidence rates since 1940 may have been due to the rising prevalence of risk factors associated with breast cancer, such as modern reproductive patterns, exposure to pesticides or other toxic chemicals and ionizing radiation. The increase in rates during the 1980s, followed by stabilization in the 1990s is believed to be related to the increased use of breast cancer screening methods. The ACS has reported that earlier detection of breast cancer via increased breast cancer screening is the main factor in the decline of breast cancer mortality rates, which have dropped 5.6% since 1990, after remaining relatively stable since 1950.

Various risk factors for the development of breast cancer have been identified, such as gender, age, environment and genetic factors (Herman, 1996). Gender is the primary risk factor, in that the disease is overwhelmingly found in women. Increasing age has been shown to be a key risk factor as well, with dramatic increases in breast cancer rates after age 45. A personal history of breast cancer is indicative of future breast cancer development. Women whose first breast cancer was diagnosed before age 50 have significant risk of having a second primary breast cancer. Any family history of breast cancer is a risk factor, though only a history of breast cancer in a first- degree relative is associated with a significant increase, indicating a heritable genetic component. Another factor is a personal history of benign breast disease, primarily women with diagnoses of atypical hyperplasia.

Factors that increase a woman's lifetime exposure to female hormones, such as early age at menarche, late age at menopause, late age at first live birth and low parity also contribute to an increased risk of breast cancer. These factors are often indicative of socioeconomic status as well. For example, women of higher formal education are likely to have their first child later and have fewer children (Herman, 1996).

Breast cancer incidence and mortality rates vary between social and ethnic groups (Figure 1). Between 1988 and 1992, white, African-American and Hawaiian women in the United States had the highest rates of incidence, with 111.8, 95.4 and 105.6 per 100,000, respectively. The lowest rates were found in Korean (28.5 per 100,000) and Native American (31.6) women, with mortality rates following the same distribution between these groups (ACS, 1997).

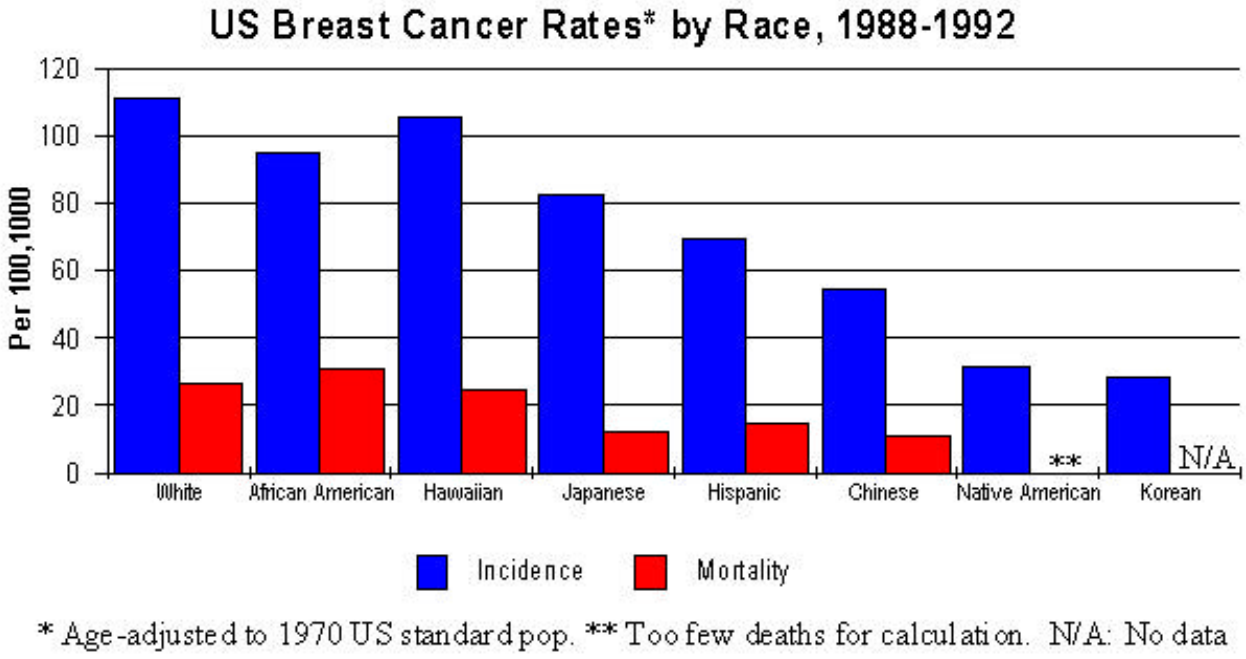


Figure 1

This ethnic variation is also apparent within the three main racial groups of New Mexico: non-Hispanic white, Hispanic and Native American (Figure 2). Breast cancer incidence and mortality rates for non-Hispanic white New Mexican women are comparable to other white women in the United States. They also have the highest breast cancer rate in New Mexico (97.3 per 100,000). New Mexico Hispanic women have a lower breast cancer incidence rate (70.9 per 100,000), and Native Americans have the lowest, with 20.9 per 100,000. While the Hispanic rate is somewhat lower than that of non-Hispanic whites, it has risen significantly since 1969. Eidson *et al.* (1994) reported that the incidence rate for New Mexico Hispanics increased by 56% from 1969 to 1987, while breast cancer mortality rates increased by 40% over the same time period. In contrast, breast cancer incidence rates for the

Native American population increased by 9%, and the non-Hispanic white by 20% (New Mexico Tumor Registry, 1997). This marked increase is probably a reflection of environmental and genetic factors, as well as from increased participation in breast screening programs.

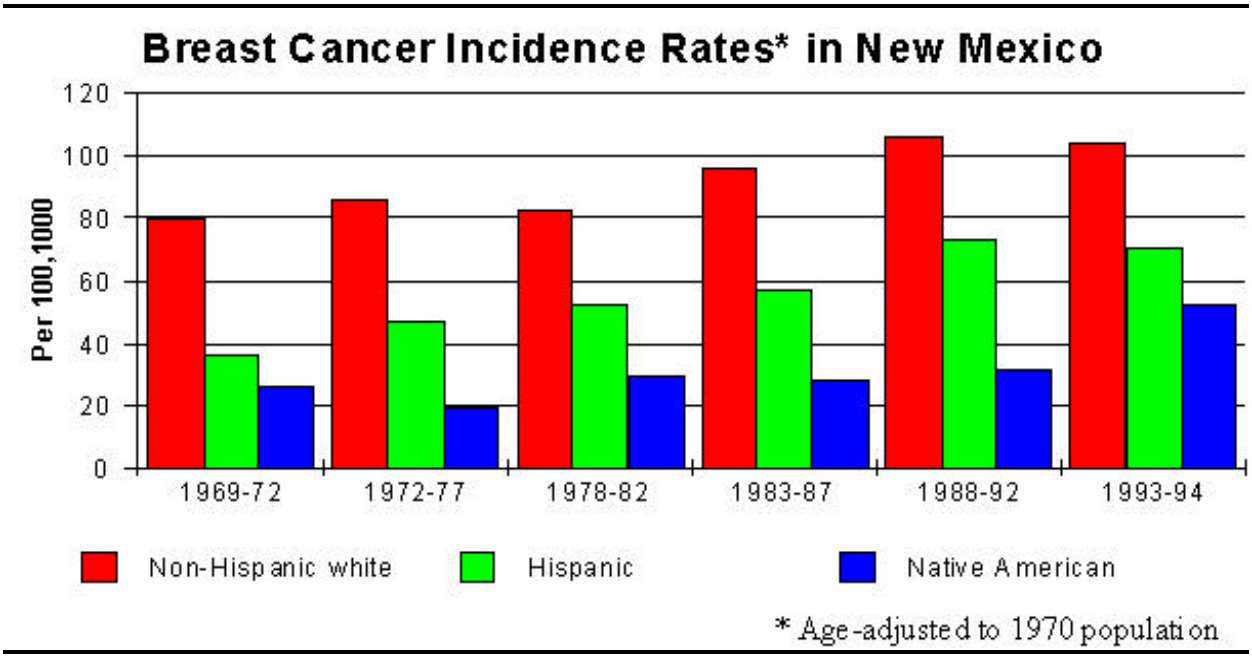


Figure 2

Cancer initiation and progression

While all of the details pertaining to the cause and mechanism of breast cancer development are not known, some key points and underlying principles have been established. Maintenance of the ducts of the mammary gland depend on the proliferative activity of stem cells, which are controlled by environmental conditions such as nutrients, hormones and growth factors. These stem cells can remain quiescent, divide into daughter cells (one of which remains a stem cell while the other differentiates), or they can form two daughter cells with little ability to differentiate but with extensive proliferative capacity. This last alternative is normally very rare, but the chance of its occurrence increases if the cell's DNA has been damaged. This may occur accidentally during mitosis or as a result of exposure to DNA damaging agents, such as radiation, oxidative hydroxy radicals or toxic chemicals. Once the genome is altered by one or more factors, there is a progressively increasing probability of further alterations (Studzinski and Godyn, 1995). These alterations are known as tumor progression, or tumorigenesis. The initial stages of tumorigenesis involve the acquisition of new properties that favor tumor growth, during which new subclones may appear with different properties and characteristics. Tumor growth results from an imbalance of cell proliferation and cell attrition by terminal differentiation and by cell death. Those cells with the ability to survive are selected for and continue to proliferate, usually becoming progressively hyperploid, decreasing in their dependence on growth factors and becoming able to grow in new environments.

This genetic instability is the force that drives tumor progression, but proliferation is a necessary prerequisite for the development of genetically damaged clonal populations (Nowell, 1976). For reasons that are not understood, one genetic defect greatly increases the probability that other events will occur which will result in further genetic damage. Abnormalities in karyotype, such as extra or unpaired chromosomes, increase the difficulty of achieving an orderly distribution of the chromosomes during mitosis, leading to even greater abnormalities of the karyotype. Many of these errors are not compatible with cell survival, but some endow the cell with an increased ability to outgrow its neighbors, and thus produce subclones that may replace the original neoplastic clone. The genetic abnormalities that promote tumorigenesis are those that lead to the activation of proto-oncogenes, loss of tumor suppression genes, alteration of growth factors and their receptors, loss of cell cycle control, inability to repair DNA, and alterations in genes that regulate programmed cell death (Studzinski and Godyn, 1995).

Oncogenes

Several dozen cellular genes receive and propagate signals for cell proliferation and differentiation (Studzinski, 1989). The malfunction of one or more of these genes is thought to contribute to the abnormal growth of neoplastic cells. When these genes are functioning normally, they are known as proto-oncogenes; they are referred to as oncogenes when they become dysfunctional. Proto-oncogenes can be activated by a somatic mutation of the gene sequence, which changes its normal function, or by amplification of the gene copy number or placement near a strong promoter of gene transcription (Studzinski and Godyn, 1995), both of which increase its level of expression.

The known activations of proto-oncogenes in breast cancer are currently limited to somatic mutations of *ras* genes, and to the amplification of *HER*-2, also known as *erbB2* or *neu* gene (Callahan and Campbell, 1989). The function of the HER-2 protein in normal tissues is not completely known, though some studies have suggested its importance in neural and neuromuscular junction development. The gene encoding HER-2 is almost invariably amplified in connection with overexpression of the corresponding mRNA and protein product. It has been estimated that 10 to 30% of breast, gastric, and ovarian cancers overexpress the c-HER-2 gene product at a sufficiently high level that the protein serves as an oncogene (Dickson and Lippman, 1996). Amplifications of *c-myc* and *int-2* are also considered by some to be responsible for the neoplastic mode of growth of breast cancer cells. Amplification of the gene for c-Myc (located on 8q24), although not yet widely appreciated, is one of the most common genetic alterations in breast cancer; approximately one third of breast cancers contain this genetic aberration. Several studies focusing on gene amplification have shown that *c-myc* activation is associated with poor prognosis, a high number of tumor cells in S-phase, and post-menopausal disease (Dickson and Lippman, 1996).

Tumor suppressor genes

Deletion of portions of chromosomes or loss of entire chromosomes is frequent in cancer cells in general, and this is equally true in breast carcinoma cells (McGuire and Naylor, 1989). An important consequence of these deletions is the loss of tumor suppressor genes, whose protein products impede the passage of cells through the cell cycle, particularly the G_1-S-phase transition. Unlike proto-

oncogenes, only one of which needs to be activated or provide the uncontrolled growth signal, both alleles of most tumor suppressor genes need to be lost, down-regulated or inactivated to allow abnormal growth.

Because the loss of both copies of a tumor suppressor gene is usually required for tumor progression, the status of these genes is more apparent in familial cancer inheritance patterns. In 1866, the French surgeon Paul Broca concluded that 5% to 10% of breast cancer cases had a familial pattern of high incidence. Such families have been thought to inherit a defective or lost allele containing a tumor suppressor gene. This supposition has been based largely on work by Knudson (1971), who postulated the inactivation of two alleles of a gene in retinoblastoma, and by Harris *et al*. (1969), who demonstrated that certain chromosomes could suppress malignancy in cell-cell hybridization studies.

Knudson proposed the two-hit hypothesis, suggesting that cancer occurred as a result of two genetic events in the same cell, inactivating both copies of a given tumor suppressor gene. In the case of sporadic, or noninherited, cancer, the likelihood that two separate events would occur in the same cell is low. Individuals who inherit an inactivating mutation in one allele of the implicated tumor suppressor gene as a germline mutation, however, only require one somatic event to inactivate the single remaining copy; thus, cancer development is much more common in individuals heterozygous for the mutated allele than in those who are homozygous for the functional one.

Within familial cancer syndromes, the loss of the remaining functional gene can often be detected in tumor samples by the loss of genetic markers in the chromosomal region of interest; this is termed loss of heterozygosity (LOH). Consistent LOH for a genetic marker at a given locus in tumors from multiple patients has been considered strong evidence of the presence of a tumor suppressor gene in that region. Nonrandom chromosome deletions and LOH at specific chromosomal regions have been identified in most common human carcinomas, including breast cancer, which supports this hypothesis. Recent work examining LOH in familial breast cancers suggests that chromosomes 8q, 16q, 17p, 17q and 19p exhibit LOH in at least 20% of tumors in this series, with 33% LOH detected (Weber and Garber, 1996). Chromosome 17 has been the focus of intense investigation because of the presence of three known tumor suppressor genes: *BRCA1* (breast cancer gene 1, on 17q), *NF1* (neurofibromatosis type 1, on 17q) and *p53* (protein 53, on 17p), as well as the metastasis suppressor gene *nm23* (17q) and the breast cancer-associated oncogene *HER-2* (17q). Detailed analysis of this chromosome suggests the presence of as many as five distinct regions that are lost frequently, two of which are known to encompass *p53* and *BRCA1* (Weber and Garber, 1996).

The first evidence of a tumor suppressor gene in the pathogenesis of human cancers was found with the isolation of the retinoblastoma gene (*Rb*). All patients with hereditary retinoblastoma carry a germline mutation in one allele of the *Rb* gene, leaving them with only one functional copy. Loss of the remaining functional allele results in the inactivation of both *Rb* alleles: the first one by the inherited germline mutation and the second by a somatic event. Thus, germline mutations in tumor suppressor genes create highly susceptible groups of individuals for whom only one somatic event is required to produce complete inactivation of tumor suppressor activity at a given locus. The *Rb* gene is inactivated in about 20% of breast cancers (reviewed in Fung and T'Ang, 1992), but of particular relevance to breast cancer are the tumor suppressor genes *p53*, *BRCA1*, and *BRCA2*.

The first tumor suppressor gene shown to have an association with inherited breast cancer is known as *p53*, located on chromosome 17p13. Genetic alterations in *p53* are associated with aggressive tumor behavior and poor prognosis (reviewed in Allred *et al.*, 1994; Elledge *et al.*, 1993). Mutations in this locus are inherited in families with the rare breast cancer and sarcoma syndrome termed LFS (Li-Fraumeni syndrome). Further investigations in patients with an inherited pattern of breast and/or ovarian cancer have identified two other tumor suppressor genes, known as *BRCA1* and *BRCA2* (located on 17q21 and 13q12, respectively). Other tumor suppressor genes, which do not appear to be mutated but rather are down-regulated in metastatic breast cancer, are *nm23*; the gene encoding the cell-cell adhesion protein E-cadherin; and the integrin-α6 subunit. The mechanisms of action of the latter three are not well understood, and each appears to have different functions from the others (Dickson and Lippman, 1996).

Growth factors, cell cycle control, genomic integrity and apoptosis

There are several growth factors and receptors that are active in breast tissue which appear to be important in the pathogenesis of breast cancer. Activation of the *HER-2* oncogene mentioned earlier has a strong association with decreased survival (Slamon *et al.*, 1987, McCann *et al.*, 1991). In transgenic mouse models, both TGF-α (tumor growth factor α) and HER-2 have been demonstrated to have important functions in the early stages of breast tissue carcinogenesis (Sandgren *et al.*, 1990, Dankort and Muller, 1996). TGF-α and FGF2 (fibroblast growth factor 2), which participate in the autocrine loops of receptor-ligand systems, also have been implicated in the early phases of breast cancer (Souttou *et al.*, 1994, Normanno *et al.*, 1994). Additionally, it is thought that, in the more advanced stages of the disease, desensitization to the growth inhibitory activity of TGF-β contributes to tumor progression (Stampfer *et al.*, 1993).

Being able to control the cell cycle is central to managing the size of the populations of cells that make up an epithelial tissue structure. One indicator of the importance of the cell cycle in cancer pathogenesis is that approximately 80% of neoplasms harbor genetic lesions impairing the G_1-S transition checkpoint. Overexpression of cyclin D1, a protein associated with cell cycle control, is seen in approximately one-third of invasive breast cancers, as well as deregulation of cyclin E (Zukerberg *et al.*, 1995, Keyomarsi *et al.*, 1995). The cell cycle inhibitor *p16*, while infrequently altered in human breast cancers, is inactivated in approximately 30% of breast carcinomas (Xu *et al.*, 1994, Herman *et al.*, 1995). Decreased expression of p27/Kip1, a cell cycle inhibitor, has been associated with poor prognosis in node negative breast cancer patients, while expression of p27 and cyclin E has been found to be associated with good prognosis (Porter *et al.*, 1997). Recently, BRCA1 was shown to transactivate expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ in a p53-independent manner, and to inhibit cell-cycle progression into S-phase following its transfection into human cancer cells (Somasundaram *et al.*, 1997). Wild type BRCA1 has been shown to inhibit growth of breast and ovarian cancer cell lines *in vitro*, and in MCF7-induced tumors *in vivo*. Mutant BRCA1 had no effect, and decreased expression of BRCA1 accelerated growth *in vitro* (Holt *et al.*, 1996, Thompson *et al.*, 1995). During the cell cycle, *BRCA1* mRNA levels have been shown to increase late in G_1 phase (Vaughn *et al.*, 1996, Gudas *et al.*, 1996). This accumulation of *BRCA1* mRNA at the cell cycle

checkpoint and its positive correlation with the proliferative status of cultured mammary epithelial cells suggests BRCA1 plays a role in controlling cell proliferation (Gudas *et al.*, 1996).

Because differentiated cells are continually replenished by stem cells, preserving genomic integrity during cell division is paramount for tissue homeostasis. The polymorphic genetic alterations found in advanced tumors would indicate that the mechanisms ensuring genomic integrity during cell division have been lost. Dysfunction in DNA repair mechanisms can promote the accumulation of genetic lesions in cells, thus driving the process of carcinogenesis (Fishel *et al.*, 1994). However, little is known about DNA repair in breast cancer. Recent studies have found the carboxyterminal BRCT (*BRCA*-related C-terminal) domain of BRCA1 is involved in DNA repair and have led to the identification of a BRCT superfamily of proteins which are putatively involved in the DNA damage response pathway (Bork *et al.*, 1997). BRCA1 also has been shown to associate with Rad51 (a RecA homolog), and has been suggested to act in concert with DNA repair enzymes to maintain genomic integrity during periods of rapid growth (Scully *et al.*, 1997, Scully *et al.*, 1997a, Thomas *et al.*, 1997).

By enhancing their chances of survival, cells also increase their opportunities to accumulate mutations. The inactivation or deregulation of apoptotic pathways appears to be important in the early phases of carcinogenesis. It also plays a role in advanced tumors, by conferring resistance to radiation and chemotherapy, which depend on triggering these apoptotic pathways. Certain growth factors appear to promote survival, and there is evidence that accessing apoptotic pathways may depend in part on depriving cells of signals for the presence of growth factors in the environment. Using gene therapy to provide a functional p53 gene product has been proposed as a possible treatment for breast cancer, as cells lacking p53 function may not be competent in accessing apoptotic pathways (Elledge and Lee, 1995). It is possible that BRCA1 is involved in p53-mediated apoptosis by regulating p53-dependent gene expression and stimulating p53's transcriptional activity (Ouchi *et al.*, 1998, Zhang *et al.*, 1998). Indeed, Shao *et al.* (1996) reported that apoptosis can be induced in *BRCA1*- transfected cancer cell lines, and Tait *et al.* (1998) have published positive results in using retrovirally-introduced *BRCA1* for gene therapy of ovarian cancer patients, while Fan *et al.* (1998) have proposed using *BRCA1* as a suppressor of prostate cancer. Wang *et al.* (1998) showed that BRCA1 binds c-Myc and inhibits its transcriptional and transforming activity in mammalian cells, indicating that BRCA1 may function as a tumor suppressor by regulating c-Myc. Cell survival also can be enhanced by overexpression of the telomerase enzyme, which counteracts chromosomal shortening and physiological cell-aging processes. Recently, Wu *et al.* (1999) demonstrated that c-Myc directly activates transcription of the human telomerase catalytic subunit, and therefore may facilitate cell survival through telomerase activation. Breast carcinomas also have been shown to express high levels of this enzyme (Hiyama *et al.*, 1996).

Breast Cancer Gene 1 (*BRCA1*)

Of all the genes described previously, *BRCA1* appears to play the biggest role in inherited breast and ovarian cancer syndromes. Given Knudson's two hit hypothesis of tumorigenesis, it is not surprising that the most consistent factor associated with a woman's risk of developing breast cancer is a family history of the disease, indicating tumor suppressor involvement. Hall *et al.* (1990) identified

BRCA1 as a gene involved in familial breast cancer and mapped it to 17q21. If this gene is a tumor suppressor gene, the Knudson hypothesis would suggest that tumors from affected family members would show loss of heterozygosity (LOH) affecting the wild type chromosome. In four multiple case breast-ovarian cancer families, Smith *et al.* (1992) indeed found that in each of nine tumors that showed allele loss, the losses were from the wild type chromosome. Kelsell *et al.* (1993) found the same for each of seven breast tumors from a single multi-affected breast/ovarian cancer pedigree.

Miki *et al.* identified a strong candidate for the *BRCA1* gene by positional cloning methods in 1994. This gene is spread across 100 kb of genomic DNA, containing 24 exons, 22 of which encode the 7.5 kb transcript that produces the 1863 amino acid BRCA1 protein (Miki *et al.*, 1994). The protein contains a zinc (RING) finger domain in its amino-terminal region, but is otherwise unrelated to previously described proteins. Since its isolation, several other functional motifs have been described, including two nuclear localization signals within exon 11 (Thakur *et al.*, 1997), cyclin-dependent kinase phosphorylation sites (Chen *et al.*, 1996), a putative cAMP-responsive element binding (CREB) site along with CpG methylation sites in the 5' UTR , and a transcriptional activation domain in the C terminus (BRCT) (Monteiro *et al.*, 1996, Mancini *et al.*, 1998) (Figure 3).

The BRCT is 217 amino acids long, comprising two repeated motifs of non-identical stretches of amino acids, with the second motif ending eight amino acids short of the carboxy terminus (Figure 3). This motif has been found to occur singly and in multiple copies in other proteins. The BRCT domain is conserved among at least 50 other proteins, including p53-binding protein 1 (p53BP1), RAD9, three eukaryotic DNA ligases, and a wide range of other proteins involved in gene regulation and DNA repair (Callebaut and Mornon, 1997, Bork *et al.*, 1997). The high acidity of the BRCA1-BRCT domain prompted the demonstration that it can activate transcription of reporter genes *in vitro* when fused to a yeast GAL4 DNA binding domain (Monteiro *et al.*, 1996, Chapman and Verma, 1996, Haile and Parvin, 1999). Furthermore, some of the *BRCA1* mutations found in *BRCA1*-associated breast cancers inactivate transcriptional activity in this assay. Recently, Hu, Hao and Li (1999) demonstrated that the acidic transcriptional activation domain of BRCA1, when tethered to a cellular replication origin, can alter the local chromatin structure and stimulate chromosomal DNA replication. Cancer-predisposing mutations in *BRCA1* that abrogate transcriptional activation also prevent chromatin remodeling and activation of replication, implying that the loss of transcriptional transactivation and rearrangement of chromatin by BRCA1 may be involved in tumorigenesis.

BRCA1 Functional Domains and Protein Interactions

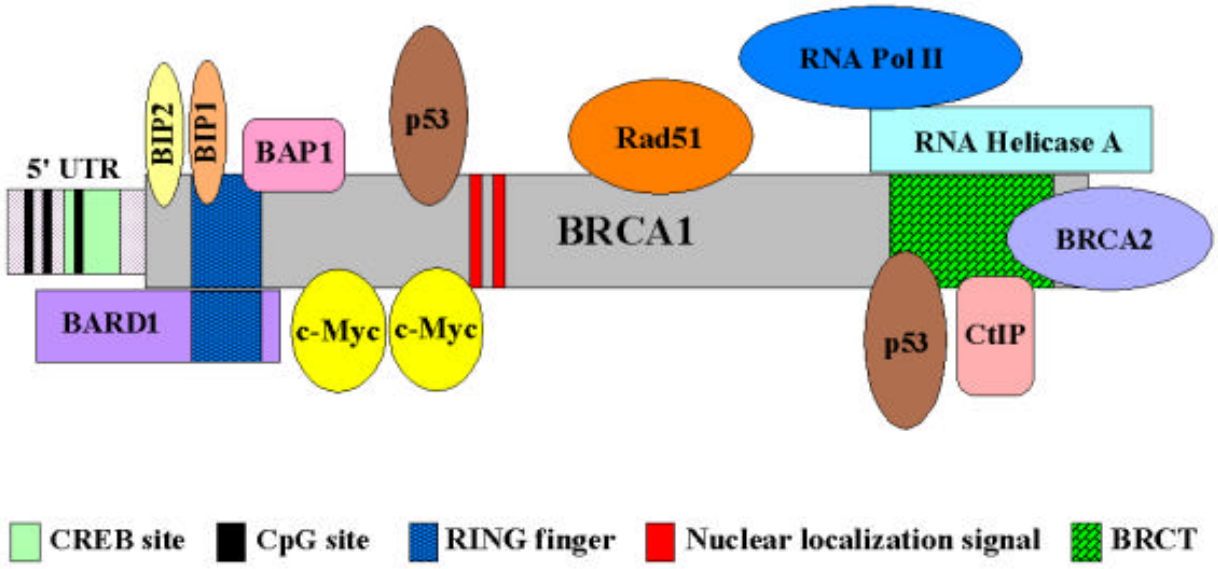


Figure 3

The promoter region of *BRCA1* has a high proportion of CpG dinucleotides, which are potential transcriptional regulation sites due to the possible methylation of cytosines (Figure 3). This methylation may then alter gene expression by preventing regulatory elements from binding to the promoter (Mancini *et al.*, 1998). Silencing of gene expression via DNA hypermethylation of regulatory regions has been documented in at least three tumor suppressor genes, namely *Rb* (Ohtani-Fujita *et al.*, 1993), *p16* (Merlo *et al.*, 1995) and *Von Hippel-Lindau (VHL)* (Herman *et al.*, 1994). Mancini *et al.* (1998) found that two out of six breast carcinoma samples and two out of five ovarian carcinoma samples had varying degrees of CpG methylation in the 5' UTR, with one of these CpG sites located within the putative CREB site (Figure 3). Further studies by Catteau *et al.* (1999) have shown that abnormal methylation of the *BRCA1* promoter may be involved in a subset of sporadic breast and ovarian cancers. Gel mobility shift assays using MCF7 nuclear extracts and radiolabelled methylated or unmethylated double-stranded oligonucleotides have shown a loss of protein binding to the methylated DNA, suggesting that the CREB site of *BRCA1* is sensitive to DNA methylation. Methylation of CREB/promoter sequence also is known to abolish specific factor binding, to inactivate transcription and to be strongly correlated with lack of estrogen and progesterone receptor expression (Iguchi-Ariga and Schaffner, 1989, Catteau *et al.*, 1999).

BRCA1 protein interactions

In addition to the studies of the functional regions in BRCA1, investigators have shown that it interacts with a multitude of proteins (Figure 3), including BARD1 (BRCA1-Associated RING-Domain protein 1), BAP1 (BRCA1-Associated Protein 1), BIP1 and BIP2 (BRCA1 Interacting Protein 1, 2), c-Myc, p53, Rad51, RNA helicase A, RNA polymerase II, BRCA2 and CtIP (CtB interacting protein) (Wu *et al*., 1996, Jensen *et al*., 1998, Zhang *et al*., 1998a, Irminger-Finger *et al*., 1999). While the function of BARD1 is still unknown, it is known that the human BARD1 protein contains a RING finger domain, three tandem ankyrin repeats and two BRCT motifs. It is likely that the interaction of BARD1 and BRCA1 occurs at the N-terminus of both proteins (Figure 3), involving the RING domain of each protein (Wu *et al*., 1996). Brzovic *et al.* (1998) have shown that the unique N-terminal structural domain found in both BRCA1 and BARD1 is capable of mediating both homodimerization and BRCA1/BARD1 heterodimerization in solution. Further evidence for a BRCA1/BARD1 interaction stems from immunofluorescence localization studies which show that both proteins co-localize to S phase nuclear dots or foci (Jin *et al.*, 1997, Scully *et al.*, 1997b). It previously had been supposed that the RING finger of BRCA1 mediated transcription. However, the BRCA1 RING finger domain does not bind DNA or polyribonucleotides by itself, as a homodimer or as a BRCA1/BARD1 heterodimer, and is not known to possess a transactivation function (Meza *et al.*, 1999). Wu *et al.* (1996) demonstrated that the BARD1/BRCA1 interaction can be disrupted by tumorigenic amino acid substitutions in BRCA1, implying that the formation of a stable complex between these proteins may be an essential aspect of BRCA1 tumor suppression.

BAP1 is a nuclear localized protein which binds to the RING finger domain of BRCA1 (Figure 3) (Jensen *et al.*, 1998). It contains motifs and activities characteristic of the ubiquitin carboxy-terminal hydrolase family, which are thiol proteases that catalyze the proteolytic processing of ubiquitin. Jensen *et al.* (1998) also demonstrated by co-immunoprecipitation and immunofluorescence studies that BAP1 and BRCA1 associate *in vivo* and have overlapping subnuclear localization patterns. However, BAP1 failed to bind to BRCA1 proteins with germline mutations of the RING finger domain found in breast cancer kindreds. Jensen *et al.* also have shown that the growth suppressive capability of overexpressed BRCA1 in MCF7 cells is augmented four-fold by the overexpression of BAP1. It is possible that *BAP1* represents a novel tumor suppressor gene, as further supported by the discovery of a homozygous deletion of *BAP1* in a lung carcinoma cell line.

Two cellular proteins, designated p64 BIP (BIP1) and p32 BIP (BIP2), bind to a GST-BRCA1 fusion protein (amino acids 1-76) *in vitro* (Figure 3, Wang *et al.*, 1997). Western blots of BIP complexes reveal binding of antibodies specific for cdc2, cdk2, cdk4, cyclin A, cyclin B1, cyclin D1, cyclin E and E2F-4, suggesting an association of BRCA1 with these proteins. BRCA1 is phosphorylated by kinases associated with cyclins D and A as well as by CDK2 *in vitro* (Chen *et al.*, 1996). Further, the splice variants BRCA1a and BRCA1b bind to GST fusion proteins of cyclin A, cyclin B1, cyclin D1, E2F-1 and E2F-4 *in vitro* (Wang *et al.*, 1997). Taken together, these results suggest that the phosphorylation status, and perhaps the activity of BRCA1, is regulated by cyclin-CDK complexes.

The product of the *c-myc* proto-oncogene binds BRCA1 in two separate locations (Figure 3) with its helix-loop-helix region in yeast two-hybrid experiments (Irminger-Finger *et al.*, 1999, Wang *et*

al., 1998). This association is also seen in co-immunoprecipitation of untransfected 293T cells, which suggests that BRCA1 may affect the transcription activation function of c-Myc. In addition, Myc-mediated transcription can be negatively regulated by BRCA1, as demonstrated by reduced expression of luciferase reporters carrying Myc-binding sites in cells overexpressing BRCA1. Furthermore, cellular transformation induced by co-transfection of *c-myc* and *H-ras* in rat embryonic fibroblasts has been shown to be suppressed by BRCA1. The phenotype of these transformants can be reversed when BRCA1 is introduced after transformation, while BRCA1 only minimally affects the transformed phenotype induced by SV40 virus. These findings raise the possibility that BRCA1 may exert its tumor suppressing activity in part through an interference of the growth-stimulatory properties of c-Myc, and that loss of this negative regulation through a *BRCA1* mutation may eventually lead to cellular transformation (Wang *et al.*, 1998).

Zhang *et. al.* (1998) have shown by co-immunoprecipitation experiments that BRCA1 and p53 physically interact *in vitro* and *in vivo* (Figure 3), requiring amino acids 224-500 (a region of exon 11) of BRCA1 and the C-terminus of p53 to do so. Tumor-derived BRCA1 protein carrying point mutations that disrupt the p53 binding domain is defective in stimulation of p53-dependent transcription, and truncated BRCA1 is a dominant negative inhibitor of p53-dependent transcription, which can be reversed by excess wild-type. Further studies by Chai *et al.* (1999) have shown that p53 also binds to the second BRCT domain *in vitro*, which shares a common domain with the p53 binding protein 53BP1 (Figure 3). Additional investigations have shown that BRCA1 serves as an activator of p53-dependent transcription, potentiating transcription of the cyclin-dependent kinase inhibitor $p21^{Waf1}$ and *BAX* promoters and increasing p53's apoptotic capability. Alternatively, BRCA1 can also activate transcription in the absence of p53, as demonstrated by the activation of the $p21^{Waf1}$ genomic promoter in p53-inactive SW480 cancer cells (Somasundaram *et al.*, 1997). The actual mechanism of transcription activation is not known, but might involve BRCA1 activating p53 by altering its conformation through direct physical binding and/or modification of the C-terminal region of p53. The role of p53 as a transcription factor has been well established, and it also appears to be important for its ability to arrest cell cycle progression as well as to induce apoptosis (Levine, 1997). The finding that BRCA1 is a co-activator with p53 and can activate the $p21^{Waf1}$ promotor independent of p53 sheds light on how mutations in *BRCA1* can affect cell cycle progression, DNA repair and the apoptotic pathway.

The investigation of BRCA1 protein function has uncovered several lines of evidence implicating BRCA1 in DNA repair. First, the BRCA1 protein co-localizes in S phase nuclear foci with the DNA-repair protein RAD51 (Scully *et al.*, 1997). Co-localization of BRCA1 and RAD51 also has been observed on developing and asynapsed (axial) elements of human synaptonemal complexes during meiosis. RAD51 shares significant homology with bacterial RecA, which mediates the pairing and ATP-dependent exchange of DNA strands in recombination (Ivanov and Haber, 1997). Second, Scully *et al.*, (1997) have shown that RAD51 associates with a specific region of BRCA1 (amino acids 758-1064) *in vitro* and *in vivo* (Figure 3). BRCA1 and Rad51 both have been observed relocating to regions of damaged, replicating DNA following UV- or hydroxyurea-induced DNA damage in S phase MCF7 cells. These regions are areas of non-duplex DNA, and are sites for proliferating cell nuclear antigen (PCNA) activity. Moreover, immunostaining for BRCA1, BARD1 and Rad51 shows that these

proteins are localized with PCNA nodules (Scully *et al.*, 1997b). It is therefore possible that BRCA1, BARD1, Rad51 and PCNA exist in a multi-protein complex at some time after DNA damage, though the existence of such a complex has yet to be shown. These findings suggest a functional interaction between BRCA1 and Rad51 in the meiotic and mitotic cell cycles, which, in turn, suggests a role for BRCA1 in the control of recombination and of genome integrity.

Association of the BRCA1 protein with the DNA repair gene RAD51 and changes in the phosphorylation and cellular localization of the protein after exposure to DNA-damaging agents are consistent with a role for BRCA1 in DNA repair. Gowen *et al*., (1998) showed that mouse embryonic stem cells deficient in BRCA1 are unable to carry out transcription-coupled repair of oxidative DNA damage, and are hypersensitive to ionizing radiation and hydrogen peroxide. These findings suggest that BRCA1 participates, directly or indirectly, in transcription-coupled repair of oxidative DNA damage. In addition, Scully *et al*. (1997a) demonstrated that the *BRCA1* gene product is a component of the RNA polymerase II holoenzyme (polII) by several criteria: first, BRCA1 was found to co-purify with the holoenzyme over multiple chromatographic steps; second, antibody specific for the holoenzyme component SRB7 specifically purified BRCA1 (SRB proteins are a key component of the holoenzyme, and were discovered in a yeast genetic screen as suppressors of RNA polymerase B mutations; hence, the designation SRB); third, immunopurification of BRCA1 complexes also specifically purified transcriptionally active RNA polII and transcription factors TFIIF, TFIIE, and TFIIH, which are known components of the holoenzyme. Moreover, the BRCT domain of BRCA1, which is deleted in about 90% of clinically relevant mutations, participated in binding to the holoenzyme complex in cells. Additional details of this interaction were reported by Anderson *et al*. (1998), who demonstrated that BRCA1 interacts with RNA polymerase II via a linkage with RNA helicase A (RHA) (Figure 3). The RHA protein itself contains domains which bind to the CREB binding protein (CRB), RNA polymerase II, and to the BRCT domain of BRCA1. It is possible that BRCA1 interacts with DNA-bound transcription factors to mediate a signal to RNA Pol II via RHA (Anderson *et al.*, 1998). This finding also provides an opportunity for crosstalk with the proteins involved in transcription-coupled repair of oxidative DNA damage (Gowen *et al.*, 1998). These data are consistent with other findings identifying transcriptional activation domains in the BRCA1 protein and linking BRCA1 to the multi-protein complex required for transcription-coupled repair via interaction with the holoenzyme.

Another protein recently discovered to interact with BRCA1 is the breast cancer susceptibility gene 2 (*BRCA2)* gene product (Chen *et al.*, 1998). The tumor suppressors *BRCA1* and *BRCA2* were previously shown to share similar properties, including the timing of expression (Rajan *et al.*, 1996), the phenotypes of knockout mice (Hakem *et al.*, 1996), and an association with Rad51 on meiotic chromosomes (Scully *et al.*, 1997, Sharan *et al.*, 1997). Through co-immunoprecipitation and *in vitro* binding studies, Chen *et al*. (1998) demonstrated that the BRCT region of BRCA1 near the C-terminus can bind BRCA2 (Figure 3). Furthermore, both BRCA1 and BRCA2 were found to co-localize in S phase nuclear foci. Upon treatment of late S phase cells with hydroxyurea, BRCA2, like BRCA1, Rad51 and BARD1, co-localized with PCNA (Chen *et al.*, 1998, Scully *et al.*, 1997b), implicating BRCA2 in a possible multi-protein repair complex. Immunostaining of human spermatocytes also showed co-localization of BRCA1 and BRCA2 on developing synaptonemal complexes, suggesting a role for these proteins in homologous recombination (Chen *et al.*, 1998). Recently, Chen *et al*. (1999)

proposed that BRCA1 and BRCA2 participate in a common DNA-damage response pathway associated with the activation of homologous recombination and double-strand break repair. Dysfunction of this pathway may be a general phenomenon in the majority of cases of hereditary breast and/or ovarian cancer, and the BRCA1/BRCA2 complex may function in post-replicational repair processes activated during the DNA synthesis stage of the cell cycle.

The BRCT domain also is implicated in transcriptional repression. Yu *et al.* (1998) found that the BRCT domain interacts *in vivo* with CtIP (Figure 3). This protein is associated with the CtBP transcriptional co-repressor, a tumor suppressor first shown to bind to E1a (Boyd *et al.*, 1993), as well as the transcriptional repression domains of three *Drosophila* transcription factors (Nibu *et al.*, 1998, Poortinga *et al.*, 1998). More recently, Li *et al.* (1999) demonstrated that the association of BRCA1 with CtIP also is abolished in cells treated with DNA-damaging agents including UV, gamma-irradiation and adriamycin, a response correlated with BRCA1 phosphorylation. They also found that the transactivation of the p21 promoter by BRCA1 was diminished by expression of exogenous CtIP and CtBP, suggesting that the binding of the BRCT repeats of BRCA1 to CtIP/CtBP is important in mediating transcriptional regulation of p21 in response to DNA damage.

Mutations in *BRCA1* and ethnic variation

Mutations in *BRCA1* initially were estimated to confer a lifetime risk of approximately 85% of developing breast cancer and a 50% risk of developing ovarian cancer (Easton *et al.*, 1995). More recent studies of the general population suggest the lifetime risk of developing breast cancer may be as low as 56% (Struewing *et al.*, 1997, Claus *et al.*, 1998). To date, more than 400 individual mutations associated with the disease have been found in *BRCA1*, spread over the entire coding sequence of the gene (Couch *et al.*, 1996, Breast Cancer Information Core (BIC) database, 1998). It is of interest that *BRCA1* mutations are extremely rare in sporadic breast cancer, though sporadic cancers often exhibit a LOH for 17q. While there appear to be no "hot spots," certain mutations have been found to be more prevalent in different populations, such as the 185delAG and 5382insC mutations in the Ashkenazi Jews, the 2595delC in Scandinavians, and the 589delCT in Chinese and Japanese women (Struewing *et al.*, 1995, Hakansson *et al.*,1997, Tang *et al.*, 1999*)*. Also, the incidence of *BRCA1* mutations has been shown to vary between ethnic groups, from 1 in 1000 for Japanese to 1 in 100 for the Ashkenazim (Tonin *et al.*, 1996, Couch and Hartmann, 1998, Szabo and King, 1997). Other reports of international ethnic variation include high frequencies found in Belgian, Dutch and Italian women (Goelen *et al.*, 1999, Peelen *et al.*, 1997, Montagna *et al.*, 1996), moderate frequencies in German, Welsh, Taiwanese and Scandinavian women (Jandrig *et al.*,1996, Lancaster *et al.*, 1998, Li *et al.*, 1999a, Hakansson *et al.*,1997) and low frequencies in Scottish, Spanish, Chinese and Icelandic women (Mullen *et al.*, 1997, Diez *et al.*, 1999, Tang *et al.*, 1999, Barkardottir *et al.*, 1995).

Investigators studying the Ashkenazi Jewish population have postulated that the high carrier frequency of specific mutations in that group are due to a founder effect (Struewing *et al.*, 1995, Labuda, *et al.*, 1997). Origination of the founder mutation has been estimated to have occurred in the early 13th century (Neuhausen *et al.*, 1996, Berman *et al.*, 1996), while others reported it to be prior to the destruction of Jerusalem in 70 A.D. (Bar-Sade *et al.*, 1998, Liu and Barker, 1999). This last estimate is probably more accurate, given the finding of the 185delAG mutation, along with a common

haplotype, in non-Ashkenazi Jews of Moroccan, Yemenite and Iranian descent- groups that have been geographically and culturally distinct from the Ashkenazim since the Jewish dispersion in 70 AD. As part of that dispersion, Jewish people migrated north into Europe, the largest group settling in Ashkenaz (medieval Germany), with others scattering over central Europe. If the 185delAG mutation was already present in the population prior to the dispersion, these small populations isolated from other Jewish communities would provide an environment for a strong founder effect to be observed in later generations (Labuda *et al.*, 1997). It is of interest that Jandrig *et al.* (1996) found the 185delAG mutation in several German families not known to be of Jewish descent. It is quite evident that there were Jewish settlements in Spain and Portugal, given the anti-Jewish actions in both countries during the Spanish Inquisition (Hauben, 1969). Over a period from 1391 to early 1600s, Jews who would not convert to Catholicism faced death or expulsion from the country (Tobias, 1990, Diez *et al.*, 1999). It is of little surprise that under such conditions, many Jewish families converted while continuing to practice their old faith surreptitiously, thus creating a significant "crypto-Jewish" population in Spain prior to Columbus's voyages (Tobias, 1990). Due to the antagonistic climate of Spain toward Jews, many left for other locations, including Mexico, from which they also migrated to New Mexico (Tobias, 1990). This migration and integration of Jews into the New Mexico Spanish population raises the question of finding Jewish influence in the present New Mexico Hispanic population (Moya, 1996). It is quite possible that the 185delAG and/or 5382insC mutations are present in New Mexico Hispanics with a family history of breast cancer, especially if those families are of European Spanish descent. This is supported by the recent finding of the 185delAG and 5382insC mutations in a European Spanish population by Diez *et al.* (1999).

BRCA1 in New Mexico Hispanics

To date, there have been no studies of *BRCA1* involving New Mexico Hispanic populations. It has been well established that BRCA1 plays an important role in breast cancer formation and that mutation frequencies vary between ethnic groups. The three main populations of New Mexico (Caucasian, Hispanic and Native American) offer a unique opportunity to study *BRCA1* sequence variations in different ethnic groups that live in the same geographic area. Breast cancer incidence rates among these three groups are strikingly different, as mentioned previously (Figure 2). While this could be the result of environmental and cultural factors, it is also possible there is an underlying genetic difference that plays an important role in the observed incidence rates.

Past and present studies have not investigated familial breast cancer in the three main populations of New Mexico, and presently there are no publications involving familial breast cancer in New Mexico Hispanics. However, investigations of a Texas Hispanic population have reported that Hispanics have a very low frequency of familial breast cancer (about 2%), while a non-Hispanic white population from the same geographic area had rates equivalent to the national average of 10% (Bondy *et al.*, 1992). Another study in the Laredo, Texas area also reported that the Hispanics of that region had a low frequency of familial breast cancer, as well as a low risk of cancer in general (Weiss *et al.*, 1986). In addition, Polednak (1996), found that Hispanics in Connecticut had lower rates of early-onset breast cancer than those of non-Hispanic whites living in the same area. Taken together, this could be evidence for a decreased involvement of *BRCA1* in Hispanic populations.

Hypotheses

1. **A reduction of *BRCA1* mutation frequencies in New Mexico Hispanics could contribute to the observed breast cancer rates**, given the clear correlation between *BRCA1* mutations and an increase in risk of familial breast carcinoma and the reported ethnic variance of *BRCA1* mutations. To test this hypothesis, blood samples obtained by the New Mexico Women's Health Study (a program established to study various health problems specific for women in New Mexico) were analyzed using various methods to search *BRCA1* exons 2, 5, 11, 13, 16, 18, 20, 21 and 24 for sequence variations in genomic DNA. No mutations were found in any of these exons, which comprise 93% of the reported mutations in the literature and the BIC database (1998). Nine polymorphisms were identified, all of which have been reported previously in women of European, Asian or Spanish descent (Miki *et al.*, 1994, Jandrig *et al.*, 1996, Durocher *et al.*, 1996, Tang *et al.*, 1999, Diez *et al.*, 1999a). Because these polymorphisms were identified, it is evident that the methodologies used would have been adequate to detect any mutations that might have been present.
2. **If there is a correlation between these polymorphisms and tumorigenesis, tumors would be expected to selectively retain them during growth**. In order to test this hypothesis, paraffin-embedded tumor tissue was obtained from the New Mexico Tumor Registry and exons 13 and 16 were sequenced for the 4427C/T and S1613G polymorphisms.

Materials and Methods

Patient selection:

In cooperation with the New Mexico Women's Health Study (NMWHS), blood was drawn from Hispanic patients presenting with breast cancer in various clinics throughout New Mexico. Selection of participants for the present study by the NMWHS was HRRC (Human Research Review Committee) approved, and based on being a Hispanic woman with breast cancer and having a first degree relative with breast cancer. Ages of the 53 selected participants ranged from 25 to 74, with the average age of diagnosis being 55 (SD= 10.91). A search of the New Mexico Tumor Registry located 32 tumor blocks from Hispanic patients diagnosed with breast cancer prior to age 45, with an average age of 38 (SD= 5.16), which were used anonymously. Information on family history was unavailable for these patients because hospital records did not contain relative disease status.

DNA isolation from whole blood:

Genomic DNA was isolated from whole blood using the Puregene^{TM} kit from Gentra Systems, Inc. (Minneapolis, MN USA). Briefly, 2 ml of whole blood was mixed with the RBC (red blood cell) lysis solution provided, held at room temperature for 10 minutes, then centrifuged at 2,000 x g for 10 minutes. The supernatant was removed, and the remaining white blood cell pellet was resuspended in the cell lysis solution included with the kit. RNase was then added and the samples incubated at 37^0 C for 15 minutes, following which protein precipitation solution provided with the kit was added, samples vortexed, and then centrifuged at 2,000 x g for 10 minutes. The supernatant was decanted into 100% isopropanol, mixed well by inversion and centrifuged at 2,000 x g for 3 minutes, after which the resulting DNA pellet was washed in 70% ethanol. Following the wash, the supernatant was removed and the samples allowed to air dry for 20 minutes at room temperature, then the DNA pellet was suspended in the DNA hydration solution provided and the final concentration adjusted to 50 ng/µl (nanograms per microliter) and stored at
-20° C.

DNA isolation from paraffin-imbedded tissue:

Genomic DNA was isolated from four 25 µm sections of paraffin-imbedded tissue using a modification of the QIAmp® DNA Mini Kit from Qiagen (Valencia, CA USA). One hundred eighty µl of ATL buffer (lysis buffer) were mixed with the 4 tissue sections in a 2 ml tube, then 20 ∶l of Proteinase K were added, mixed well and the samples incubated in a water bath at 56^0 C for approximately 16-18 hours. Following the digestion, 20 ∶l of RNase A (20 mg/ml) were added, mixed well and the samples incubated at room temperature for 2 minutes. Two hundred ∶l of AL buffer (detergent) were added, the tubes mixed well and incubated at 70^0 C for 10 minutes, following which 200 ∶l of 96% ethanol were added. The tubes were then centrifuged briefly at 8,000 rpm in an Eppendorf 5415C centrifuge, then the samples were loaded onto QIAmp® spin columns and centrifuged at 8,000 rpm for 4 minutes. The filtrate was discarded (also discarded in the following 2 spins), new collection vials attached and 500 ∶l of AW1 buffer (wash buffer 1) were added to the columns, which were spun at 8,000 rpm. Five hundred ∶l of AW2 buffer (wash buffer 2) were added and the samples centrifuged for 3 minutes,

after which the columns were placed in a labeled collection tube. Two hundred µl of 70^0 C AE buffer (collection buffer) were added, the column incubated at 70^0 C for 5 minutes and spun for 2 minutes at 8,000 rpm to elute DNA, then repeated using a new collection tube. The collected DNA was then combined for a final concentration of approximately 50 ng/µl and stored at -20^o C

PCR and sequencing primer table:

Primer sequences were obtained from Myriad Genetics (Salt Lake City, UT USA), the BIC database (1998), supplied by Ambion, Inc. (Austin, TX USA) or designed individually. All were produced in the Protein Chemistry Laboratory of the University of New Mexico. Sequences are written 5' to 3'.

Exon	Forward Primer	Reverse Primer	Sequencing primer
2	2F- aaaccttccaaatcttcaaa	2R- gtcttttcttccctagtatgt	2F
5	5F- ctcttaagggcagttgtgag	5R- atggttttataggaacgctatg	5F
11a	11aF- ctccaaggtgtatgaagtatgta	11aR- tctgctccgtttggttag	11-1FS- tagcaaggagccaacataacag
11b	11bF- aaagcgtaaaaggagacctac	11bR- tcatgtcccaatggatactta	11-3F- gtgttcaaataccagtgaactta
11c	11cF- cactctagggaaggcc	11cR- cccctaatctaagcatagcat	11-bRF- taagtatccattgggacatga
11d	11dF- caagcaatattaatgaagtagg	11dR-accagaagtaagtccaccagtaa	11-cRF- atgctatgcttagattagggg
11-4	11-4F-ccaagtacagtgagcacaatta	11-4R- gtgctcccaaaagcataaa	11-37F- tagtgaggatgaagagcttcc
13	13F- aatggaaagcttctcaaagta	13R- tgttggagctaggtccttac	13F
16	16F- aattcttaacagagaccagaac	16R- aaaactctttccagaatgttgt	16FS- ctttgtaattcaacattcatcg, 16FS1- ccttacctggaatctggaatca
18	18F- ggctctttagcttcttaggac	18R- ctcagactcaagcatcagc	18F
20	20F- atatgacgtgtctgctccac	20R- gggaatccaaattacacagc	20F
21	21F- aagctcttccttttgaaagt	21R- gtagagaaatagaatagcctct	21F
24	24F- atgaattgacactaatctctg	24R- gtagccaggacagtagaagga	24F

Polymerase Chain Reaction with tumor-extracted genomic DNA:

Because of the poor quality of the DNA extracted from the paraffin (as determined by agarose gel electrophoresis), separate PCR conditions specific for use with the tumor-extracted genomic DNA were developed. The cycle times were as follows: Hold 95^o C, 5 minutes; Cycle 35X: 95^o C 20 s, 56^o C 30 s, 72^o C 1 minute and hold at 72^o C 5 minutes. The reaction conditions were 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.001% w/v gelatin, 1X Q solution (Qiagen), 3 units *Taq* DNA polymerase, 0.625 mM forward and reverse primers, 150 ng genomic template and 0.25 mM dNTPs (deoxynucleotide triphosphates) in a final volume of 20 µl.

Polymerase Chain Reaction prior to DNA sequencing:

Touchdown PCR was used to amplify the individual *BRCA1* exons from genomic DNA with intronic primers, so all of the exon and part of the introns on either side of the exon were amplified. This method decreases the chance of random priming by using a high annealing temperature initially, then gradually dropping it over the course of the reaction cycles. Primer sequences were obtained from Myriad genetics, the BIC database (1998) or designed individually, as listed in the previous table. The cycles were as follows: Hold 95° C, 2 minutes; cycle 4X 95° C, 10 s; 68° C, 10 s; 72° 10 s. The next 12 cycles were the same, except the annealing temperature (initially 68°) was decreased by two degrees every four cycles, and then 30 cycles at 95° C, 10 s; 60° C, 20 s; 72° C, 30 s. The reactions consisted of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 2 units *Taq* DNA polymerase, 0.12 mM forward and reverse primers (see previous table), 50 ng genomic template and 0.25 mM dNTPs in a final volume of 40 µl.

Solid-phase sample preparation and sequencing:

For solid-phase sequencing, biotin was attached to the 5' end of the reverse primer used in the initial PCR reaction to facilitates the isolation of single strand antisense PCR product with paramagnetic, streptavidin- coated Dynabeads®, following the protocol provided by Dynal (Oslo, Norway). Briefly, Dynanbeads® were washed and re-suspended in 2X binding and washing buffer (B&W) (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl, 0.1% Tween 20), following which the PCR product was bound to the beads at room temperature for 30 minutes with continual gentle shaking to keep the beads suspended. A magnet was then used to immobilize the bound DNA while the supernatant was removed, and the bound template was washed again in 2X B&W, after which 0.2 M NaOH was mixed in, then held at room temperature for 5 minutes. The bound DNA was immobilized with a magnet, and the supernatant removed. The immobilized DNA was then washed twice with 1X B&W to remove the unbiotinylated strand, and once with TE buffer (10 mM Tris-HCL, pH 7.5, 1 mM EDTA), then re-suspended in 12 µl of dH_2O. For solid-phase sequencing, the immobilized, single-strand PCR products were sequenced using the standard PRISM™ Ready Reaction DyeDeoxy™ Terminator T7 Sequencing kit (PE/Applied Biosystems, Foster City, CA USA), on a standard 6% polyacrylamide / 8.3M urea gel (FMC, Chicago, IL USA) poured onto 24 or 36 cm length plates. The samples were electrophoresed for 6-10 hours in 1X TBE at 30 W, with an ABI 373a or ABI 377 automated sequencer for detection. In some cases, results were also obtained using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Taq FS Sequencing kit (PE/Applied Biosystems). The PRISM™ dGTP Big Dye™ Ready Reaction Terminator Taq FS Sequencing kit (PE/Applied Biosystems) was used to sequence double-stranded PCR products directly, without the use of biotinylated primers. Prior to sequencing, the excess dNTPs and primers were removed from the PCR products using Microcon® YM-100 centrifugal filters from Millipore (Bedford, MA USA). Genomic DNA obtained from Myriad Genetics, containing both the *BRCA1* 185delAG and 5382insC mutations, was used for positive mutation control reactions.

Sequence Analysis:

Since the net rate of dideoxynucleotide vs. deoxynucleotide incorporation by *Taq* or T7 Sequenase™ is highly dependent on the local sequence environment, dideoxynucleotide terminator sequence traces

tend to have very uneven peak heights. This uneven peak pattern makes it difficult to properly identify bases, because sometimes a small peak representing a real base may be construed as background, or background signals may be erroneously considered bases. Additionally, in the case of a polymorphism, it may be difficult to distinguish if the DNA sequence is from a homozygote or heterozygote. In order to address these problems, the sequence output files were analyzed using software provided with the ABI 373a or 377 automated sequencer, as well as Factura™, Sequence Navigator™ and PolyPHRED. The raw sequencing data was analyzed first with Factura™, which removes unreadable/unreliable sequence (usually from the extreme 5' and 3' ends of the sequence), normalizes peak spacing and marks unidentified bases and possible heterozygous sites. The Sequence Navigator™ was used to look manually at the electropherogram of all the questionable base assignments, align multiple sequences for pattern identification and mutation detection, edit base assignments and create consensus sequences. PolyPHRED examines heterozygous sites in the electropherogram and compares the peak areas at and around the site in question, using the rules established by Kwok *et al*. (1994) and Parker *et al*. (1995) to determine if it is homo- or heterozygous. It also performs a Clustal V alignment of the sequences so variations between the individual samples can be quickly located.

Non-Isotopic RNase Cleavage Assay (NIRCA):

Exon 11 of *BRCA1* was analyzed completely by NIRCA, using the Mismatch Detect™ II kit from Ambion, Inc. (Austin, TX USA), with the primers supplied in the *BRCA1* exon 11 genomic DNA screening module (see previous primer table) and the procedure as given by Ambion. Briefly, exon 11 was amplified from genomic DNA by PCR using 4 overlapping primer sets (designated 11A, 11B, 11C and 11D) with the following conditions: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 2 units *Taq* DNA polymerase, 0.3 µM forward and reverse primers (see sequencing primer table), 50 ng genomic template and 0.16 mM dNTPs in a final volume of 40 µl. Cycle times: Hold 95° C, 5 minutes; cycle 30X: 95° C 20 s, 54° C 30 s, 72° C 1 minute; Hold at 72° C 5 minutes for final extension. The product was then reamplified in a nested reaction with the above conditions, using 2 µl of the first PCR product as template and nested primers with T7 and SP6 promoter sequences attached to the 5' ends (see NIRCA primer table). The size, purity and concentration of the nested PCR products were confirmed by agarose gel electrophoresis. Two µl of the nested PCR product were used as template for *in vitro* transcription reaction containing 1X transcription buffer (provided), 0.5 mM rNTPs and 1 U each of T7 and SP6 RNA polymerase in a final volume of 10 µl. Following transcription, 10 µl of mismatch hybridization buffer (provided) was added to each reaction and the RNA product was heated to 95° C for 3 minutes, then cooled to room temperature for 5 minutes to form heteroduplexes. Four µl (x3) of the hybridized reactions were then digested in three separate reactions with the three different RNase enzymes (provided by Ambion) for 45 minutes at 37° C. Fifteen µl of the digestion reaction were then analyzed on a 2% agarose gel in 1X TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) and photographed. The positive control, consisting of a 1032 bp insert of segment 11a containing an 11 deletion was supplied as part of the screening module by Ambion.

Nested NIRCA primer table:

The primers listed in this table were used in the nested PCR reaction described in the NIRCA section. Primers were provided as part of the *BRCA1* genomic DNA screening kit from Ambion. Sequences are written 5' to 3', bold type indicates T7 or SP6 promotor sequence.

Exon 11	Forward internal primer	Reverse internal primer
11a	T7aF- ga**taatacgactcactatagggt**tttttgagtaccttgttattt	SP6aR- gc**atttaggtgacactatagga**cgtttggttagttccctgatt
11b	T7bF- ga**taatacgactcactatagggt**attttatcaagaaagcagatttg	SP6bR- gc**atttaggtgacactatagga**tacttaaagccttctgtgtc
11c	T7cF- ga**taatacgactcactatagggc**aaaaacagaaccaaataaatg	SP6cR- gc**atttaggtgacactatagga**ttcaattttggccctctgtt
11d	T7dF- ga**taatacgactcactatagggc**attaatgaaataggttccagtg	SP6dR- gc**atttaggtgacactatagga**gctccccaaaagcataaa

Base Excision Sequence Scanning (BESS):

Parts of *BRCA1* exons 13 and 24 were screened for mutations using a protocol adapted from the BESS T-scan™ Mutation Detection kit from Epicentre Technologies (Madison, WI USA). Forward primers were labeled with [γ-^{32}P]-ATP (adenosine triphosphate) using T4 polynucleotide kinase (PNK). Six µl of [γ-^{32}P]-ATP (3,000 Ci/mmol) were added to a master mix containing 2 µM primer (sequencing primers from the previous table), 1x T4 PNK buffer and 4 U of T4 PNK enzyme (New England Biolabs, Beverly, MA USA) in a final volume of 50 µl. The reaction was incubated at 37º C for 30 minutes, then inactivated at 70º C for 5 minutes. The labeled primers were added directly to the PCR master mix consisting of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 0.2 mM dNTPs, 20 µM dUTP (deoxyuradine triphosphate), 0.4 µM reverse primer and 2 U *Taq* DNA polymerase (Qiagen) in a final volume of 575 µl. Twenty-three µl of the master mix were then dispensed into PCR tubes containing 2 µl template. Thermal cycles were as described in the polymerase chain reaction and DNA sequencing section previous. The PCR product was then subject to excision and cleavage using Uracil *N*-Glycosylase and Endonuclease IV (Excision enzyme mix, Epicentre Technologies) with the following parameters: 50 mM Tris-HCl pH 9, 20 mM $(NH_4)_2SO_4$, 10 mM EDTA, 0.5 µl excision enzyme mix and 5 µl PCR product in 10 µl total volume. The mixture was prepared on ice, then incubated at 37º C for 30 minutes. Loading buffer (95% (v/v) formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, pH 11) was added, the tubes heated to 90º C for 3 minutes and 5 µl loaded on a Burst-Pak™ 6% acrylamide / 8.3M urea sequencing gel (Owl Scientific, Portsmouth, NH USA). The gel was electrophoresed in 1X TBE for 1.5-2 hr at 60W, then was dried at 45º C for 30 minutes under vacuum and exposed to Kodak X-OMAT AR film for 12-36 hrs.

Single Strand Conformational Polymorphisms (SSCP):

Genomic DNA was used as template, or in the case of the tumor cell samples, PCR was performed as described previously prior to the SSCP reactions and the PCR product was used. The SSCP-PCR

cycles were as follows: Hold 95° C, 5 minutes; Cycle 35X: 95° C 10 s, 56° C 10 s and 72° C 20 s Hold at 72° C 2 minutes. The final reaction mix contained 50 ng genomic DNA (or 2 µl PCR product for the tumor cell samples), 0.2 mM of each dNTP, 0.25 µM [F]dUTP (R110 or R6G label, PE/Applied Biosystems), 0.625 µM of both primers, 2.5 U *Taq* DNA polymerase (Qiagen), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% w/v gelatin. After PCR, 100 µl of dH_20 were added to each sample prior to dNTP and excess primer removal using Microcon® YM-100 centrifugal filters, following the provided protocol. One µl of the resulting 40 µl was mixed with 5 µl of loading buffer consisting of a 100% formamide:50 mM EDTA:GENSCAN-500 TAMRA size standard mix (PE/Applied Biosystems). The sample was heated to 90° C for 3 minutes and placed on ice prior to loading on a 0.5X MDE gel (FMC) poured onto 36 cm length plates. The ABI 377 Genescan system used was modified by the attachment of a VWR model 1160 heating/cooling water bath containing 5% anti-freeze/distilled water to control the gel temperature. Electrophoresis was performed in 1X TBE buffer for 6-14 hours at 30 W, with gel temperature strictly maintained at 10°, 15°, 20° or 25° C. Data collection and analysis were done with the standard Genescan® 672 software.

CEL 1 cleavage reaction:

The CEL 1 enzyme was graciously provided by Dr. Anthony Yeung.
Genomic DNA was used as a template for PCR as given in the SSCP section above. PCR product (5 µl) was digested at 45° C for 30 minutes with CEL 1 in a 20 µl reaction containing 20 mM Hepes pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Qiagen) and 0.12 pg CEL 1 enzyme. The digestion reaction was then dried down to approximately1 ml with a Labconco Centrivap concentrator, after which 5 ml of loading buffer consisting of 100% formamide:50 mM EDTA:Genescan-500 TAMRA size standard (PE/Applied Biosystems) was added. The sample was heated to 90° C for 3 minutes and placed on ice prior to loading on a 6% polyacrylamide / 8.3M urea gel (FMC) poured onto 36 cm length plates. The samples were electrophoresed on an ABI 377 for 4-6 hours in 1X TBE at 30 W. Data collection and analysis were done with the standard Genescan 672 software. However, results obtained by this method were not used in this study, due to the inconsistent results obtained in control reactions under varying conditions.

Results

I. *BRCA1* sequence variations in somatic cell DNA:

The genomic DNA extracted from the 53 blood samples obtained from the NMWHS was analyzed by various methods for DNA sequence variations. A summary of methods used to analyze these samples is shown in Table 1. Table 2 contains a summary of the methods by which specific polymorphisms (sequence variations not associated directly with disease) were identified. A summary of the polymorphisms identified and their frequencies in the population studied are shown in Table 3. There were no mutations (i.e., sequence variations associated with disease) detected in any of the exons examined, which contain 93% of the reported sequence variations in the literature and the BIC database. A total of 1,008,677 nucleotides were analyzed during the course of this investigation.

Sequencing

Direct sequencing was used to search for possible DNA sequence variations in exons 2, 5, 20 and 21. In addition, portions of 18, 11 and 24 were also sequenced to confirm potential variations inferred from NIRCA, BESS T-scan and SSCP results. Exons 13 and 16 were sequenced in all 53 samples to verify SSCP results and determine allele frequency. Figure 4 shows representative sequencing results from individuals in this study and displays the three possible genotypes for the 4427C/T polymorphism in exon 13. Figure 5 contains typical sequencing results from individuals with the three possible genotypes of the S1613G polymorphism identified in Exon 16. Figure 6 shows electropherograms of a wild type sequence trace, and that of a positive control sample showing the 5382insC mutation in exon 20 (supplied by Myriad Genetics). These results indicate that both single base substitutions and length polymorphisms can be detected accurately by this method.
Summary: No sequence variants were identified in exons 2, 5, 20 and 21 by DNA sequencing (Table 2).

SSCP (Single Strand Conformational Polymorphism)

Figure 7 shows typical SSCP analysis results for exon 16, showing the different peak patterns for PCR products from the three possible genotypes of the S1613G polymorphism. Figure 8 pictures results of exon 13 PCR products containing the three possible genotypes for the 4427T/C polymorphism. These results demonstrate that SSCP is able to distinguish the single-base substitutions involved in these sequence variants. While no mutations were identified by this method, the 4427C/T, S1613G and 5272+66A/G polymorphisms were detected. All potential sequence variants identified by SSCP analysis were confirmed by sequencing (Tables 1 and 2).
Summary: While no mutations were found by SSCP, three previously reported polymorphisms were identified in exons 13, 16 and intron 18 (Table 2).

NIRCA (Non-Isotopic Rnase Cleavage Assay)

Figure 9 shows NIRCA products on a 2% agarose gel under UV light. Using NIRCA to screen exon 11, no samples with mutations were detected, although 3 potential polymorphisms in the exon 11-B, C and D sections were identified. The B and C sections contained multiple polymorphisms

that were not detected individually until these samples were analyzed by DNA sequencing (Table 2). Sequencing results for these polymorphisms are very similar to those in Figures 4 and 5, and so are not shown.

Summary: Six previously reported polymorphisms were identified in exon 11 segments B, C and D by NIRCA (Table 2).

BESS (Base Excision Sequence Scanning) T-scan

Figure 10 is a representative autoradiograph of a BESS T-scan analysis of exon 24. All potential variants were sequenced to confirm results. No mutations or polymorphisms were detected by this method (Table 2).

Summary: No sequence variants were identified in exon 24 by BESS T-scan.

Polymorphisms 4427T/C and S1613G and age distribution in somatic cells

The age of onset was analyzed in relation to the genotype of the patient for the 4427T/C and S1613G polymorphisms in the 53 patients from the NMWHS. Both the mean and median ages of women with the three possible genotypes were not significantly different ($p= 0.37$ and $p= 0.44$, respectively) as compared by ANOVA 1-way analysis of variance.

Summary: See Table 4.

Comparison of Methods Used for *BRCA1* Analysis in Somatic Cell DNA Samples

BRCA1 exon #	Sequenced	SSCP	NIRCA	BESS T-scan
2	X	CO	ND	CO
5	X	ND	ND	ND
11a	CO	ND	X	ND
11b	CO	ND	X	ND
11c	CO	ND	X	ND
11d	CO	ND	X	ND
13	X	X	ND	ND
16	X	X	ND	ND
18	CO	X	ND	ND
20	X	CO	ND	CO
21	X	ND	ND	ND
24	CO	CO	ND	X

Table 1: Summary of methods used to analyze individual *BRCA1* exons.
"X" indicates all 53 somatic cell DNA samples from the NMWHS were tested.
"CO" indicates confirmatory analysis only.
"ND" indicates the specified method was not used in analysis.

Summary of *BRCA1* Polymorphisms Identified in Somatic Cell DNA Samples

	Polymorphisms identified			
***BRCA1* Exon #**	**Sequencing**	**SSCP**	**NIRCA**	**BESS T-scan**
2	None	None	ND	None
5	None	ND	ND	ND
11a	None	ND	None	ND
11b	D693N, 2201C/T, 2430T/C	ND	D693N, 2201C/T, 2430T/C	ND
11c	L871P, E1038G	ND	L871P, E1038G	ND
11d	K1183R	ND	K1183R	ND
13	4427T/C	4427T/C	ND	ND
16	S1613G	S1613G	ND	ND
18	5272+66A/G	5272+66A/G	ND	ND
20	None	None	ND	None
21	None	ND	ND	ND
24	None	None	ND	None

Table 2: Summary of polymorphisms identified by the four methods used to analyze individual *BRCA1* exons. No mutations were detected.
"None" indicates no sequence variations were found.
"ND" indicates analysis was not done.

BRCA1 Polymorphisms Identified in New Mexico Hispanics

BRCA1 nucleotide	Nucleotide variant	Amino acid variant	Less common allele freq.	Heterozygosity Observed	Heterozygosity Expected[a]	*P* value (HWE)[b]
Polymorphisms in somatic cell DNA samples						
2196	G to A	D693N	0.10	0.20	0.18	NS
2201	C to T	silent	0.32	0.52	0.44	NS
2430	T to C	silent	0.32	0.52	0.44	NS
2731	T to C	L871P	0.28	0.45	0.41	NS
3232	A to G	E1038G	0.27	0.43	0.40	NS
3667	A to G	K1183R	0.33	0.55	0.44	NS
4427	T to C	silent	0.31	0.47	0.43	NS
4956	A to G	S1613G	0.31	0.47	0.43	NS
5272+66	A to G	non-coding	0.33	0.46	0.44	NS
Polymorphisms in tumor cell DNA samples						
4427	T to C	silent	0.48	0.76	0.50	0.005
4956	A to G	S1613G	0.48	0.76	0.50	0.005

Table 3: Summary of polymorphisms identified in the 53 somatic cell DNA samples and 32 tumor cell DNA samples.

[a]Expected heterozygosity = 2q(1-q).

[b]Significance level calculated by χ^2 test of Hardy-Weinberg equilibrium.

"NS" Indicates not significant.

Sequencing Results for Exon 13

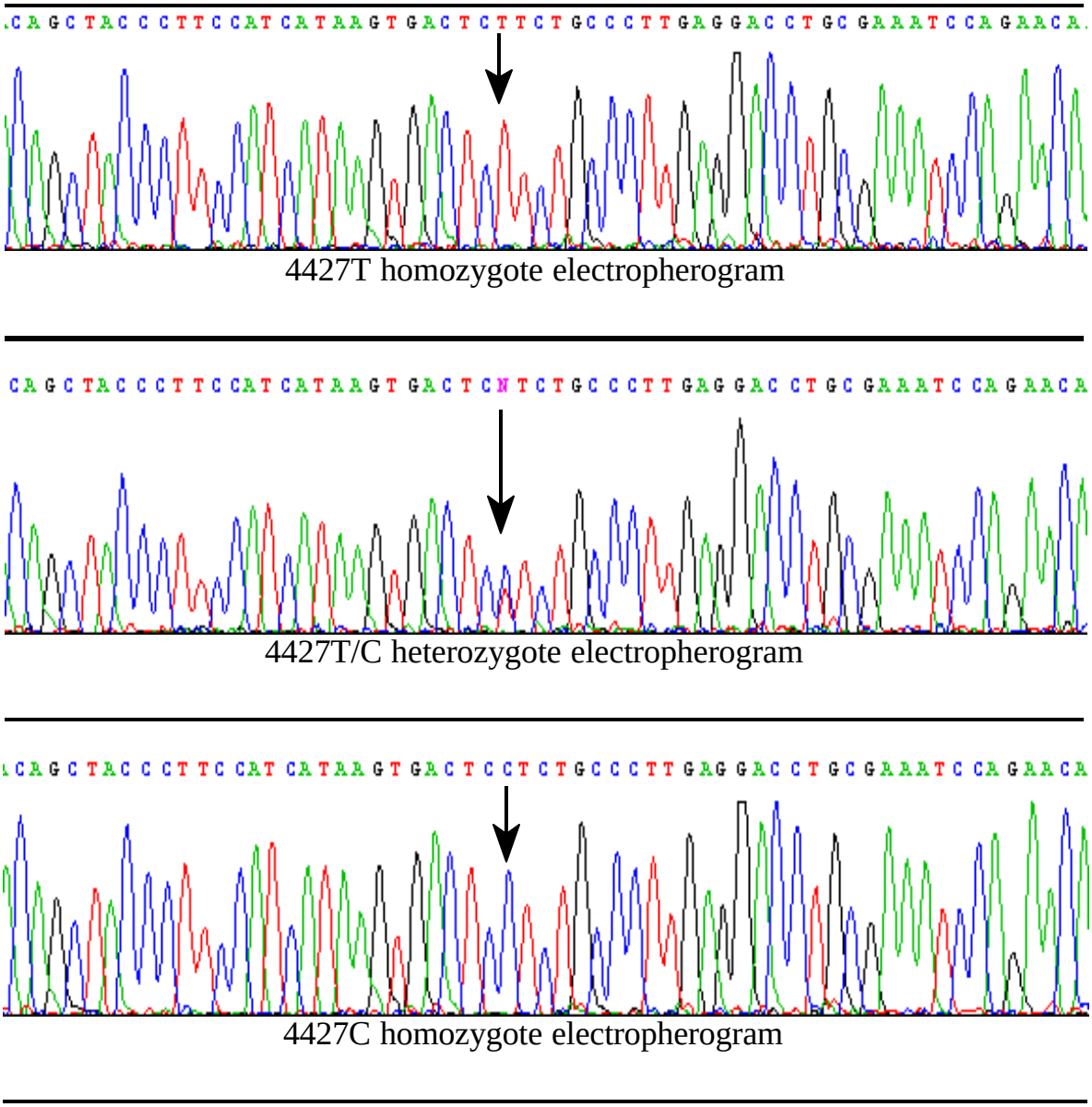
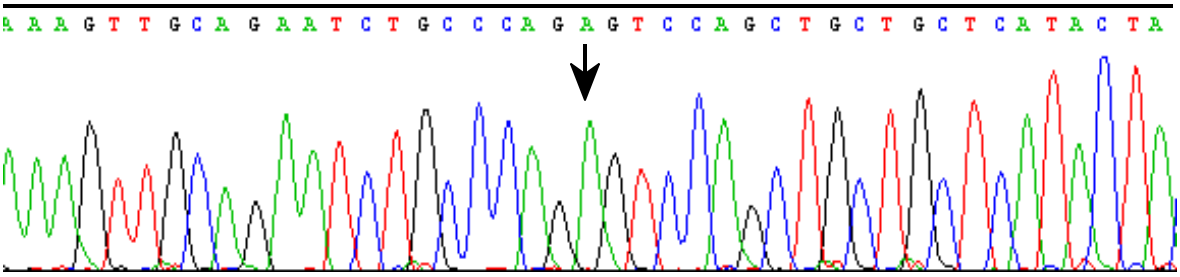
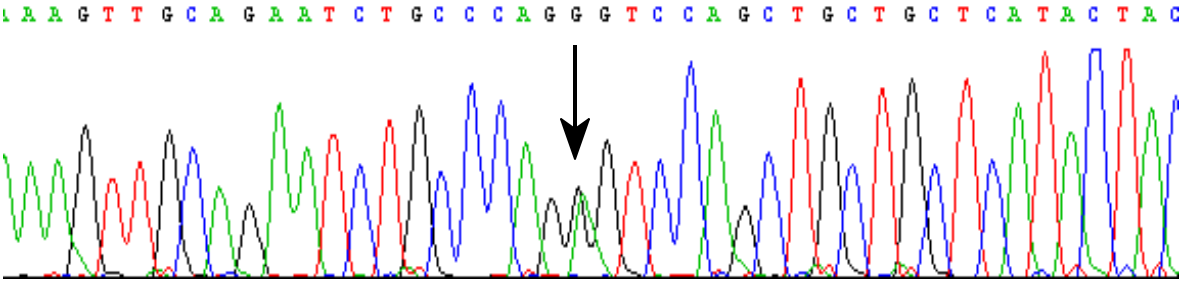


Figure 4: Electropherograms of sequencing results showing the three possible genotypes for the 4427T/C polymorphism in exon 13.

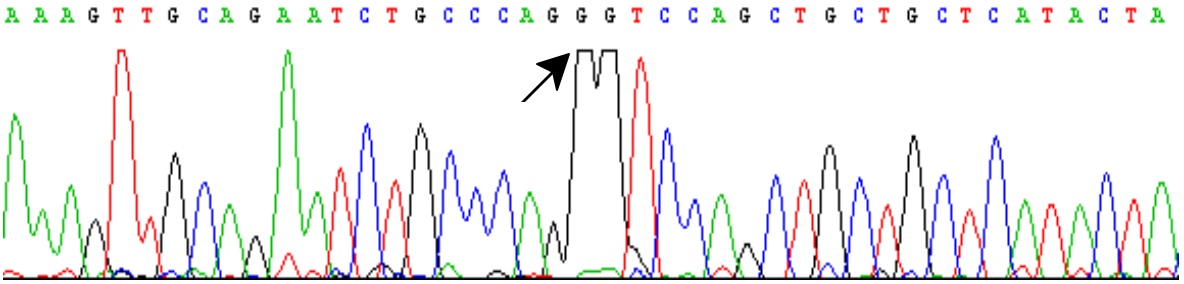
Sequencing Results for Exon 16



S1613 homozygote electropherogram



S1613G heterozygote electropherogram



1613G allele homozygote electropherogram

Figure 5: Electropherograms of sequencing results showing the three possible genotypes for the S1613G polymorphism in exon 16. The 1613G electropherogram displays a different peak pattern overall because an alternate sequencing chemistry was used.

Sequencing Results for Exon 20

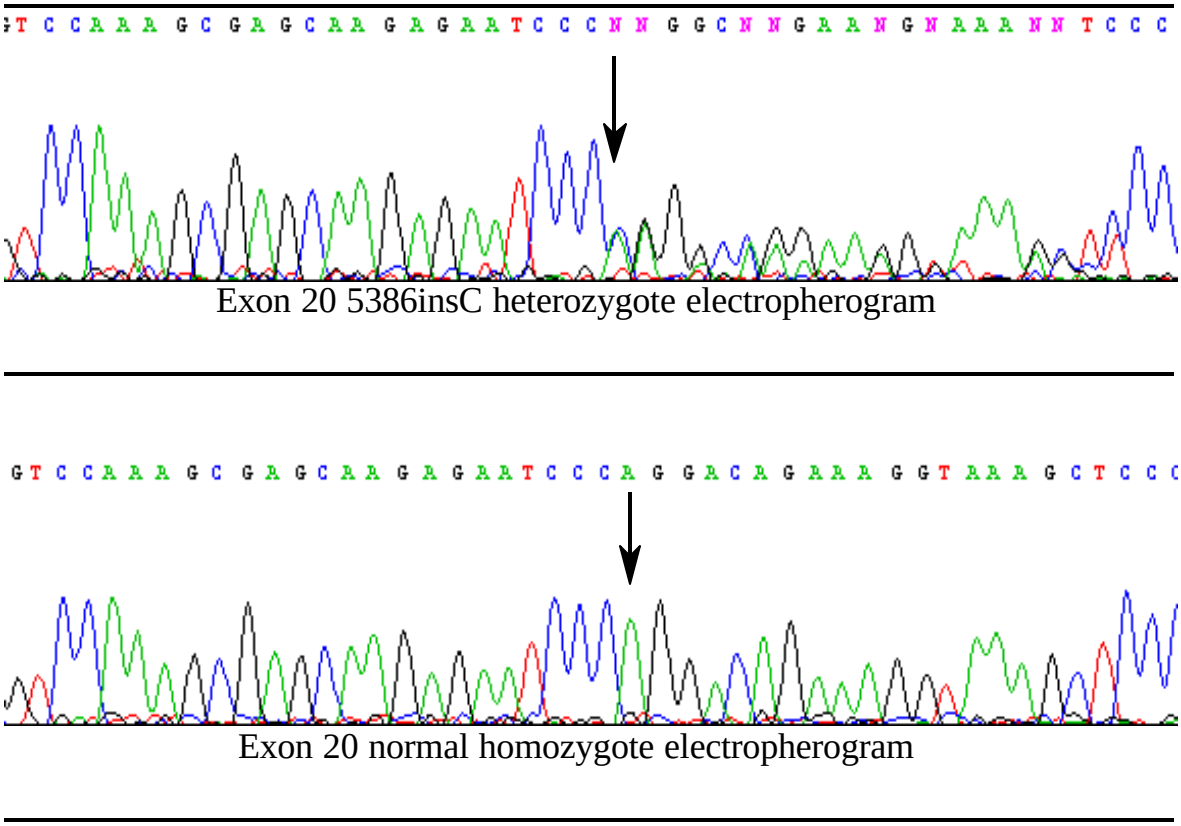


Figure 6: Sequencing results showing electropherograms for the 5386insC mutation and the wild type allele. Note that the sequence trace downstream of the insertion site in 5386insC contains several double peaks. This is due to the one base frameshift in the chromosome with the mutation.

SSCP Analysis: S1613G Polymorphism

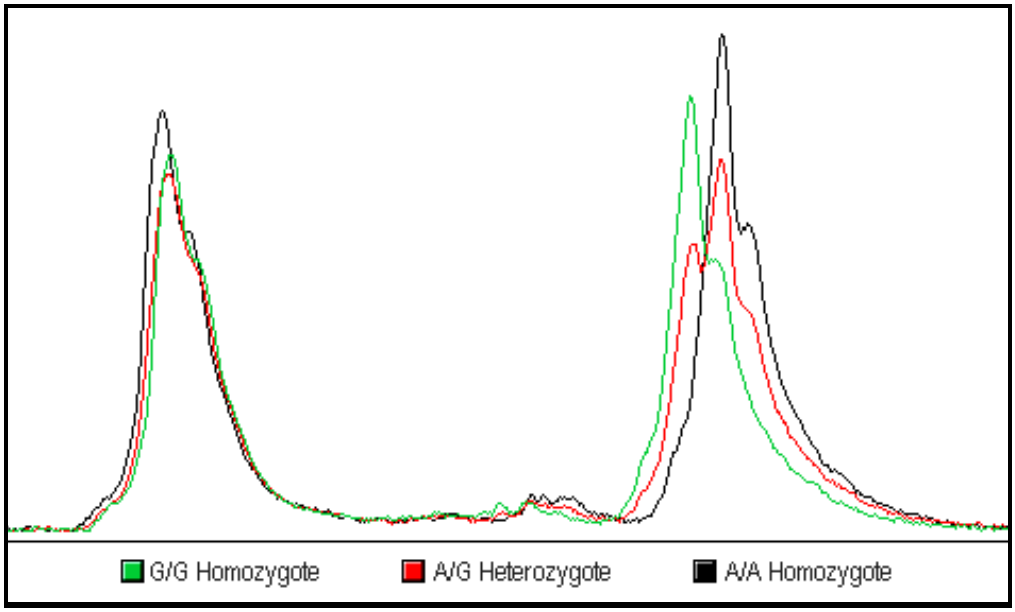


Figure 7: SSCP results for exon 16 showing the different peak patterns of the three possible genotypes.

SSCP Analysis: 4427T/C Polymorphism

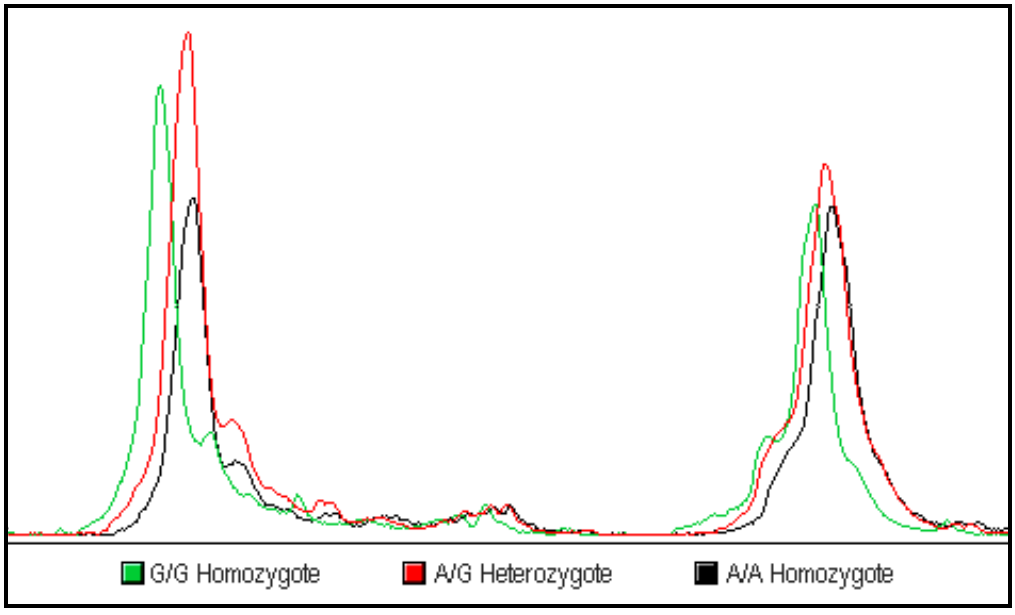
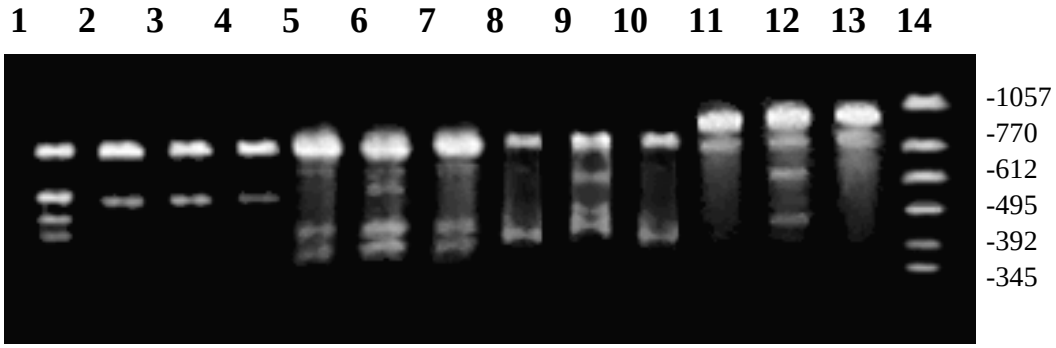


Figure 8: SSCP results for exon 13 showing the different peak patterns of the three possible genotypes.

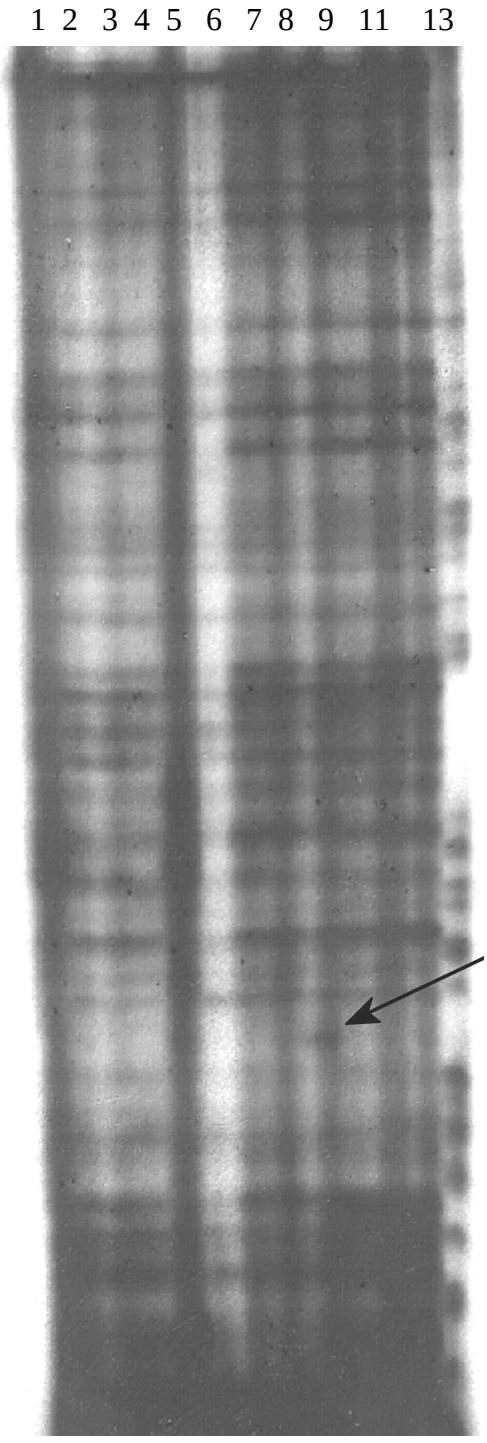
Composite NIRCA Results for Exon 11



Lane #	Sample description
1	Ex. 11-A positive control
2	Ex. 11-A #1
3	Ex. 11-A #2
4	Ex. 11-A negative control
5	Ex. 11-B #1
6	Ex. 11-B #2 (heterozygote)
7	Ex. 11-B negative control
8	Ex. 11-C #1
9	Ex. 11-C #2 (heterozygote)
10	Ex. 11-C negative control
11	Ex. 11-D #1
12	Ex. 11-D #2 (heterozygote)
13	Ex. 11-D negative control
14	ϕ X-174 RF DNA-Hinc II digest size standard

Figure 9: Typical NIRCA agarose gel results, compiled from representative gels. Samples #1 and #2 are from different patients; #1 has wild type sequence, while #2 has the 2430T/C, L871P, E1038G and K1183R polymorphisms. All samples were digested as described with RNase #2 prior to gel analysis. Note that all exon 11 segments contain cleavage sites, resulting in bands that could be interpreted as a sequence variation. However, the presence of a matching pattern in the negative controls indicate that the cleavage is nonspecific. Further evidence for this conclusion is that treatment with RNase #1 and #3 do not result in cleavage products in exon 11a or 11b products (results not shown).

BESS T-Scan Results for *BRCA1* Exon 24



Lane #	Sample description
1-12	Exon 24, samples 1 through 12
13	Positive control

Figure 10: Representative autoradiograph of BESS T-scan results for *BRCA1* exon 24. The positive control was supplied by Epicentre, and has the expected banding pattern. The extra band in lanes 9 and 12 were proven to be spurious by sequencing.

Exon 13 and 16 Genotype and Age of Onset Distribution in Somatic Cell DNA Samples

Genotype (Exon 13 and 16)	Number of patients	Mean age of diagnosis	Median age of diagnosis
T/T and A/A homozygote	20	59	61.5
T/C and A/G heterozygote	28	54.7	57
C/C and G/G homozygote	4	53.2	49.5

Table 4: Somatic cell DNA samples were divided by genotype and the mean and median ages of diagnosis compared by ANOVA. No significant differences were found (p= 0.37 for mean and p= 0.44 for median). Note that the 4427T/C and S1613G polymorphisms (exons 13 and 16, respectively) are in complete pair-wise linkage disequilibrium (Table 3).

II. *BRCA1* sequence variants in tumor cell DNA:

A large number of heterozygotes for the 4427C/T and S1613G polymorphisms were observed in the somatic cell DNA samples from the NMWHS. Therefore, exons 13 and 16 were completely sequenced in the 32 tumor cell DNA samples, without prior SSCP analysis. Figures 4 and 5 show representative sequencing results for these two exons. The two polymorphisms identified (4427T/C and S1613G) were significantly out of Hardy-Weinberg equilibrium in these DNA samples (Table 3). In order to determine if this lack of equilibrium was specific for the tumor tissue, paraffin blocks containing peripheral normal tissue (determined by cytological evaluation) from the same patients that had supplied the tumor tissue were located and sequenced. Of the 7 normal tissue blocks available, there was 100 percent agreement in genotype between the normal and paired tumor tissue. Comparison of the genotype distribution between all non-paired tumor and normal peripheral tissue by Fisher's exact test indicated no significant differences (p= 0.58). No additional sequence variations were found in exons 13 or 16 in the tumor cell samples, with 37,400 nucleotides sequenced.

Summary: The two polymorphisms previously identified in exons 13 and 16 of the somatic cell DNA samples were also found in archival tumor cell DNA samples and normal peripheral archival tissue. These two polymorphisms were not in Hardy-Weinberg equilibrium (Table 3), and the lack of equilibrium was not correlated with samples being from tumor tissue. No other sequence variations were identified in exons 13 and 16 of the tumor cell samples.

Comparison of genotype distributions in somatic and tumor cell DNA samples

Somatic cell DNA samples were found to be in Hardy-Weinberg equilibrium for the 4427T/C and S1613G polymorphisms, while the tumor cell DNA samples were not. Fisher's exact test was used to determine if the somatic and tumor cell DNA samples had the same distribution of the three possible genotypes at the two polymorphic sites (Table 5). This comparison showed a slightly significant difference between the two groups (p= 0.04).

Summary: The 4427T/C and S1613G genotype distribution between the somatic and tumor cell DNA samples is significantly different (Table 5).

Comparison of Genotypes in Somatic and Tumor Cell DNA Samples

	Polymorphism			Number of tumor samples	Number of somatic samples
	4427T/C	S1613G			
Genotype:	**T/T**	**A/A**	**Observed:**	**6**	**24**
			Expected:	11.3	18.1
			Percent of Total:	18.8%	45.3%
Genotype:	**T/C**	**A/G**	**Observed:**	**23**	**25**
			Expected:	18.1	30.4
			Percent of Total:	71.9%	47.2%
Genotype:	**C/C**	**G/G**	**Observed:**	**3**	**4**
			Expected:	2.6	4.5
			Percent of Total:	9.4%	7.6%

Table 5: Comparison of the distribution of the 4427T/C and S1613G genotypes between the somatic and tumor cell DNA samples. Fisher's exact test indicated a slightly significant difference (p= 0.04). Expected values and percent of total calculated with SAS. Note that the two loci are in complete linkage disequilibrium in somatic and tumor cell DNA samples.

III. *BRCA1* polymorphisms in age-adjusted samples:

The patients from the NMWHS were Hispanic women living in New Mexico who had breast cancer and at least one first degree relative with breast cancer. The tumor paraffin blocks came from Hispanic women living in New Mexico who had breast cancer diagnosed before the age of 46. In addition, the 4427T/C and S1613G polymorphisms were both in 100% linkage disequilibrium in these two groups (Table 3). Thus the two groups were deemed similar enough to combine for analysis of these two alleles.

Polymorphisms 4427T/C and S1613G and age distribution

The archival tumor tissue was significantly out of Hardy-Weinberg equilibrium (Table 3) for the 4427T/C and S1613G loci, while the somatic cell samples were not. The mean age of diagnosis for the

somatic samples was 55, while the mean for tumor samples was 38. Therefore, the relationship of these polymorphisms in respect to age of onset was investigated further by pooling the somatic and tumor cell DNA results and distributing by age of onset. Table 6 summarizes the comparison between women with an age of onset after 45, and those diagnosed at 45 years or younger. There is a significant difference (p= 0.0018, Fisher's exact test) in the distribution of the three genotypes between those diagnosed at or prior to 45 years and those diagnosed after 45 years of age. The comparison between the three genotypes and age of onset (as done in the somatic cell samples, see Table 4) for all patients (tumor and somatic DNA) is shown in Table 7. The mean ages of diagnosis for the three genotypes are significantly different (p= 0.036, 1-way ANOVA). Non-parametric methods used to analyze the median ages gave similar results. Further analysis of the data by Tukey-Kramer HSD revealed that the significant difference within the comparison of the three groups was between the T/T-A/A and T/C-A/G genotypes. The odds ratios of early diagnosis in relation to genotype at the 4427T/C and S1613G loci are shown in Table 8, using the common genotype T/T-A/A as the reference. Women who have the T/C-A/G genotype have odds 4.91 times the odds of the reference genotype to be diagnosed with breast cancer before the age of 45 (p= 0.003), while those who have the C/C-G/G genotype have an odds ratio of 7.66 (p= 0.051). Exact logistic regression was used to calculate the odds ratio because of the small sample size (LogXact 2.0 from Cytel Software Corp.).

Summary: The distribution of the 4427T/C and S1613G alleles in women who are diagnosed with breast cancer at or before 45 years is significantly different (p= 0.0018) from those diagnosed after age 45. Women with the T/T-A/A genotype are diagnosed at a mean age of 56.5, while those with the T/C-A/G genotype are diagnosed at 48.7 and those with the C/C-G/G genotype at age 46. Women with the T/C-A/G genotype have odds 4.91 times more than the odds of the T/T-A/A genotype to be diagnosed early.

Age Distribution of the 4427T/C and S1613G Polymorphisms

	Polymorphism			Number diagnosed	Number diagnosed
	4427T/C	S1613G		<45 years	>45 years
Genotype:	**T/T**	**A/A**	**Observed:**	**7**	**23**
			Expected:	14.5	15.0
			Percent of total:	17.1%	52.3%
Genotype:	**T/C**	**A/G**	**Observed:**	**29**	**19**
			Expected:	23.2	25.2
			Percent of total:	70.7%	43.2%
Genotype:	**C/C**	**G/G**	**Observed:**	**5**	**2**
			Expected:	3.4	3.7
			Percent of total:	12.2%	4.6%

Table 6: Somatic and tumor cell DNA samples were pooled and then divided into two groups according to age of onset. Expected values and percent of total calculated with SAS. The genotype distribution between these two groups is significantly different (Fisher's exact test, p= 0.0018).

Exon 13 and 16 Genotype and Age of Onset Distribution in combined DNA Samples

Genotype (Exon 13 and 16)	Number of patients	Mean age of diagnosis	Median age of diagnosis
T/T and A/A homozygote	30	56.5	59
T/C and A/G heterozygote	48	48.7	45
C/C and G/G homozygote	7	46	44

Table 7: Somatic and tumor cell DNA samples were divided by genotype and the mean and median ages of diagnosis compared by ANOVA. A significant difference was found (p= 0.036) in the age of onset between the three groups.

Odds Ratios of Early Diagnosis in Relation to Genotype

	Polymorphism		Exact odds ratio of	95% confidence	P-value
	4427T/C	S1613G	diagnosis <45 years	interval	
Genotype:	T/T	A/A	1.00	NA	NA
Genotype:	T/C	A/G	4.91	1.64 - 16.39	0.003
Genotype:	C/C	G/G	7.66	1.0 - 97.27	0.051

Table 8: Comparison between the three observed genotypes and the odds ratio of early breast cancer diagnosis. The T/T-A/A genotype was used as the reference.
"NA" indicates not applicable.

Discussion

Mutations in *BRCA1*

The finding that there were no mutations in the 53 somatic cell DNA samples examined supports the hypothesis of a lower *BRCA1* mutation frequency in Hispanics. It also supports the findings of others who have studied breast cancer in Hispanic women of other geographic locations (Bondy *et al.*, 1992, Weiss *et al.*, 1986, Polednak, 1996). The study by Bondy *et al.* concluded that Hispanics referred to the University of Texas M. D. Anderson Cancer Center had a deficit of familial breast cancer cases when compared to White and Black patients from the same hospital. Of the Hispanic patients enrolled in the study, only 2 percent reported an affected first-degree relative, while 10 percent of the White and 15.7 percent of the Black patents reported having an affected first-degree relative. Weiss *et al.* (1986) found that, while there was a degree of familiality in Hispanic breast cancer cases, it was much less pronounced than in other ethnic groups. More recently, Diez *et al.* (1999a) reported that Spanish breast cancer families have an overall lower frequency of *BRCA1* involvement than other ethnic groups studied in the same geographic area.

While the current study supports these findings, the possibility of observing a false negative result cannot be excluded due to the small sample size. Another factor contributing to the ambiguity of the results is the relatively late age of onset of the women studied. It is not likely that mutations were missed in the areas of *BRCA1* that were analyzed, since other sequence variations were easily detected in those regions. However, it is possible that other genes are involved as well, such as *BRCA2, HER-2* or *p53*, or that there are mutations in the non-coding areas of *BRCA1* that were not analyzed. Thorlacius *et al.* (1996) reported that only nine percent of Icelandic women with familial breast cancer have *BRCA1* mutations, while 62 percent have mutations in *BRCA2*. An investigation of Dutch breast cancer patients found 36 percent of the *BRCA1* mutations identified in their population were large genomic deletions that could not be detected by conventional methods that look at relatively short genomic segments, such as SSCP, sequencing, or other PCR-based assays, due to the inability to determine if a patient was homozygous or hemizygous (Petrij-Bosch *et al.*, 1997). Further investigation of New Mexico Hispanic populations will be required to evaluate these possibilities.

The observations of the present study support the hypothesis that *BRCA1* mutation frequencies vary between different populations. This is not unexpected, given the ethnic variance of breast cancer rates in general (Figure 1). Other studies have found higher frequencies of breast cancer than the current study in other ethnic groups using patients with similar parameters, as shown in Table 9. The estimated frequency of *BRCA1* mutations in moderate risk Caucasian families with more than one affected sister is between 7 and 21 percent (Couch and Hartmann, 1998, Langston *et al.*, 1996, Malone *et al.*, 1998, Fitzgerald *et al.*, 1996). The findings of the present study imply that the mutation frequency could be significantly less for the population of New Mexico Hispanics sampled (Table 9). Of particular interest are the findings from Japan and Iceland; both populations that are relatively homogeneous, have a low frequency of breast cancer, and a correspondingly low frequency of *BRCA1* mutations (Emi *et al.*, 1998, Inoue *et al.*, 1995, Barkardottir *et al.*, 1995, Thorlacius *et al.*, 1996). The findings of Diez *et al.* (1999) and Newman *et al.* (1998) would indicate that there are some populations that have a very low or undetectable *BRCA1* involvement in breast cancer (Table 9). Diez *et al.* also proposed that the ethnic influence of the population studied has direct bearing on the type and frequency of *BRCA1* mutations. Taken together, it is clear that a woman's ethnic background

needs to be considered when genetic testing of *BRCA1* is undertaken. However, the findings of this study indicate that this population probably would not benefit from routine genetic screening for *BRCA1* mutations.

It was anticipated that the 185delAG mutation found in the Ashkenazim might be encountered in the present study, given that there is a possibility of crypto-Jewish ancestry in New Mexico Hispanics (Tobias, 1990, Moya, 1996). This is supported by the recent finding of the 185delAG and 5382insC mutations in a European Spanish population by Diez *et al*. (1999). The absence of this specific mutation would indicate that either the population sampled did not have crypto-Jewish ancestry, or that this ancestry is probably not extensive enough to be identified in a population sample as small as this one. Both of these situations are likely, therefore any definite conclusions cannot be drawn from a negative result in this instance.

Germline *BRCA1* Mutations in Select Populations

Population Studied	Number Of Cases Screened	Percent With Germline Mutations	Reference
New Mexico[a]	53	0	Present Study
Seattle, WA[ab]	208	7.2	Couch & Hartmann, 1998
North Carolina, White[a]	104	6.6	Newman *et al.*, 1998
North Carolina, Black	88	0	Newman *et al.*, 1998
Boston, MA Jewish women[b]	39	21	FitzGerald *et al.*, 1996
Paris	103	15	Stoppa-Lyonnet *et al.*, 1997
Belgium	30	10	Sibillehoang *et al.*, 1998
Italy[a]	43	16	Montagna *et al.*, 1996
Italy[a]	53	9	Santarosa *et al.*, 1998
China	130	3.8	Tang *et al.*, 1999
Spain[b]	159	1.9	Diez *et al.*, 1999

Table 9: A compilation of the percentage of *BRCA1* mutations found in other populations by studies with parameters similar to the present investigation.
[a] Cases had first-degree relative with breast cancer.
[b] Cases were diagnosed with breast cancer before 45 years of age.

Polymorphisms in *BRCA1*

This is the first sequence analysis of *BRCA1* to be done in New Mexico Hispanics, so there are no previous reports of polymorphisms or rare sequence variants with which to compare results. Other investigators have reported the same polymorphisms that were found in this study (Table 2), as well as several that were not observed. The allele frequencies of the 9 different polymorphisms found in the somatic cell DNA samples (Table 3) are similar to the frequencies found in other studies, such as those by Durocher *et al.* (1996), Dunning *et al.* (1997) and Inoue *et al.* (1995). However, more recent investigations have found these alleles to have a much lower frequency in other populations (Southey *et al.*, 1999, Tang *et al.*, 1999). It is also apparent that not only do the allele frequencies vary between populations, but the pattern of linkage between the individual polymorphisms differs as well. While some of the linkages found in this New Mexico Hispanic population (Table 3) have been reported previously (Southey *et al.*, 1999), no other population has shown the same pattern of linkages. However, this appears to be true of other populations as well, with all sharing some polymorphisms, but each population having a unique set of patterns within itself. The main patterns that were observed in this study were the 100 percent linkages between the 2201C/T and 2430T/C polymorphisms, the

4427T/C and S1613G polymorphisms, and the K1183R and 5272+66A/G polymorphisms. In most cases, these three pairs were also in linkage disequilibrium with each other, as shown by their allele frequencies in Table 3. Overall, there were fewer polymorphisms in the Hispanic population, though this could be an artefact of the population sample studied. The design of this study is not such that linkage analysis could be performed using the nine polymorphisms identified (Table 2), so no real conclusions can be made regarding the various patterns of polymorphisms identified and their possible role, if any, in breast cancer development. However, such a study was performed by Dunning *et al.* (1997), who found evidence that a unique polymorphism pattern was associated with a reduced frequency of breast cancer, indicating that a follow-up study of the specific linkage patterns of the polymorphisms identified here would be prudent.

During the process of sequencing the tumor cell DNA samples, it became apparent that there was an unusual abundance of heterozygotes for the 4427T/C and S1613G polymorphisms. These alleles have previously been shown to be in Hardy-Weinberg equilibrium (HWE) in populations of European descent by Durocher *et al.* (1996). As shown in Table 3, the heterozygosity found in the somatic cell DNA samples is what would be expected of a population in HWE. However, the heterozygosity of the tumor cell DNA samples, which were from women with early onset breast cancer, was well above what would be expected, indicating that the population was not in HWE for these genotypes.

The use of HWE allows the observed heterozygosity of a population to be compared with what would be expected if the population is sufficiently large, mating randomly, has no net change in the gene pool due to mutation, no migration into or out of the population, and has no selective pressure for the allele in question. There is no reason to suspect that the population sampled was not sufficiently large or not mating randomly for the genotypes studied, and the lack of observed mutations would suggest that the third assumption is being met as well. Thus the only assumptions in question are if there is a large amount of migration into or out of the population, or if there are selective pressures for the 4427T/C and S1613G genotype. Because the amount of migration in the population studied was not measured, it is difficult to say if this assumption is satisfied. However, given that both the somatic cell and the tumor cell DNA samples came from the same general population, it would be expected that any migratory effects would be the same for both groups. It is possible that the increase in heterozygosity is the result of admixture in the population sampled, which is consistent with an observed deviation from HWE due to an increase in the number of heterozygotes. However, it is not likely that any admixture present would only be apparent in the tumor and not the somatic cell samples, given their common source. Therefore, the assumption that most likely is not being satisfied is that of possible selective pressures exerted on these alleles in this population. If this assumption is false, it could explain the observed lack of HWE in the tumor cell DNA samples.

The apparent association of increased heterozygosity and early age of onset prompted the investigation of the 4427T/C and S1613G polymorphisms in relation to the patient's age of onset in the previously sequenced somatic cell DNA samples, which is summarized in Table 4. Because these two polymorphisms are completely linked in this population, they are grouped together for data analysis. It is apparent that there are differences in the age of diagnosis between the three genotypes, though this difference is not statistically significant. This lack of significance does not, however, mean the results are not clinically relevant. It is likely that the lack of significance is related to the sample size, for when the somatic and tumor cell DNA samples are grouped together, a clearly significant difference is observed

(Table 7). As this table summarizes, The T/C-A/G genotype is diagnosed 7.8 years earlier, and the C/C-G/G genotype 10.5 years earlier, than those with the T/T-A/A genotype, on average (p= 0.036). Further analysis of the three genotypes and age of onset by exact logistic regression (Table 8) revealed that women who have the T/C-A/G genotype have odds 4.91 times the odds of the T/T-A/A genotype to be diagnosed with breast cancer before the age of 45 (p= 0.003), while those who have the C/C-G/G genotype have an odds ratio of 7.66 (p= 0.051). It should be noted that the very small number of patients with the C/C-G/G genotype limits the conclusiveness of these results in relation to that particular group. A larger sample size will be needed to confirm these results. However, the results obtained when comparing the other two genotypes are clearly significant, as shown by the one-way ANOVA, Tukey-Kramer HSD analysis and exact logistic regression to estimate odds ratios. Non-parametric analysis using the median ages of these groups also supports this finding.

At this point, it is unclear if it is the loss of the T-A allele or the gaining of the C-G allele that is responsible for the earlier age of onset. While the parameters of the present study cannot address this question directly, it appears that there is no real difference between the T/C-A/G and C/C-G/G genotypes, indicating that either the C-G allele is dominant in its effects, or that the loss of one T-A allele is sufficient to reduce the functionality of BRCA1 and decrease the age of onset. The process by which these effects are accomplished is beyond the scope of this investigation, though it has been shown that breast tumors with no apparent *BRCA1* mutations have significantly lower levels of *BRCA1* expression (Thompson *et al.*, 1995), indicating that there could be a gene dosage effect with the loss or inactivation of one good allele. The data presented in Tables 4 and 5 suggest that it is the C-G allele which increases the risk of early onset breast cancer. In Table 5, there is an obvious lack of T/T-A/A genotypes in the tumor samples (those with early onset), comprising 18.8 percent of the total, while in somatic samples (late onset) 45.3 percent of the genotypes are T/T-A/A. This difference is more pronounced in Table 6, where the T/T-A/A genotype is only 17.1 percent of the total in the early onset group, and 52.3 percent of the late onset while the T/C-A/G genotype comprises 70.7 percent and 43.2 percent, respectively. Unfortunately, the small number of patients with the C/C-G/G genotype makes it impossible to determine if there is a clear distinction between that group and the T/C-A/G group as well. The observation that there are still a fair number of T/C-A/G genotypes in the late onset group supports the theory that the C-G allele is linked to other factors that effect age of onset, and is not the causative agent alone. Thus some women can have the C-G allele, but not be at risk for early onset of breast cancer. It is therefore more likely that it is the C-G allele that increases the risk of breast cancer development prior to age 45, though it is probable that other linked factors are important, or that the risk is influenced by gene dosage.

Other studies have found borderline correlation with some of the polymorphisms identified in *BRCA1* and an increased risk of breast cancer, while others have found no relation, or even a slight protective effect (Dunning *et al*., 1997, Durocher *et al.*, 1996, Monteiro *et al.*, 1997). No other investigators have reported such a striking linkage between these polymorphisms and early age of onset in breast cancer, though a recent report by Janezic *et al.* (1999) has identified a significant association with the Q356R polymorphism and a family history of ovarian cancer. Previous studies that have looked specifically at these polymorphisms have used populations that have a higher frequency of breast cancer in general, and usually have chosen families out of those populations that are at even greater risk. By looking at a population that has a much lower frequency of breast cancer, it is possible that subtle linkages and associations that would go unnoticed in a “high risk” population are more

apparent. Indeed, epidemiological data suggest that the majority of breast cancer cases might be caused by common variants that confer a moderate risk of developing the disease rather than a few highly penetrant genes (Ford *et al.*, 1995). These common variants would be more apparent when looking in populations that have a low frequency of highly penetrant genes such as *BRCA1*.

If there is a correlation between these polymorphisms and tumorigenesis, it would be expected that tumors would protect them from loss or inactivation, and thus assist tumor progression. As discussed earlier, *BRCA1* functions as a classic tumor suppressor, and is often involved in LOH at 17q21 in breast carcinomas (Szabo and King, 1995). The selection of one genotype over another indicates that genotype is permissive to tumor formation. By comparing genomic DNA extracted from somatic and tumor tissue in the same patient, it is possible to determine if LOH for a given loci has occurred. When LOH is determined by a sequencing reaction, a heterozygote for the given genotype in somatic DNA would appear to be a homozygote in the tumor DNA, though the tumor would actually be hemizygous due to the loss of one allele. As shown in Tables 4 and 6, there was no increase in observed homozygosity in the tumor samples, indicating that the 4427T/C and S1613G polymorphisms are not involved in tumorigenesis through LOH due to genomic deletions. It is possible that the LOH actually occurs through another mechanisms, such as inactivation or down-regulation, though this is not commonly observed in *BRCA1*-mediated tumors.

The apparent association of the 4427T/C and S1613G polymorphisms with early-onset breast cancer would appear to be at odds with the lack of LOH occurrence in the tumor samples. If the association is real, then why are these polymorphisms not selected for? The most apparent explanation is that these polymorphisms are in fact benign in nature, as has been previously suggested (Durocher *et al.*, 1996), but are in tight linkage with one or more carcinogenic factors located nearby on chromosome 17q21. Liu and Barker (1999) identified several variants within the *RNU2* locus that are very strongly associated with a collection of polymorphisms in exon 11 of *BRCA1*, over 150 kb away. This theory also is supported by a multitude of studies that have found a paucity of *BRCA1* mutations when studying women with familial breast cancer who showed convincing evidence of linkage to chromosome 17q21 (Friedman *et al.*, 1994, Narod *et al.*, 1995, Szabo and King, 1995, Greene, 1997, Sorlie *et al.*, 1998, Catteau *et al.*, 1999a). If these polymorphisms are indeed linked to a mutation, then they would appear to be associated with early onset through their linkage disequilibrium to the actual causative loci, but would not necessarily be involved in any LOH events involving that loci.

The extreme lack of recombination in this area of chromosome 17q21 has been discussed by Liu and Barker (1999), who postulate that the suppression of recombination in this area might be related to the involvement of BRCA1 in genomic recombination and DNA repair pathways, and that transcriptional activity during meiosis may prevent recombination, or specific mechanisms may exist to regulate recombination in regions encoding proteins directly involved in meiosis and genetic recombination. Many reports of *BRCA1* mutations in a wide variety of ethnic groups also have included descriptions of the almost complete linkage disequilibrium found in this region (Dunning *et al.*, 1997, Durocher *et al.*, 1996, Tang *et al.*, 1999, Inoue *et al.*, 1995, Couch *et al.*, 1996, Li *et al.*, 1999a, Southey *et al.*, 1999), mainly involving common polymorphisms such as the ones described here. It is possible that, individually, these polymorphisms have little effect, but when acting in combination with other sequence variants these polymorphisms are etiologically significant. Several of the polymorphisms involve amino acid changes that could affect how BRCA1 interacts with other proteins, without changing the structure of BRCA1 itself. Given the complexity of protein-protein interactions involving

BRCA1, it is likely that while these polymorphisms do not seem to affect normal BRCA1 structure *in vitro*, they could affect the interacting sites of the many proteins that are involved directly or indirectly with BRCA1 *in vivo*. As discussed earlier, BRCA1 contains several methylation sites that have been implicated in transcription regulation. Another possibility is that one or more of these polymorphisms could change the methylation status, and thus the regulation, of *BRCA1*.

As shown in Table 7, there is a distinct difference in the age of diagnosis between women with the more common T/T-A/A genotype and those who have the T/C-A/G or C/C-G/G genotype. Furthermore, there is a significant risk associated with these two genotypes (Table 8). While the involvement of these genotypes in this decrease in age is not known, the nucleotide differences can still be used as biomarkers to assist in genetic testing for those at risk of developing breast cancer. Use of these markers could alert physicians to women who need more rigorous screening procedures, though it is likely that the ethnic background of the patient would dictate how useful these markers would be. However, such tests would require extensive testing of the ethnic groups involved before these polymorphisms could be utilized in such a manner. A more feasible and direct application of these polymorphisms would be in screening for germ-line deletions of large portions of *BRCA1*. As reported by Petrij-Bosch *et al.* (1997) and Puget *et al.* (1999), these large germ-line deletions appear to be common in some populations, making the more common screening methods, such as SSCP and sequencing, impractical due to the inability to distinguish a homozygote from a hemizygote. The extreme pair-wise linkage disequilibrium of these and other polymorphisms in this region (Table 3) make them useful not only as SNPs (Single Nucleotide Polymorphism), but as markers to determine LOH. If a deletion involving one linked polymorphism occurred in a heterozygote, it would be readily apparent in the discrepancy of one of the linked polymorphisms being hemizygous and the other heterozygous. LOH would not be as easily detected in the case of a homozygote, but this could be addressed by picking additional SNPs in the region that are heterozygous, of which there are several.

Comparison of methodologies

As shown in Table 1, in every instance where a method other than sequencing detected a sequence variation, it also was confirmed by sequencing. This ensured adequate sensitivity and provided a common procedure to validate results acquired by several different methods. By using direct sequencing methods with PCR product samples, it is possible to scan the individual exons of *BRCA1* for known mutations, as well as search for new ones. This method is more labor-intensive than others currently in use, such as single-strand conformational polymorphism analysis (SSCP) or the protein truncation test (PTT), but it has the ability to detect any type of mutation that might be present in the PCR product, so that the likelihood of false negative or positive results are minimal. The small size of most of the *BRCA1* exons (Figure 11) makes sequencing of individual exons feasible, if somewhat expensive.

Analysis of the sequencing electropherogram directly, instead of the sequence text output, made detection of sequence variances simple, and allowed for comparison of electropherogram patterns to verify the sequencing reaction integrity. As can be seen in Figures 4, 5 and 6, the electropherogram peak pattern is very consistent between individual samples. This inter-sample consistency is a good indicator that the sequence is valid, and also accentuates any sequence variation, such as the double peak that indicates a substitution (Figures 4 and 5) or the sudden drop in quality and change in peak pattern following an insertion or deletion (Figure 6). Any variations in electropherogram pattern from

the majority readily indicate that the sequencing result for that sample is suspect. This peak pattern consistency does not hold true when sequencing results are obtained using different reaction conditions. As is shown in Figure 5, the peak pattern of the bottom panel, which was obtained using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Taq FS Sequencing kit (PE/Applied Biosystems), bears little resemblance to the peak pattern of the two above it, which were sequenced using the PRISM™ dGTP Big Dye™ Ready Reaction Terminator Taq FS Sequencing kit (PE/Applied Biosystems). It is therefore imperative, when using peak pattern analysis to identify heterozygotes, only to compare electropherograms obtained using the same sequencing chemistry.

Exon Map of *BRCA1*

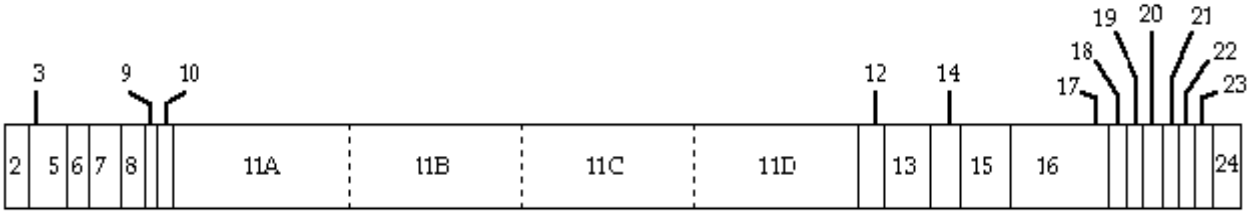


Figure 11: Graphic representation of *BRCA1* exon size, roughly to scale. Exon 11 is divided into four sections according to the four primer sets used for NIRCA (see methods). Exons 1 and 4 are spliced out of normal *BRCA1* mRNA, and so are not included.

SSCP was chosen to screen exons 13, 16 and 18 as a less expensive and time-consuming alternative to sequencing. While SSCP is not as sensitive as other methods, it is the mutation detection method of choice in most *BRCA1* studies because of the ability to quickly screen large numbers of samples of small PCR products. The employment of a fluorescent dNTP-based SSCP (F-SSCP) protocol further simplified the basic SSCP method by allowing for use of non-labeled primers, rapid detection and automated analysis. In this study F-SSCP proved to be a viable mutation detection technique, readily detecting polymorphisms in exons 13, 16 and intron 18 (Figures 7 and 8). The ability to look at small-sized PCR products makes SSCP superior to NIRCA for all of the *BRCA1* exons except 11 (Figure 11). By using the ABI 377 automated sequencer and Genescan® collection and analysis software, data analysis time was greatly reduced, and the complications of using radioactivity were avoided. It was also possible to control the gel temperature precisely from 4º C to 35º C, making multiple runs at various temperatures possible without changing any other conditions.

In Figures 7 and 8, representative SSCP results show typical peak patterns observed for exons 13 and 16. The three unique peak patterns for exon 16 PCR product are easily distinguished, as seen in Figure 7. The G/G and A/A homozygotes are distinguished by shifts in mobility, with the G/G homozygote having a slightly faster mobility, while the A/G heterozygote has a distinguishing precursor peak that forms a shoulder on the left side of the main peak. Data analysis in the case of exon 13 is not so straight-forward, with the distinguishing characteristic being a mobility shift and no unique peak pattern (Figure 8). In most instances it was possible to determine genotype by varying the run conditions, but for a few samples it was impossible to determine which genotype was present from the

exon 13 SSCP data alone, requiring more samples to be sequenced because of the SSCP result ambiguity.

NIRCA was used to screen for mutations in exon 11 (Table 1). Other investigators have used PTT or SSCP to scan this exon; however, PTT is only able to detect mutations that result in a truncated protein, and is estimated to be close to 80 percent accurate. SSCP involves extended gel running times as well as multiple gels per analysis with varying run conditions, though the main drawback in this case is that it requires ~25 reactions to cover exon 11 (Figure 11). It is estimated to be 80 to 90 percent accurate. In contrast, NIRCA has been shown to be greater than 95 percent accurate in identifying all types of sequence variances as small as one base, can cover exon 11 in only 4 reactions, and uses standard molecular biology lab equipment. The basis for the assay is that RNase will cleave single-stranded RNA, such as that found in the heteroduplex "bubble" that is formed when there is a mismatch in double-stranded RNA. These mismatches are created when a heterozygous RNA sample is heated and then quickly re-annealed. Three different RNases are used to increase sensitivity, since they have different mismatch recognition and cleavage properties (Ambion). Unfortunately, NIRCA does not work well for detecting mismatches in DNA fragments smaller than 400 base pairs, eliminating most of the *BRCA1* exons from this method (Figure 11).

The main disadvantages observed in the NIRCA methodology were the need to do three separate RNase digestions for each sample analyzed and the high background associated with some templates. Having to digest each sample in triplicate slowed down throughput significantly when large agarose gels were not used, and combining the three RNase digests into one reaction resulted in an unacceptable background of nonspecific cleavage. Satisfactory results were obtained using an agarose gel box capable of separating 60 samples (20 x 3) in approximately 45 minutes. As can be seen in Figure 9, there is a degree of nonspecific cleavage in all the exon 11a segments that were analyzed. Fortunately, the cleavage patterns are very reproducible, so sequence aberrations are apparent by comparing the gel pattern of the negative control to that of the samples (Figure 9).

BESS T-scan (Base Excision Sequence Scanning) was used to screen exon 24 as a low-cost method that was relatively rapid and simple. This is a relatively new method that has only been used once in published studies of *BRCA1* sequence variations (Hawkins and Hoffman, 1999). BESS T-scan relies on using a labeled primer and incorporation of dUTP during PCR. This is followed by treatment with a Uracil *N*-Glycosylase that removes the uracil base from the nucleotide, creating an abasic site. Subsequent treatment with Exonuclease IV cleaves the phosphodiester bond at the abasic site, generating a series of DNA fragments that produce a ladder pattern when separated by PAGE (polyacrylamide gel electrophoresis), as shown in Figure 10. Polymorphisms are indicated by a change in the ladder pattern when compared with one of a known sequence. While all insertions, deletions, and substitutions involving a Thymine are easily detected by this method, substitutions involving Adenine are more difficult. A base change is undetectable when it only involves both Cytosine and Guanine (G to C or C to G). Even with these limitations, BESS is estimated to detect 90 percent of all possible mutations. While BESS is technically simple and requires few steps, the use of radiolabeled primers makes it more time-consuming to do and requires a radioisotope use area within the lab. Also involved are the additional cost of use, storage and clean-up of radioactive material, as well as the extended film exposure time, often 16-24 hours, that is required before data can be analyzed. These draw-backs can be alleviated by the use of dye-labeled primers; however a specialized detection system is then required, such as is used in automated sequencing, as well as primers made and labeled specifically for

each PCR reaction. Initially, the original protocol was used to determine the usefulness of BESS in scanning for polymorphisms in exon 24 (Figure 10). After several attempts, the protocol was modified to give better results; however, false positive results were still quite frequent, increasing the number of samples that were sequenced. Another factor adding to the difficulty of the data analysis is the high thymine content of exon 24, which resulted in a large number of digestion products, increasing the time needed for analysis. After duplicating several BESS experiments, it was also apparent that PCR conditions heavily influenced the appearance of the final PAGE product, and that some poor-quality templates would not yield satisfactory results. Overall, BESS T-scan proved to be a moderately difficult protocol that required further optimizations and resulted in difficult data analysis. Modifying the protocol for use with flourescent dyes used in automated detection instead of radioisotope would facilitate easier data analysis.

Cel1 enzymatic cleavage was attempted for exon 11-a and exon 13, using the protocol published by Oleykowski *et al*. (1998), who reported that CEL 1 was able to detect all types of sequence variants in PCR products. This enzyme is a novel plant endonuclease isolated from celery that has promising characteristics, such as the ability to function at neutral pH and a high specificity for regions of DNA helical destabilization. In these studies, however, CEL 1 appeared to have sporadic activity, giving results that were not easily reproduced, and varied between identical samples within the same experiment. For example, a template of known sequence that was used as a positive control did not exhibit identical cleavage patterns when treated with CEL 1 in parallel experiments under identical conditions (data not shown). It is possible that the particular CEL 1 enzyme used was of sub-optimal quality, or was contaminated in some fashion. However, due to time constraints, another lot of CEL 1 was not able to be acquired and tested. It is also possible that the experimental conditions were not properly optimized for the control template that was used. Several preliminary experiments using CEL 1 on various PCR products resulted in cleavage products that appeared to be in the expected size range, indicating that the enzyme was able to identify and cleave some PCR products. Further method development will be needed to determine if CEL 1 is indeed a useful endonuclease for use in sequence variation detection protocols.

Overall, it was a combination of methods that yielded the best results with minimal effort. F-SSCP for small exons and NIRCA for larger ones, combined with confirmatory sequencing, produced the most consistent results with the easiest data analysis. However, this was not the most thrifty combination of methods due to the required use of an ABI 377 for the F-SSCP and sequencing. However, the offset in cost was easily compensated for by the increased speed of data acquisition and analysis.

Conclusions

This investigation has searched for mutations in *BRCA1* in a population of New Mexico Hispanic women with breast cancer using automated sequencing, SSCP, BESS T-scan and NIRCA. Exons 2, 5, 11, 13, 16, 18, 20, 21 and 24 were analyzed, along with their corresponding intron/exon boundaries, which comprise 93% of the reported mutations in the literature and the BIC database. Nine polymorphisms have been identified, all of which have been reported previously, while no mutations were found. The absence of mutations in *BRCA1* indicates that the observed variance in breast cancer rates between the New Mexico Caucasians and Hispanics is most likely influenced by genetic as well as environmental factors, and that this population is not likely to benefit from routine genetic screening

of *BRCA1*. Because polymorphisms were able to be identified, it is evident that the methodologies used would have been adequate to detect any mutations that might have been present. Of the nine polymorphisms that were identified, a novel association was observed with the heterozygosity of the two polymorphisms 4427T/C and S1613G and women who were diagnosed with breast cancer before age 45. Women with the T/C-A/G genotype are diagnosed 7.8 years earlier, and the C/C-G/G genotype 10.5 years earlier, than those with the T/T-A/A genotype, on average ($p= 0.036$). Women who have the T/C-A/G genotype have odds 4.91 times the odds of the T/T-A/A genotype of being diagnosed with breast cancer before the age of 45 ($p= 0.003$), while those who have the C/C-G/G genotype have an odds ratio of 7.66 ($p= 0.051$). A comparison of genotypes between DNA from somatic and tumor tissue indicated that the 4427T/C and S1613G polymorphisms were not selected for by LOH, indicating that the polymorphisms are likely in tight linkage with some other locus that is facilitating an early onset of breast cancer. These polymorphisms could potentially be used as markers for the detection of genomic deletions in *BRCA1* or in SNP linkage analysis.

Future Plans

In order to validate the initial observation that the 4427T/C and S1613G polymorphisms are linked in some fashion to an earlier age of onset, a study involving a significantly larger sample size of matched female cases and controls should be undertaken. This sample would be selected from the general population of New Mexico, and include cases and controls of all ages, with equal representation of both Hispanic and non-Hispanic whites. Particular attention must taken in recruiting women diagnosed with breast cancer prior to age 45, so that there is an equal representation of women with early and late onset.

The use of a high-throughput method, such as the ELISA- based oligonucleotide ligation assay (OLA) or allele-specific oligonucleotide hybridization (ASOH), specific restriction enzyme digestion or PCR using polymorphism-specific primers would make screening large numbers of samples quick and inexpensive.

Complete pedigree information should also be collected from those who show possible association between the two polymorphisms and age of onset. This pedigree information could then be used for linkage analysis to confirm the current study, as well as to determine if there are particular patterns of linkage that are more tightly associated with age of onset than others. By collecting samples from both Hispanic and non-Hispanic whites, it may be possible to determine if there are particular patterns of polymorphisms specific for either ethnic group, as has been suggested in the current study.

If some associations between age of onset and the two polymorphisms in question are elucidated in this proposed study, a follow-up should be done to determine if there is any linkage between the two polymorphisms and some other locus on chromosome 17q21. There are several genetic markers in this area that have been used successfully for linkage analysis by Liu and Barker (1999). By identifying markers that appear to have strong associations with the two polymorphisms in question, it would be possible then to do a more localized search near the linked marker for additional genes that show linkage to the polymorphisms as well. This would address the theory that the 4427T/C and S1613G polymorphisms are not the causative loci themselves, but are in linkage with another locus that is.

Due to the lack of *BRCA1* mutations in this population, it would be interesting to look for mutations in *BRCA2*. As mentioned previously, some studies have identified populations that have a high degree of linkage to *BRCA2*, with almost no association to *BRCA1* (Thorlacius *et al*. 1996). Also, no studies of *BRCA2* have been done in any Hispanic population. This would be a very labor-intensive and time consuming project, given that *BRCA2* is even larger than *BRCA1*. However, given the lack of information regarding *BRCA2* involvement in Hispanic women, it would certainly be beneficial.

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