PRODUCTION OF RECOMBINANT HUMAN BRCA2 ENCODED PROTEINS in E.coli

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BİLKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

BY
EMRE SAYAN
AUGUST 1997

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Emre Sayan.

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I certify that I read this thesis and in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

Prof. Dr. Mehmet Öztürk

I certify that I read this thesis and in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

Assoc. Prof. Dr. Tayfun Özçelik

I certify that I read this thesis and in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

Prof. Dr. Semra Kocabıyık

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Director of Institute of Engineering and Science

ABSTRACT

BACTERIAL CLONING AND EXPRESSION OF BRCA2 ENCODED PROTEINS.

Emre Sayan

M.S. in Molecular Biology and Genetics

Supervisor: Prof. Dr. Mehmet Öztürk

August 1997, 109 pages.

Breast cancer is known to be the most common cancer among women in the world. It is assumed that one in ten women will develop breast cancer till the age 80. Heredity is the major risk determinant in breast cancer. 30% of the women with breast cancer have a positive family history and 10% of women is defined to have high risk of early onset breast cancer who have multiple family members of breast and ovarian cancer. A novel breast cancer susceptibility gene, BRCA2 was confirmed to account more than 40% of the mutations in familial breast cancer patients. BRCA2 tumor supressor gene encodes a 3418 amino acid protein with little or no homology to other known proteins. There is no significant data concerning the cellular functions of BRCA2 and many facts about BRCA2 are waiting to be uncovered. The gaps in the knowledge about BRCA2 must be filled by generating new points of views and technical approaches. Such studies require the cloning of BRCA2 and the presence of purified protein products. In this study we cloned a fragment of exon 11 and exons 19 to 27 as 2 overlapping fragments. Since more than 80% of the mutations result in the loss of the C-terminal of the protein and produce cancer phenotype, we expressed and purified the extreme C-terminus of the BRCA2 protein.

ÖZET

BRCA2 GENİNİN BAKTERİYE KLONLANIP EKSPRESYONU

Emre Sayan

Moleküler Biyoloji ve Genetik Yüksek Lisans

Tez Yöneticisi: Prof. Dr. Mehmet Öztürk

Ağustos 1997, 109 sayfa.

Meme kanseri dünyada kadınlar arasında en yaygın görülen kanserdir. Her on kadından birinin 80 yaşından önce meme kanserine yakalanacağı düşünülmektedir. Kalıtım meme kanseri riskinin en önemli belirleyicisidir. Her on kadından üçünün ailesinde meme kanseri geçmişi vardır ve her on kadından birinin ailesinde birden çok meme ve over kanserli birey olduğu sanılmaktadır. BRCA2 yeni bulunmuş bir kansere yatkınlık genidir ve ailesel meme kanserlerinde yüzde kırk ihtimalle mutasyona uğrar. BRCA2 tümör baskılayıcı geni, hiçbir bilinen genle benzerliği olmayan 3418 aminoasit uzunluğunda bir protein kodlar. Şu ana kadar BRCA2 proteininin fonksiyonları ile ilgili tatmin edici bilgiler elde edinilememiştir ve bu genin özellikleri keşfedilmeyi beklemektedir. Bu genle ilgili eksiklikler, yeni düşünceler ve yeni teknik yaklaşımlarla bulunulabilir. Bu tip çalışmalar için BRCA2 geninin klonlanması ve proteininin saflaştırılması gerekmektedir. Biz bu çalışmada BRCA2 geninin ekzon 11'inden bir parça ve ekzon 19`dan 27`ye kadar olan kısmı 2 birleştirilebilir parça olarak klonladık. BRCA2 genindeki mutastonların çoğu karboksil ucu olmayan proteinler oluşturduğu ve kansere sebeb olduğu için karboksil uç kısmının proteinini bakteride ürettik ve saflaştırdık.

TO MY PARENTS EROL AND ZEYNEP SAYAN

AND

TO MY GRANDPARENTS ALİ AND REVMAN ERKILIÇ

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Thanks to other instructors and post-docs of our department, especially to Assoc. Prof. Dr. Tayfun Özçelik for understanding my loneliness in Ankara and Cengiz Yakıcıer, for their constructive comments.

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ABBREVIATIONS

AI	Allelic Imbalance
amp	ampicilin
bisacrylamide	N, N, methylene bis-acrylamide
bp	base pairs
BSA	bovine serum albumin
c-terminus	carboxyl terminus
cDNA	complementary deoxynucleic acid
kDa	kilo daltons
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ds	double strand
DTT	dithiothreitol
EDTA	diaminoethane tetra-acetic acid
EtBr	ethidium bromide
HBC	Hereditary Breast Cancer
HMECs	Human Mammary Epithelial Cells
IPTG	isopropylthio- β-D-galactoside
kan	kanamycin
LFS	Li-Fraumeni syndrome
LB	Luria-Bertani media

LOH	Loss Of Heterozygosity
MQ	MilliQ water
nm	nanometer ($1/10^9$ of a meter)
N-terminus	amino terminus
MW	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
ss	single strand
TAE	tris-acetic acid-EDTA
TBE	tris-boric acid-EDTA
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	tris (hydroxymethyl)-methylamine
TSG (s)	Tumor Supressor Gene (s)
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl- β-D-galctoside

1- INTRODUCTION

1.1- Quick review on Breast Cancer

Breast cancer is the most frequent cancer in women worlwide. It is estimated that one of each ten women will develop breast cancer in their lifetime in the industrialized countries. In the United States, more than 180000 new cases were observed in 1996 and 45000 women had died of breast cancer (Parker *et al.* 1996). Breast cancer is 100 times less frequent in men and it is also very rare in young women (<20 years). Its frequency increases progressively from 25 to 45 years of age, it stabilizes around 55 years, then increases abruptly in older age. About the 85 % breast cancers occur after 40 years of age. Genetic alterations that take place in the progression from normal breast epithelium to metastatic carcinoma are summarized in figure 1.

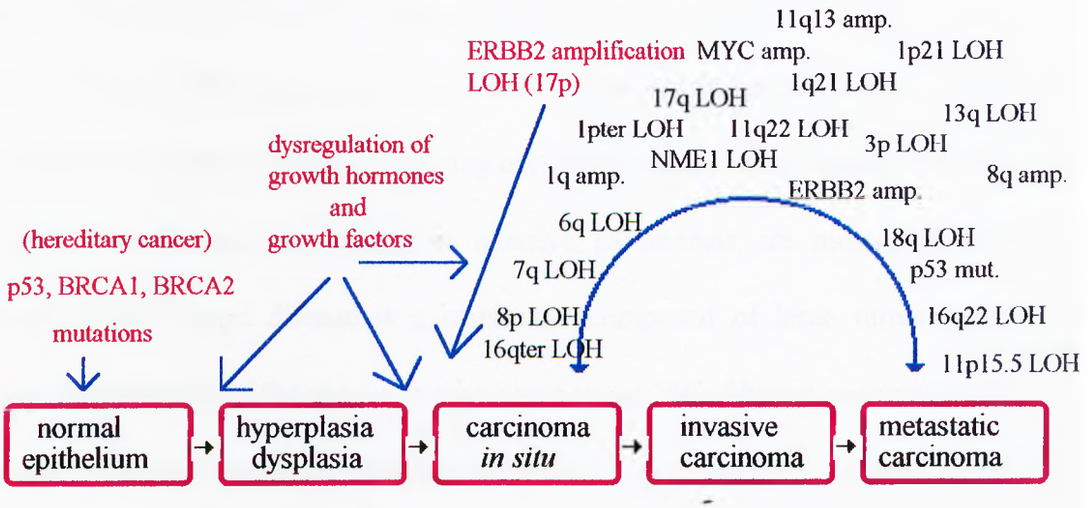


Figure 1: Genetic alterations that take place in the progression from normal breast epithelium to metastatic carcinoma

Breast cancer is a tumor of the mammary gland. The anatomy of breast is shown in figure 2. The breast is composed of a duct epithelium with surrounding fibrous stroma. The ducts and lobules, together with interlobular fibrous tissue are called breast parenchyme. This parenchyme is diffusely distributed within the adipose tissue of breast. With the onset of menarche, pituitary-ovarian hormones allow the differentiation and development of terminal duct lobular units. Unlike the intermediate duct systems which are stable and unaffected through hormonal fluctuations, the terminal lobular units are dynamic structures and undergo marked alterations during regular menstrual cycles. The 17β-estradiol (active estrogen) and progesterone play a critical role in the physiological regulation of mammary gland. Estrogen acts mostly on the terminal ducts and induce mitotic activity of epithelial cells. The progesterone, which is secreted periodically during the second phase of menstrual cycle, blocks mitotic activity induced by estrogens.

Breast tumors are classified as benign and malignat tumors. Among benign tumors, fibroadenomas are the most frequent forms which develop before the age of 30. They are chracterized by a glandular proliferation with a variable fibrous component. More than 98% of malignant breast tumors are carcinomas. In situ carcinomas or non infiltrating carcinomas occur in the epithelium of ducts or the lobuls. They do not infiltrate the neighbouring connective tissue. They represent about 7% of breast carcinomas. Infiltrating or invasive carcinomas are mostly 'ductal carcinomas' (70%). Paget disease is a carcinoma composed of large tumor cells infiltrating the epidermis of the nipple. It represents about 2% of breast cancers and it is associated to another mammary carcinomas (Rubin E. and Farber JL. 1988).

The anatomy of the breast is shown in figure 2.

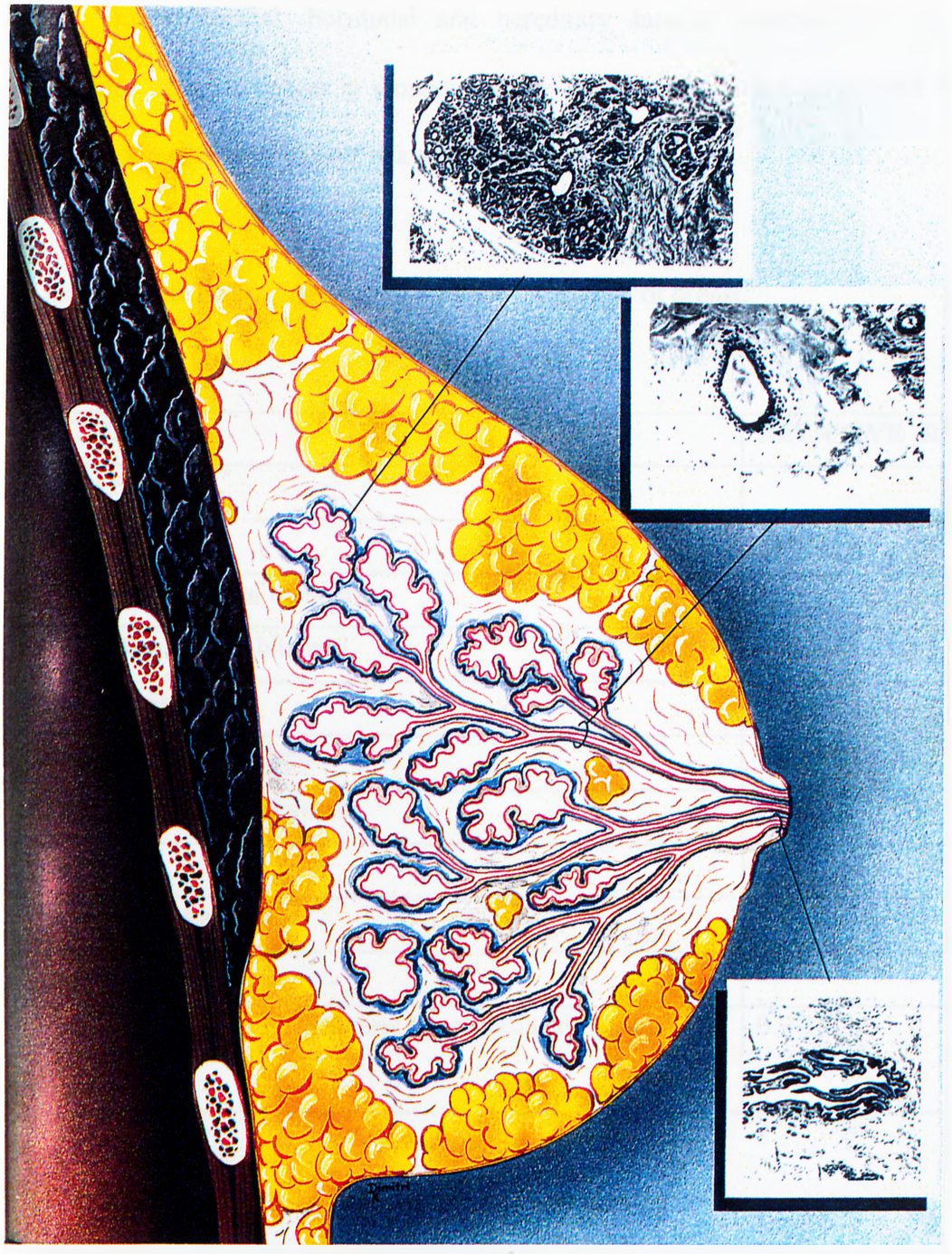


Figure 2: The anatomy of the breast.

1. 2 Etiology of Breast Cancer

Environmental, hormonal and hereditary factors contribute to the high frequency of beast cancer in women (Daudt. *et al.* 1996). Some of the risk factors associated with breast cancer risk are summarized in table 1.

Table-1: Risk Factors associated with the carcinoma of breast.

FACTOR	RELATIVE RISK
Family History	
Primary relative	1.2-3.0
Postmenapausal unilateral	1.5
Premenapausal bilateral	8.5-9.0
Menstrual History	
Age at menarche<12 years	1.3
Age at menapause>55 years	1.48-2.0
Preganacy	
First child after 35	2.0-3.0
Nulliparous	3.0
Other Breast Diseases (including hyperplasia and in situ carcinoma)	0.9-10

1. 2-1 Hereditary predisposition to breast cancer:

About one third of women with breast cancer have a positive family history of one or more first degree relatives (Marcus *et al.* 1996) and one tenth of all breast cancers are attributed to familial breast cancer, which is seen in high risk families. There are two breast cancer succeptibility genes identified (BRCA1 and BRCA2) that define more than 80% of the familial breast cancer cases after age 80 (Ford *et al.*

1995). Considering the 10% lifetime risk of breast cancer, 10% risk of familial breast cancer among all breast cancers and 80% of the mutations to be in BRC genes, one in 120 women is in access of breast cancer after the age of 80 due to BRC genes.

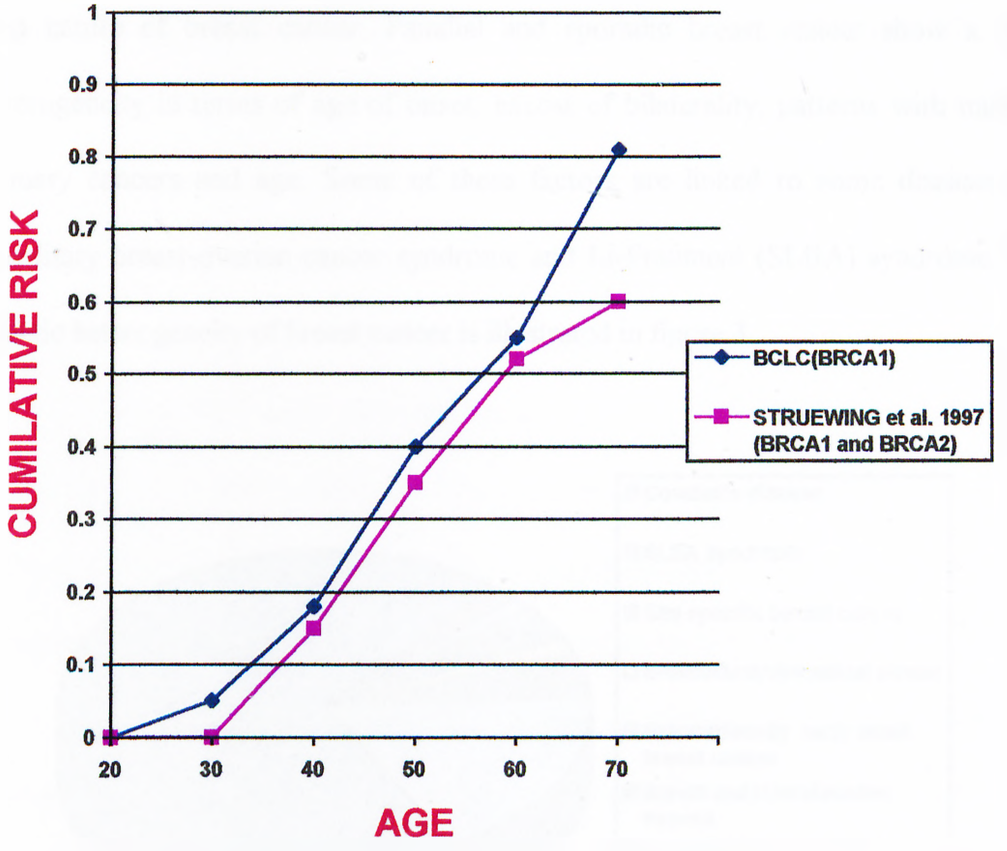
1. 2-2 Environmental Factors:

Diet and exercise: Sevaral factors including socioeconomic and nutritional status were underlined to be associated with increased risk of breast cancer. Because of improved health conditions in industrialized countries, the average age of menarche decreased whereas the average age of menopause increased. The breast epithelium is mitoticly active during this period, so it is vulnerable to carcinogenic influences for a longer time period. Women, who had migrated to US. from countries where the breast cancer incidence is low, quickly attain a breast cancer risk close to the inhabitants of the area (Rubin E. and Farber JL. 1988). Statistical studies defined that dietary intake monounsaturated and polyunsaturated fats, might reduce the risk of premenopausal bilateral breast cancer (Witte *et al. 1997*). Olive oil and milk consumption were observed to be associated with decreased risk of breast cancer (Knekt *et al.* 1996) as well as exercise and daily uptake of vitamin A (Bertstein *et al.* 1994, Kushi *et al.*1996).

Alcohol and smoking: In 38 statistical epidemiologic studies it was shown that women drinking three or more drinks daily had a 40% higher risk of breast cancer when compared with non-drinkers . The risk factor is most elevated at around age 50 (Homberg *et al.* 1995). When smoking is concerned, there is no direct evidence between breast cancer, but typical smoking related DNA adducts were seen in the tissue samples of smokers and (possibly passive but) non-smokers (Li *et al.* 1996).

Age: Strong statistical data describing the cumilative breast cancer risk due to mutations in BRCA1 (calculated by Breast Cancer Linkage Consortium) and due to both BRCA1 and BRCA2 mutations (Struewing *et al.* 1997) were available and shown in table 2 (Easton *et al.*1997).

Table-2: Age versus cumilative breast cancer risk in familial breast cancer patients.



Based on the fact that both genetic and environmental factors play a role in breast cancer, the tumors can be classified as familial and non-familial. Since the familial breast cancer is only 5-10% of total breast cancers and there is a gradual increase in breast cancers since 1940, environmental factors can be thought to play a

bigger role, but the most important thing concerning this fact is the genes that couse sporadic and familial breast cancer are mostly common.

There are 3 genes known to contribute both familial and sporadic breast cancer: p53, BRCA1 and BRCA2.

1.3 Molecular Basis of Breast Cancer

As many cancers, breast cancer is a multi-factorial and multi-step disease. Hereditary and somatic mutations on different genes were shown to define the multi-step nature of breast cancer. Familial and sporadic breast cancer show a great heterogeneity in terms of age of onset, excess of bilaterality, patterns with multiple primary cancers and age. Some of these factors are linked to some diseases like hereditary breast-ovarian cancer syndrome and Li-Fraumeni (SLBA) syndrome. The genetic heterogeneity of breast cancer is illustrated in figure 3.

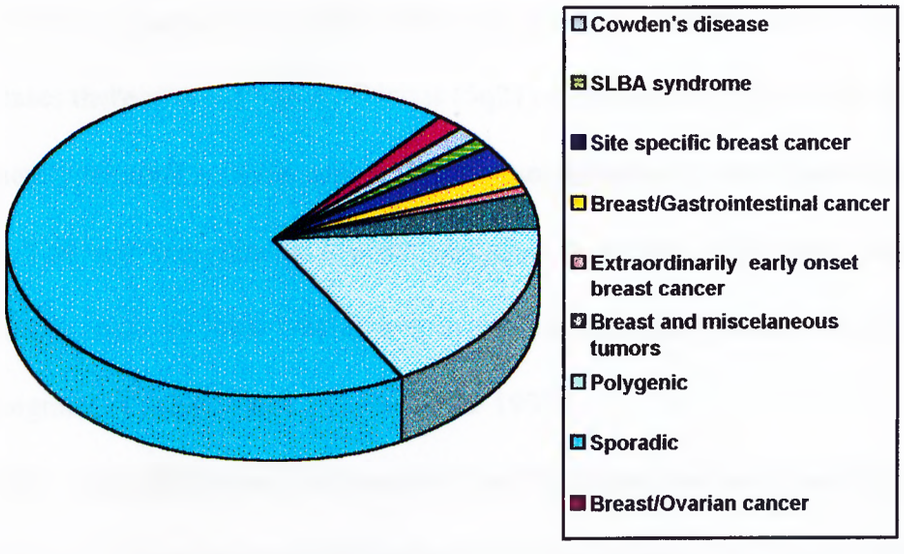


Figure-3: Genetic heterogeneity of breast cancer (Lynch *et al.* 1994).

synthesis at G1/S phase of cell cycle. In a study of n=223, loss of Rb expression was defined at 21% of primary breast tumors (Anderson *et al.* 1996).

P53 : p53 gene which is considered to be the guardian of the genome, is the most common mutated TSG in all types of cancers (~40% average). Of about 6.5 million cancer cases worldwide each year, 2.4 million tumors were estimated to contain p53 mutation. It is also very important to consider that breast cancer is more frequent in developed countries, being the most common in US. The estimated incidence of breast cancers in US. with p53 mutations is 25-30% (44000 in 183000 new breast cancer cases) in 1995 (Harris C. Curtis 1996).

1.3-2 Oncogenes:

BCL1 (cyclin D1) : Cyclins are common markers for breast cancer progression. Especially cyclin E and D1 (BCL1) expressions were seen to be elevated in such tumors. Immunohistochemical staining of cyclin D is a good technique since the detection of BCL1 gene amplification may not result in high expression, but uncontrolled expression. BCL1 overexpression is seen at approximately 30% of breast cancers (Gillett *et al.* 1994).

BCL2 : This is a gene which belongs to a family that is involved in the control of cell fate. BCL2 is an inhibitor of cell death and acts as an antagonist of bax gene, which encodes a protein inducing programmed cell death. In a recent study the expression pattern of BCL2 in breast carcinoma was investigated and found to be altered in 33% of them (by immunohistochemical methods), especially in tumors where p53 expression was altered (Siziopikou *et al.* 1996). In another study BCL2 and p53 mutations were used to differentiate male and female breast cancer. It was shown that p53-/BCL2+ phenotype is more associated with male breast cancer (Weber-Chappuis. *et al.* 1996).

ERB-B2 : ERB-B2 (neu/Her-2) is a cell surface growht factor receptor with receptor tyrosine kinase activity and its overexpression is an indicator of tumor progression. In a controlled study of 295 primary breast tumors, it was shown to be amplified in 28% of the cases (Berns *et al.*1995)

MDM2 : This cellular oncogene may play a role in tumorigenesis by inactivating the p53 protein which was defined as the guardian of the genome. Mdm2 expression levels were defined to be elevated up to 8 folds in 4 to 9% of breast tumors with a decreased imuunostaining of p53 (McCann *et al* 1995, Marchetti *et al.* 1995).

MYC : The myc proto-oncogene is first chracterized as a viral homologue of a cellular protein and it is essential for transcriptional activation of DNA polymerase subunits and cyclins to allow the cell to propogate through cell cycle. Its amplification and overexpression is seen in about 17% of breast tumors (Berns *et al.*1995). Myc gene is amplified in about 15% (range 6-32%) breast cancer cases (Marc *et al.* 1993).

1.4 Hereditary Breast Cancer Genes

BRC genes and p53 : The p53 tumor supressor gene, which is localized to 17p encodes 53 kDa phosphoprotein with multiple important functions concerning cell fate. These functions include cell cycle arrest, apoptosis and induction of DNA damage response genes upon DNA damage (Vogelstein *et al.* 1992). The loss of functional p53 produces genetic instability and being prone to new mutations. This is why p53 mutations were seen more than 40% of all tumors (Harris C. Curtis

1996). In table 3, the results otained by detection of LOH at p53 locus in breast cancer (Lindblom A.. *et al.* 1993) were summarized.

Table-3: Loss of heterozygosity at p53 locus.

Loss of Heterozygosity at p53 locus		
Breast tumors from families of type :	LOH in p53 locus :	
Breast cancer families	3/13	25-30%
Cancer families	1/3	30-35%
Predominant breast cancer mean age<50	4/9	45-50%
Predominant breast cancer mean age>50	1/8	10-15%
Breast cancer and other tumors mean age<50	2/5	40%
Breast cancer and other tumors mean age>50	2/6	30-35%

These and similar type of experiments showed that there are 30% LOH at p53 locus at breast cancer families. The incidence gets higher especially in breast cancers occuring before the age 50 (50%), which are called as familial breast breast cancer. Germline p53 mutations were detected in many families with Li-Fraumeni syndrome (LFS). The tumors associated with LFS are breast cancer, osteosarcoma, soft tissue sarcoma, brain tumors and leukemia. Several studies conformed the increased incidence of breast cancer in LFS families (Malkin *et al.* 1990). Interestingly, different investigations done in defining the mutation frequency of p53 in male breast cancer had conflicting results. The percentage of loss of functional p53 changes from 5-10% (Weber-Chappu *et al.* 1996) to 40% (Anelli *et al* 1995, Soussi *et al.* 1996). These

results may indicate a probable interaction of p53 with BRCA1 or BRCA2 since BRCA2 mutations were most commonly seen in families with a positive history of male breast cancer unlike BRCA1.

When BRC genes are concerned it is possible to say that they account for 80% of the cases for the breast cancer patients in high risk families. About one third of women with breast cancer have a positive family history of one or more first degree relatives with the disease. A smaller fraction (5-10%) of breast cancer is hereditary breast (and ovarian) cancer and strictly defined to be :

- Having 3 or more first degree relatives with breast and/or at least 2 ovarian or male breast cancer cases in the family.
- The age of onset of the disease must be less than 50.

BRC genes were proven to be very important in the development of familial breast cancer but it is possible that they have also a role in sporadic breast cancer. The experiments done for defining the involvement of BRCA1 in sporadic breast tumors showed that BRCA1 is rarely involved in sporadic breast tumors but in sporadic ovarian tumors (Merajver *et al.* 1995, Cropp *et al.* 1994). The involvement of BRCA2 in sproradic breast tumors is not well defined for now. Allelic imbalance (AI) was found at 13q loci at 52-63% of 78 sporadic primary breast tumors, 9 of which showed AI at BRCA2 locus but not Retinobastoma (Rb) and 6 of which showed AI at Rb locus but not in BRCA2 (Hamann *et al.* 1996). This data suggested that Rb and BRCA2 are distinct targets in sporadic breast cancer. In a more definitive study, Lancaster *et al.* (1996) found 2 mutations in BRCA2 gene, which of 1 was a sporadic missence mutation (n=70) in primary breast tumors. The mutation rate of BRCA2 in sporadic ovarian cancer seems to be higher. Foster *et al.* (1996) defined 2 sporadic and 2 germline mutations in 55 primary ovarian tumors. These results

indicate that the mutation incidence of BRCA2 in sporadic breast and ovarian tumors is significantly higher than BRCA1, but still rare.

High risk breast cancer families helped the identification of first breast cancer succeptibility gene (BRCA1) in 1990 (Hall *et al.* 1990) which is cloned in 1994 (Miki *et al.*1994). The cloning strategy of BRCA1 required extensive study on 23 high risk families with 146 cases of breast cancer. The families include individuals with sporadic and familial breast cancer with early onset of the disease and bilateral progression as well as late onset (probably some sporadic cases). Hall *et al.* (1990) found a LOD score of 5.98 for linkage of breast cancer succeptibility in early onset families to marker D17S74, which is located at 17q21. Negative LOD scores were observed in families with late onset disease. Candidate genes included HER2 (Erb-b2), a cluster of homeo box 2 genes, retinoic receptor alpha and estradiol-17-beta-dehydrogenase. Claus *et al.* (1991) reported the same location harboring a rare autosomal dominant allele (q=0.0033) for succeptibility to breast cancer. This work based on a data set of 4730 histologically confirmed breast cancer patients aged 20-54 and 4688 controls. The cumilative lifetime risk of breast cancer for carriers of this allele was turned out to be 92% and 10% for non-carriers, which was quite close to the lifetime risk of BRCA1 defined by current studies. Studies done in families with hereditary breast and ovarian cancer showed that LOH in this locus was at 70% (Narod *et al.* 1991, Lynch HT and Watson P. 1992).

Although p53, BRCA1 and BRCA2 may account for a small fraction mutations in all breast cancer (10 % of all cases), their trait of inheritance is highly penetrant and autosomal dominant in Hereditary Breast and Ovarian Cancers (HBOC).

1.5 BRCA2

1.5-1 Review on BRCA2

A small portion of breast cancers, especially that occured at a young age is specifically due to mutations at autosomal dominant inherited genes. Germline mutations in p53 gene (Malkin *et al* 1990) and BRCA1 (Gayther *et al.* 1995) were defined to confer the 10% to 55% of the breast and ovarian cancers in predisposed families. One of the probable remaining breast cancer succeptibility genes, BRCA2 waited to be discovered until late 1994 (Wooster *et al.* 1994). In figure 4 the ideogram of human chromosome 13 is shown.

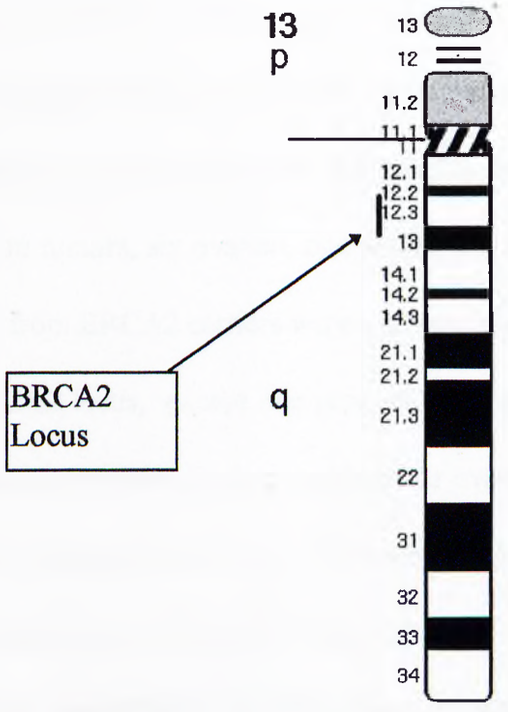


Figure-4: Ideogram of chromosome 13

For localizing BRCA2, a genomic linkage search was performed in 15 families with multiple cases of early-onset breast cancer that were not linked to BRCA1. Out of the 162 total cancer cases there were 8 male breast cancers and 16 ovarian cancers. Families were genotyped with microsatellite repeat markers and the second breast cancer succeptibility gene was roughly localized at a 6-cM interval on chromosome 13q12-q13, around marker D13S260. This study defined a 87% risk of breast cancer by the age 80, which is similar to BRCA1 phenotype but unlike BRCA1, ovarian cancer risk associated with this loci is not as much. The further localization is done by Schutte *et al.* (1995). They tried to define the fine LOHs in pancreatic adenocarcinomas using a new technique called representational difference analysis and showed a homozygous deletion in a 1-cM region of 13q12.3. The presence of this deletion suggested the presence of a TSG within the 6-cM region which was identified as BRCA2 locus. Localization of a new TSG further increased the effort on chracterization the gene and mutations at that loci and Gudmundsson *et al.* (1995) reported involvement of LOH in different tumor types in 5 families of 50 members that show strong evidence of linkage to BRCA2. These tumors (58 sample from 50 patients) include mostly female breast cancer and LOH is defined in 28 of 33 breast tumors. Seven prostate tumors, six ovarian, one colon, one cervical, one male breast and one ureter tumor from BRCA2 carriers were analyzed and all were defined to lose heterozygosity at BRCA2 locus, except one prostate and one ovary tumor. The high incidence of male breast cancer and the low incidence of ovarian carcinoma associated with this locus also proves the presence of a new breast cancer gene. Thorlasius *et al.* (1995) draw the attention to breast cancer risk of females in the families with male breast cancer. Familial susceptibility to early onset breast and ovarian cancer in women has been linked to BRCA1 gene in some extent, but the low frequency of male

breast cancer risk in such families was also defined (Stratton *et al.* 1994). In localization studies of BRCA2, Wooster *et al.* (1994) defined a positive LOD score in BRCA2 linkage in families with female and male breast cancer. The work of Thorlasius *et al.* (1995) defined a definite linkage to BRCA2 locus in male breast cancer families, but interestingly there was no increase in female breast cancer. The fine localization of BRCA2 to 13q12-q13 was unexpectedly enhanced by the work of Shutte *et al.* (1995) who defined a homozygous somatic deletion at this region and narrowed down the region to 600kb around marker D13S171. Wooster *et al.* (1995) reported the identification of a gene that had six different germline mutations in breast cancer families that were likely be due to mutations in BRCA2. Each mutation was also confirmed to disturb the open reading frame of the transcriptional unit. Wooster *et al.* (1995) generated yeast artificial chromosome (YAC) and P1 artificial chromosome (PAC) contigs to extend 700 kb centromeric and 300 kb telomeric to D13S171 and identified minimally overlapping 14 PACs. Transcribed sequences lying in these PACS were defined by either exon trapping or hybridization with complementary DNA and screened for crucial mutations like truncation or splice site destruction in breast cancer families showing linkage to BRCA2 (families including male breast cancer or families showing negative LOD score in BRCA1 linkage). The definition of six disease associated mutations like truncation of the encoded protein showed the expressed sequences at that region belong to the second breast cancer susceptibility gene. With this approach a 7.3 kb cDNA is defined (including 300 bp of 3 prime untranslated region) to encode 2239 aminoacids, which was not full BRCA2 cDNA. The identified portion of BRCA2 showed no significant homology with any known proteins except a weak homology at residues 1783-1863, with BRCA1 residues 1394-1474. The work done by Wooster *et al.* was completed by Tavtigian *et*

al. (1996) and the complete coding sequence, exon-intron structure and the expression pattern of BRCA2 was examined. The features of BRCA2 defined by this study were as follows:

BRCA2 lies near the centre of a 1.4 megabase interval flanked by markers D13S1444 and D13S310. Translation of the cDNA starts at nucleotide 229 to encode for a 3418 aminoacid protein. Hybridization of the labeled cDNA revealed a 11-12kb transcript and high expression was detected in breast, testis and thymus with less amounts in spleen, lung and ovary. The identified cDNA was 11385bp without poly adenylation signal and poly A tail. When gene structure was concerned, cDNA and genomic sequences were compared. The gene was composed of 27 exons and distributed over roughly 70kb of genomic DNA. Southern blot analysis revealed no close homologue of the gene in the human genome. The cDNA consists of >60% A/T unlike most of the human genes. There was a CpG rich region at the 5 prime end of the gene suggesting a regulatory region, but there was no clue of a signal sequence or membrane spanning regions on the coding sequence. One of the unique property of BRC genes were revealed with this study: a huge exon 11, 3426 bp for BRCA1 and 4932 bp for BRCA2. BRCA2 protein was found out to be highly charged, roughly one quarter of all amino acids were acidic or basic. The discovery of BRCA2 allowed the comparison between BRC genes and define their role as a tumor supressor. Here are the similarities of BRC genes:

- Highly charged proteins
- Have a huge exon 11
- Distributed on a 70kb genomic DNA
- Translation starts at the second exon

- Expression patterns are similar (highest in testis and breast)
- Most of the mutations destrupt the reading frame so produce early termination of translation
- Unlike mammalian genes, they are A/T rich

Tavtigian *et al.* (1996) not only found out the features BRCA2 gene, but also screened 18 kindreds (for 9 kindreds exon 15 was not available) for germline BRCA2 mutations to further define some mutations that disable the function of the protein, so understand the functional domains of this novel protein. Mutations were detected in 9 of the 18 kindreds, and all except one seem to produce immature stop of translation. The exceptional mutation was a three base deletion at position 4132 (del thr1302), which didn't alter the reading frame. This mutation was not observed in 36 other unrelated breast tumors and the function of thr1302 could not be determined. Most of the mutations detected in BRCA2 were microdeletions of 1-6 bp whereas point mutations and microinsertions dominate the mutation profile of BRCA1. There were also 9 polymorphisms, 2 in the 3 prime, 1 in the 5 prime untranslated region in BRCA2.

After the discovery of the gene, the attention was drawn on BRCA2 mutation profile to define early detection and prevention methods for breast cancer. Lancaster *et al.* (1996) screened entire gene using a combination of SSCA, denaturing gel deletion analysis and PTT techniques in primary breast and ovarian tumors. LOH was observed at 34 of breast and 18 of ovarian tumors but no mutation was identified in ovarian tumors and only 2 of the 70 breast tumors displayed a mutation. One of the mutations was a germline mutation that caused a frameshift (4710del AG) and the other one was a somatic missense mutation (Asp 3095 Glu) of unknown function. The results were similar to ones obtained in BRCA1 in sporadic breast and ovarian tumors

and the authors indicated the infrequent mutation of BRC genes in sporadic cases. Teng *et al.* (1996) had similar results. In this study, complete BRCA2 gene was screened for mutations in 12 different cell lines, representing different tumors. A germline mutation in a pancreatic carcinoma cell line was detected proposing a role to BRCA2 in that neoplasm. A more convincing experimental result came from Miki *et al.* (1996) in which the study was done in 100 sporadic Japanese breast cancer patients with no family history. One somatic missense (His 2415 Asn) and 2 germline frameshift mutation were identified. One of the germline mutation was a 346 bp insertion to exon 22. A 64 bp polyadenylate tract a 8 bp duplication showed the presence of an active Alu element insertion at this site. The Alu element didn't cause a frameshift mutation but terminated translation by an internal stop codon. The other germline mutation was a 4 bp deletion at codon 252, producing an early truncation.

Population-based studies define new mutations in BRCA2. Gudmundsson *et al.* (1996) reported the first study amond Icelandic population and showed a strong likage to BRCA2 region. The interesting point of the study was the same haplotype observed in 5 of the 7 families. Thorlacius *et al.* (1996) studied 21 Icelandic families which were selected on the basis of frequent breast cancer cases including male breast cancer. 16 of 21 families showed a strong linkage to BRCA2 locus. A common mutation of a 5 bp deletion at exon 9, starting at nucleotide 999 in codon 257 was found. This common mutation and the same haplotype suggests a founder effect. 999 del 5 was also defined in different tumors such as prostate, pancreas, ovary, colon, thyroid, cervix and endometrium (Gudmundsson *et al.* 1996). Neuhausen *et al.* (1996) and Couch FJ. *et al.* (1996) reported a novel mutation in highly inbreed Ashkenazi Jewish population. This mutation was 6174 del T and the frequency of the

mutation appeared to be 3 per 1000 in the general population. Six of 80 Ashkenazi Jewish women diagnosed with breast cancer before the age of 42 were heterozygous for the mutation (8%) and the non-Ashkenazi control group showed no sign of that mutation (n=93). In 2 of 27 additional Jewish families, in which breast cancer occurred at age 42-50 the same mutation was detected (7%). These results propose the recurrent BRCA2 mutation in an inbreed population resulting about a quarter of all familial breast cancers. The observations of Neahusen e*t al.* were partially refuted by Berman *et al.* (1996), who described the particular mutation to be encountered in Jewish and non-Jewish individuals. The study was done on 83 individuals with breast and 93 individuals with ovarian cancer and 6174 del T was observed in 8 patients (4.5%). 7 of the 8 patients were Ashkenazi origin but only 2 of them showed a common haplotype indicating several independent origins of this common mutation. Two more reports define the importance of revealing the specific mutations in specific populations like Ashkenazi Jews (Roa *et al.* 1996, Oddoux *et al.* 1996) and giving exact numbers for carrier frequency and penetrance of 6174 del T. It was supposed that the mutation was the most frequent mutant allele (carrier frequency=1.52%) predisposing breast cancer among Ashkenazi Jews but the penetrance was not as high as the most frequent mutant allele of BRCA1 (185 del AG) in this population. The relative risk of developing breast cancer with this mutation was calculated to be 9.2% by the age 42.

1.5-2 Known mutations in specific populations

In 1997, numerous studies were done to define the mutation profile of BRCA2 in different populations. Most of these studies rely on high risk breast and/or ovarian families with male breast cancer (for BRCA2) except for the ones that family history is omitted. The general outcome of these reports on BRCA1 and BRCA2 indicate that BRCA1 mutations couse a higher percentage of breast and ovarian cancer in the world (Szabo CI. and King MC, 1997), with the exception of some isolated populations like Icelanders. In the families with male breast cancer cases, BRCA2 mutations predominate. This is also the case in U.S. extensive studies done in different ethnic groups, BRCA2 was defined to be responsible for 19% of familial male breast cancers but for a very small fraction of male breast cancer in the general population. Fewer number of mutations and the low penetrance of BRCA2 mutations, define a 25% reduced risk for BRCA2 compared with BRCA1 (except Iceland). In about 30% of high risk families there were no BRCA1 or BRCA2 alterations detected. These included 3 of 4 Hungarian families with at least 6 cases of breast or ovarian cancer (Ramus *et al.* 1997) and 2 of 6 male breast cancer families and 15 of 23 female breast cancer families of midwestern American origin (Serova *et al.* 1997). The data led several groups to suggest the existence other BRC genes.

Most of the mutations in BRCA2 are microdeletions and microinsertions of 1-6 bp whereas there were very little missense and nonsense mutations. There are few, one aminoacid deletions (3 bp) suggesting the importance of specific aminoacids (see figure 5 and above). The general outcome of the mutations in BRCA2 is the immature stop of translation due to a frameshift in the gene.

Except for a few, most of these studies were reviewed in Am. J. Hum. Genet. volume 60, 1997. In tables 4 and 5, the incidence and country specific distribution in various populations of BRCA2 mutations were summarized (Modified from Csilla I. Szabo and Mary-Claire King 1997).

Table-4: BRCA2 mutation incidence in different countries.

POPULATION	No. of mutation/ No. of patients	Reference (s)
Families with three or more cases of female breast and/or ovarian cancer:		
Britain	25/290 (9%)	Gayther *et al.* (1997)
Canada	8/49 (16%)	Phelan *et al.* (1996)
Finland	8/100 (8%)	Vehmanen *et al.* (1997)
France	14/77 (18%)	Serova-Sinilnikova *et al.* (1997)
Hungary	4/32 (13%)	Ramus *et al.* (1997)
Iceland	7/11 (64%)	Thorlacius *et al.* (1996)
Israel	8/34 (24%)	Levy-Lahad *et al.* (1997)
Sweden and Denmark	12/106 (11%)	Hakansson *et al.* (1997)
United States	24/94 (25%)	Couch *et al.* (1996), Serova *et al.* (1996, 1997), Tavtigian *et al.* (1996), Schubert *et al.* (1997)
Families with male and female breast cancer:		
United States	12/64 (19%)	Couch *et al.* (1996), Serova *et al.* (1997)
Hungary	2/6 (33%)	Ramus *et al.* (1997)
Iceland	9/10 (90%)	Thorlacius *et al.* (1996)
Breast and/or ovarian cancer patients not selected for family history		
Iceland	42/497 (8%)	Johannesdottir *et al.* (1996)
Israel	14/243 (6%)	Abeliovich *et al.* (1997)
Japan	2/103 (2%)	Miki *et al.* (1996)

Table 5: Specific BRCA2 mutations in specific populations.

BRCA2 mutations in breast and ovarian cancer families and patients

		No of mutations								
Exon	BRCA2 nucleotide change	Brit.	Fran.	Hun.	SweD.	Fin.	Ice.	Isr.	US.	Can.
11	6503delTTT	5								
11	5573delA*	2	1							
11	3034del4	3	1						1	2
10	1529del4	1							1	
10	2034insA	1							1	
11	6174delT	1		1				11	5	1
2	277delAC			1					1	
23	9326insA		1	1	1					
11	4486delG				2					
9	999del5					2	61			
18	8555T->G					2				
I-23	9346 (-2)A->G						2			
9	982del4								3	
20	8764delAG									2
	Other	12	13	3	10	2	0	NT	19	3

Brit.: Britain, Fran.:France, Hun.:Hungary, SweD.:Sweeden and Denmark, Fin.:Finland Ice.: Iceland Isr.:Israel, US.:United States Can.: Canada
*: This mutation was defined as 5573del/insA/AA NT: Not tested.

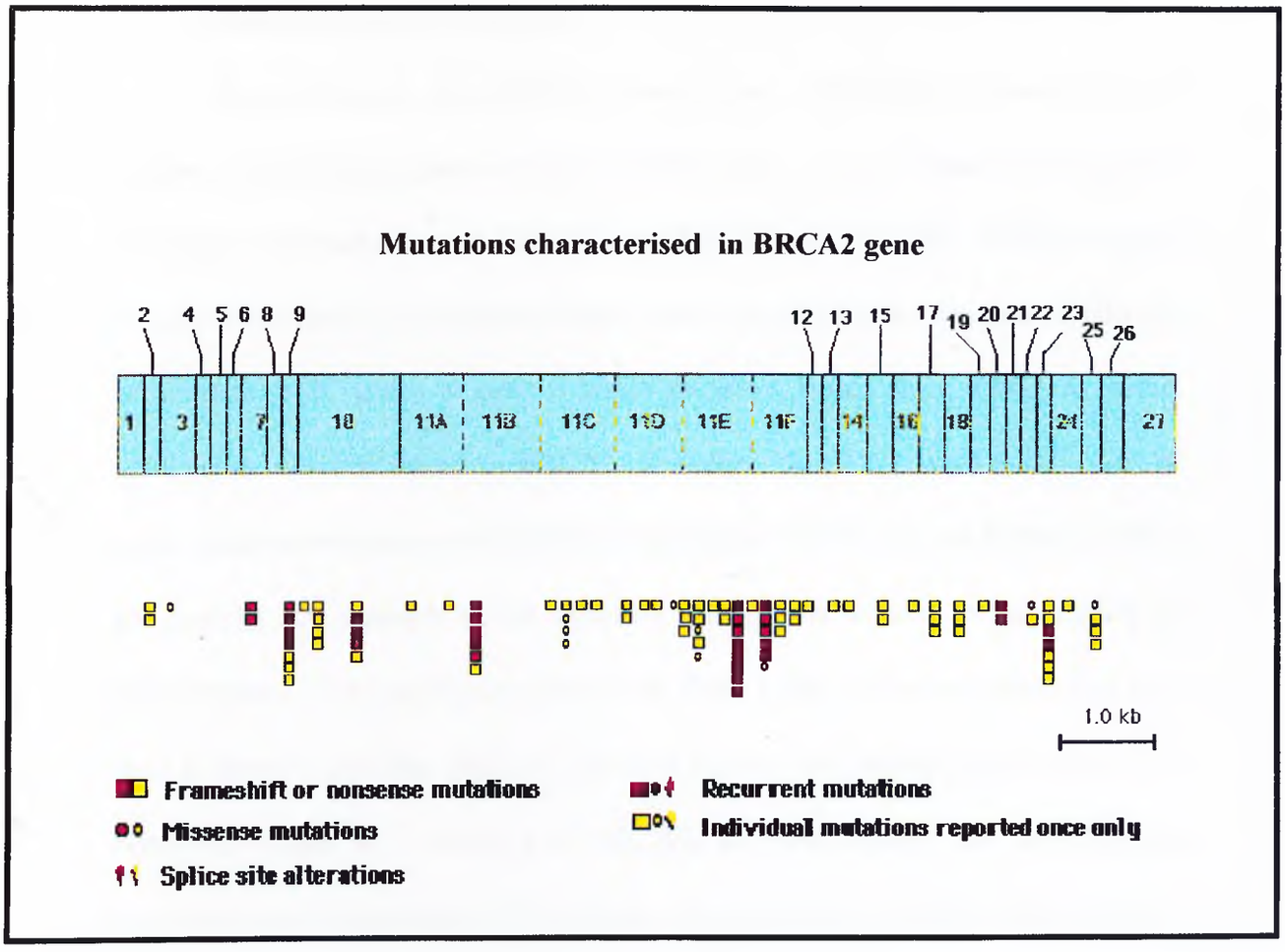


Figure-5: The mutation profile of BRCA2.

1.5-3 Computer based studies

1.5-3-1 Homology with granin family, with BRCA1 and conserved internal repeats in BRCA2

As a novel gene, identified by Wooster *et al.* (1995), BRCA2 was defined to confer no homology region with other known genes, except a weak homology at a restricted region (amino acids 1394-1474 of BRCA1 and 1783-1863 of BRCA2) with an unknown function. A computer based study considering the function of BRCA2 revealed the BRC genes to carry a granin domain (Jensen *et al.* 1996). A perfect granin consensus was found in BRCA1 at residues 1214-1223 and a match of six of seven constrained amino acids in BRCA2 at residues 3334-3344 was defined. Granins are proteins that assemble in the secretory vesicles and thought to help folding of other proteins. They are highly charged and share a 90% conserved granin box near their C-terminal just like BRCA2. Jensen *et al.* not only defined the presence of a homology region at C terminus of BRCA2, but also showed that BRCA1 was localized to membrane fraction of cell lysate, in the secretory vesicles and Golgi body. The presence of a secreted tumor supressor gene is fascinating since it opens the way of variety therapeutic studies. However these speculations were ruled out by the discovery of a polymorphic stop codon leading to production of the protein without the granin domain which was fully functional (Mazoyer *et al. 1996).*

BRCA2, as a very big gene producing a 390 kd protein may have lots of functions. Such proteins produce the constructural framework of the cell, cytoskeleton. Although these proteins are very big, their aminoacid structure propose a modular structure of repeating domains which are essential for binding other proteins. Dystrophin can be a good example of such proteins having multiple copies

of conserved motifs called spectrin like repeats and hinge sites. Expression pattern of BRCA2 defines a functional, rather than a structural role, but the functions of BRCA2 is not identified for now. The work of Bork *et al.* (1996) showed the presence of 8 conserved repeats in the exon 11 of BRCA2. The average of the lenght of these repeats were 60 (30-80) aminoacids and 13 of these residues in these repeats were 80% conserved. One of the repeats even coincided with the BRCA1 homology region which demonstrated the evolutionary importance of this domain. Also these modular regions were identified to confer homology with a C. *elegans* protein of unknown function and called **BRC repeats**. If a protein is important, it is most probably conserved throughout evolution. The structure and homology of BRC repeats were further studied by Bignell *et al.* (1997) and it was found that they were conserved in 8 mammals including dog, swine, hamster and mice. It is of importance that the BRC repeats were conserved but the intervening regions were not.

1.5-3-2 Homology with c-jun transcription activation region

In a search for a similarity to proteins with known functions, BRCA2 was found to have a sequence similariry to the transcripton activation domain of c-jun (Milner *et al.* 1997). This region was included in exon 3 of BRCA2, aminoacids 48 to 105, which was well conserved among mice and human indicating a functional role. The transcription activation role was studied by fusing this region with the DNA binding domain of lexA protein. The results showed a positive transcription activation capacity in yeast system. BRCA2 exon 3 sequences (aminoacids 18-105) also had a transcription activation potential in two different mammalian cell lines, namely U20S and NMuMG when linked to the GAL4 DNA binding domain. The minimum c-jun homology region (residues 60-105), contributed to this activation, but the adjacent region (residues 18-60), have higher activation capacity alone. When assesed alone,

the primary activation region (residues 18-60) have a 5 fold increased activity compared with auxilary activation region (residues 60-105), but still lower than the full activation region (residues 18-105). This consequence led the authors to define the transcription activity of adjacent regions, and two inhibitory regions at each side of activation site (residues 1-18 and 105-125), which was a similar regulation of some transcription factors, like c-fos.

C-jun was shown to be regulated by jun-N terminal kinase (JNK), which was shown to bind to the BRCA2 homology region (Derijard *et al.* 1994). An identified missense mutation in exon 3 of BRCA2 (tyrosine 42 cysteine) defines a probable target for such kinases and tyrosine 42 might be regulating the activity of this transcription activation domain.

1.5-4 Mouse homologue of BRCA2

Mouse homologue of BRCA2 is very important since mouse is the most commonly used experimenal animal and a good model system. Connor *et al.* (1997) described the mouse BRCA2 gene. The gene was mapped to chromosome 5 in mouse which was consistent with its localization to human chromosome 13. The cloning and the sequncing of mouse BRCA2 showed the gene to be relatively poorly conserved between human and mice although the size of the protein was similar (3329 amino-acids). There are gaps in mouse gene when compared with human homologue, explaining its shortness. The overall identity between two sequences was 59.2% with 72.6 similarity. The mouse BRCA2 gene was expressed at a wide range of tissues with the exception of lung which could not be detected after 40 cycles of PCR amplification from cDNA. The highest expression was seen at testis, thymus, eye, ovary and mammary gland. These results demonstrate the striking smilarity of mouse

and human BRCA2 expression as well as the similarity of BRCA2 and BRCA1 expression in mouse.

1.5-5 Expression studies and the role of BRCA2 in cell fate

In 1996, several studies were done considering the role of BRC genes in cell proliferation and differentiation. The lack of these genes is known to produce tumors especially at tissues that are highly controlled by hormones (breast, ovary and prostate). The hormonal induction in these tissues produce a proliferative response, like the mensturational increase and decrease of women's breast size. Such alterations in these tissues are the result of extensive proliferation and apoptosis so it is possible that BRC genes have a role in cell fate. Vaughn *et al.* (1996) reported the first functional study on BRCA2 concerning its expression pattern to implicate the role of BRCA2 in tumor suppression. G_0 and G_1 synchronized normal human mammary epithelial cells (HMECs), ovarian epithelial cells (NOSE1) and MCF-7 cells were used to experiment the expression pattern of BRCA2 (and BRCA1). The cells arrested at G_0 and early G_1 were having low levels of BRC expression whereas when they were stimulated to proliferate, the expression increases reaching a maximum at late G1 and retained throughout the S-phase. G_0 arrest of these cells were achieved by growth factor deprivation and G_1 arrest of cells were achieved by the drug Lovastatin. When released from the G_0-phase the maximum expression was detected at 9^{th}, 12^{th} and 15^{th} hours, lagging one time interval of Histone2A (H2A) which reached maximum at 12^{th} and 15^{th} hours, indicating cells entered S-phase. This result also indicates that the expression of BRC genes were not coupled to bulk DNA synthesis like H2A. When Lovastin blocked cells were released by adding fresh medium containing mevanolic acid, a high BRC expression was seen after 23 hours (at the

time when 21% of the cells were in the S-phase) and retained throughout the S-phase. Levels of mRNA from the estrogen inducible pS2 gene was very high through Lovastatin block and retained at 5th and 23rd hours, but declined at later points, showing an estrogen induced gene not coupling from BRCA1 and BRCA2. Rajan *et al.* (1996) clarified the expression pattern on BRCA2 by concerning differentiation and proliferation together. The results obtained in the case of proliferative stimulus were the same with the observations of Vaughn *et al.* (1996). The cell cycle arrested, transformed and non-transformed mammary epithelial cells did not express BRC genes, whereas the expression peaked at late G_1 and S-phase upon proliferative stimulus. Upon induction with lactogenic hormones, BRCA1 and BRCA2 expression was found to be steady and high although the cells were postconfluent. This data strongly suggested that the upregulation of BRC genes during differentiation occurred in a proliferation-independent manner. The parallel regulation of BRC genes suggested a similar control mechanism of induction of both of the genes but it is not true to call these genes to be redundant since the tumor incidence and tumor type associated with each gene are different.

These studies above indicate the BRCA2 expression pattern in cell culture, which is not a *in vivo* system so might be affected by some artifacts. Rajan *et al.* (1997) defined a controlled *vivo* system for studying BRCA2 expression. As the first part of experiment, the spatial and temporal expression pattern of the murine homologue of BRCA2 in fetal development, adult tissues and in mammary gland during postnatal development were investigated. The results indicated that BRCA2 was expressed at very high amounts in proliferationg cellular compartments, particularly those undergoing differentiation. Several studies defined the expression pattern of BRCA1 in mice (Lane *et al.* 1995, Marquis *et al.* 1995), which was

virtually indistinguishable from that of BRCA2, although these genes confer no homology. This study and previous ones thus described the importance of BRCA2 expression in proliferation and differentiation which could be similarly regulated with BRCA1. The only difference in expression of these two genes was identified by Rajan *et al.* (1997) at endocrine target tissues (testis and mammary gland), so the probability of differential control of BRC genes by sex hormones was proposed. The differences were significant especially in testis (during spermatogenesis) and breast (during preganacy).

In testis, various differentiating cell types appear during spermatogenesis, which occur during the first weeks of life in mice. The mRNA level in testis was investigated by RNase protection assay as a function of age from 6 to 27 days following birth. This analysis revealed a progressive increase in BRCA2 mRNA levels beginning at day 12 and reaching a peak by 4 weeks of age and remaining high in adult life. This was not the case for BRCA1, which was constantly expressed during the same period.

In situ hybridization was used to determine the spatial and temporal expression pattern of BRC genes in mammary gland during postnatal development. High levels of BRCA2 expression was seen in immature mice, predominantly at breast epithelium, with decreasing levels as the mice got matured. As assayed by RNase protection, BRCA2 mRNA levels in the female breast increased sharply early in pregnancy, when alveolar buds begin to differentiate to form, mature milk producing alveoli. During the rest of pregnancy, expression decreased, reaching the pre-pregnancy values at day 20. When BRCA1 was concerned, the expression patern was similar of BRCA2, but the magnitude of upregulation in pregnancy and the steady state expression was at least two times of BRCA2.

As the second part of the experiment, the expression of BRCA1 and BRCA2 was investigated in ovariectomized animals with external supply of 17 β-estradiol and progesterone. Steady state levels of BRCA1 and BRCA2 were significantly lower in mammary glands of overiectomized animals when compared with the normal littermates. When either of 17 β-estradiol or progesterone, or both was supplied to operated animals, there was a significant increase in BRCA1 and/or BRCA2 expression. The up-regulation of mRNA of BRCA2 was very low when compared with BRCA1 supporting the higher incidence of BRCA1 mutations in ovarian and mammary cancers.

These observations partially explain the gender and tissue specific cancers that are due to mutations in BRCA1 and BRCA2. This may be the couse of difference in the phenotype of families with breast and ovary cancer (due to mutations in BRCA1) and families with male and female breast cancer (due to mutations in BRCA2)

1.5-6 Gene knock out studies

In vivo experiments are essential for especially functional studies. Sharan *et al.* (1997) reported the first knock-out mice model for studying the function of BRCA2. Mouse embryonic stem cells were targeted by a targeting vector with a selection marker, which was designed to delete 2.8 kb of BRCA2 genomic locus, including the splice acceptor site and 812 aminoacids of exon 11. The vector was electrophorated into embryonic stem cells and 4 of the 8 clones were injected into blastocycts. Most of the chimaeric mice, born to carry the mutant BRCA2 were fertile and produced BRCA2 heterozygous progeny. Being a model for predisposed people, BRCA2-/+ mice were thought to have high risk of developing breast tumors, but none of the heterozygous mice developed any tumors until at least eight months of age. Six of the

BRCA2-/+ mice intercross failed to produce any offspring, indicating the importance of BRCA2 for embryonic development. Such heterozygous mice carrying a homozygous mutant embryo were dissected at various stages of development. At E6.5 (embryonic day 6.5), the BRCA2 null embryo was not seem to be different than the normal embryos, but at E7.5 (embryonic day 7.5), 25% of the null embryo exhibited a mutant phenotype. At this time of development BRCA2 expression was detectable in normal embryos, which was coupled with the formation of mesoderm and migration of cells of mesodermal origin into ectoderm to form structures such as amnion and chorion. BRCA2 homozygous mutated mice displayed very little, if any mesodermal tissue and no sign of migration, although the presence of mesodermal tissue was confirmed by the expression of brachury gene.

The interpretation such a result was hard, since the phenotype of BRCA2 null mice might be due to some differentiation or proliferation defects. As the null embryo were functionally equivalent to those carrying no mutations at E5.5, a heterozygous embryonic stem cell line was targetted by another similar targetting vector at prenatal day 6. 12 clones were obatained, hoewever the Southern blot analysis revealed that none of the clones distrupted the remaining allele of BRCA2, instead, the clones had targetted the mutant allele. Even the presence of positive selection didn't helped to isolate BRCA2 -/- cells indicating the importance of at least a single allele of BRCA2 for survival and proliferation of ES cells. The authors also concluded for the role of BRCA2 in development to be in proliferation, rather than differentiation since BRCA2 expression began at E6.5, increased to high amounts at E7.5, at the time when the entire embryo was undergoing its most rapid phase of cellular proliferation.

In another study done by Ludwig *et al.* (1997), the phenotypes of BRCA1, BRCA2, BRCA1/BRCA2, BRCA1/p53 and BRCA2/p53 knock out mice determined.

BRCA1 was targetted at its exon 2, which was shown to contain a ring finger motif, and BRCA2 was targetted at its exon 11, by insertion a stop codon. BRCA1 and BRCA2 heterozygous mice were indistinguishable from their littermates. Similar results were also observed by Sharan *et al.* (1997). One of the BRCA2-/+ mice developed a squamus cell carcinoma of skin at 4 months of age, but the heterozygosity was defined at BRCA2 locus and the tumor had no relevancy with BRCA2. The results of intercrosses between BRCA2 mice and between BRCA1 mice produced no BRCA null progeny indicating homozygous mutations in these genes result in embryonic lethality. For analyzing the lethal phenotypes, pregnant females were sacrificed at different times. A high incidence of empty decidua was observed in BRCA1-/+ intercrosses (about 50%), containing only giant cells and extraembryonic membranes. This was not the case for BRCA2-/+ intercrosses (6%) and all the nullizygous embryos were seen to be quite uniform especially before embryonic day 9.5 (E9.5). The histological and morhological analysis revealed that BRCA1 null embryos were hard to develop 3 germ layers and head fold, reduced in size and the most advanced ones had a yolk sac with blood islands. Another feature of BRCA1 null embryos was inconsistency, since the gross morphology of these embryos differ very much. In contrast to BRCA1 mutants, the E9.5 BRCA2 nullizygotes were all similar and exhibited a reduced embryonic size with head-folds, a large allantois and an expanded yolk sac with visible blood. Athough, slightly reduced in size, BRCA2-/- embryos developed 3 germ layers at E5.5 and possessed an anteroposterior axis at E8.5. The developmental problems of BRCA2 nullizygotes were reduction in size, poor development of amnion, loose structure of mesodermal layer and lacking somites.

The growth defficiency of BRCA2 knock out embryos were previously reported by Sharan *et al.* (1997) and linked to hypoproliferation although no experimental evidence was shown. In the study of Ludwig *et al.* (1997), a cell proliferation assay was done to qualify the growth of the nullizygotes by injetion of 5-Bromo-2'-deoxyuridine (BrdU) 1 hour before the sacrefice of mother. Incorporation of BrdU into DNA was assayed for counting the labeled and unlabeled nuclei. Embryos at E6.5 were found out to be no different than the normal controls whereas, at E7.5, the BrdU positive nuclei in normal embryos appear to be 15% higher than the nullizygotes. Interestingly, this was the time when BRCA2 expression became siginificant, so growth retardation could be related directly to hypoproliferation and uninduced BRCA2 expression.

To examine the combined effect of BRCA1 and BRCA2 mutations, BRCA1 (-/+) BRCA2 (-/+) double heterozygotes were crossed. Fifthy one decidual swelling were reported and the mothers were dissected at E9.5. Nine of the decidua were empty, whereas in the remaining 42, all expected genotypes were chracterized at expected rates. Embryos with at least one wild-type allele of each gene were normal and embryos lacking only one of the gene showed the phenotypic chracteristics of each mutation. Two of the 42 recovered decidua contained double nullizygous mutants. One of these had developed an allantois and severely reduced in size whereas the other consited of only yolk sac and giant cells. The double nullizygotes were phenotypically more similar to the BRCA1 null mutants.

In the study of Ludwig *et al.* (1997), the phenotype of p53/BRCA2 knock out mice was also defined. The majority of E9.5 embryos possesing either one wild type of each gene or lacking only p53 were normal (with 15-25 somites), although they were exencephalic. Of eight embryos lacking BRCA2 (all heterozygous for p53),

two were smaller than their most advanced littermates, but otherwise normal with 15-20 somites (other six were abnormal with variable phenotypes). Two double nullizyguos recovered at E9.5 were similar to the most advanced BRCA2 knock-out littermates, except exencephaly. The investigations done at E10.5 showed that, the embryos having at least one wild type allele of each gene and most of the embryos lacking p53 were normal (some p53 null embryos were exencephalic). There were five embryos lacking BRCA2 and heterozygous for p53,and they were all abnormal with variable phenotypes. All of the double nullizygotes (n=5), except one were more developed than the most advanced BRCA2-/- littermates with some exhibiting exencephaly. The exceptional double mutant embryo was indistinguishable from normal except that it also exhibited exencephaly.

Such results were also obtained in another knock-out study, which showed the partial restoration of embryonic lethality of MmRAD51 in p53-/- background (Lim *et al.* 1996). These results may indicate a possible antagonistic effect of p53 induced cell cycle arrest and BRCA2 mediated dsDNA repair. In p53 positive background BRCA2 knock-out embryos had extensive growth retardation. This growth retardation is mostly due to growth arrest instead of apoptosis because increased cell death is not chracterized as a feature of BRCA2 knock-out mice (Sharan *et al.* 1997). It could be the case that BRCA2 null, p53 positive cells can not repair dsDNA breaks, so undergo p53 mediated cell cycle arrest, whereas double (p53 and BRCA2) knock out cells progress through the cell cycle without any control of damaged DNA. This definition of partial restoration of embryonic lethality in p53-/-BRCA2-/- mice system also explains the rare involvement of BRCA2 in sporadic breast tumors. Kinzler and Vogelstein (1997) defined the two different subgroups of TSGs as 'caretakers' and 'gatekeepers'. 'Gatekeepers' have a negative regulatory role in cell cycle progression

and positive role in promoting cell death. Each cell type has one or a few 'gatekeepers' and by the inactivation of specific 'gatekeeper', a very specific tissue distribution of cancer arises. Retinoblastoma, neurofibromatosis type I and adenomatous polyposis coli are well chracterized 'gatekeepers', leading to the specific tumors of retina, Schwann cells and colon. The mutations in 'gatekeepers' directly promote tumor initiation, since the control of cell cycle progression and cell death was diminished.This is the opposite for 'caretakers', since they have role in maintaining the integrity of the genome. If a 'caretaker' has loses its function, it promotes an increase in the mutation rate, so the second hit at a 'gatekeeper' is more probable. Secondary mutations are essential for neoplastic transformation. Known caretakers include mismatch and nucleotide excision repair genes. The definition of BRCA2 null cells to be vulnerable to DNA demage and a possible interaction of BRCA2 with a known dsDNA break repair gene (Sharan *et al.* 1997) promotes the idea that BRCA2 is a 'careteaker', rather than a 'gatekeeper'.

1.5-7 Protein-protein interactions and functional studies

BRCA1 protein was shown to interact with HsRad51, a protein likely to be involved in the repair of double-strand DNA breaks (Scully *et al.* 1997). For identifying the proteins interacting with MmRad51, Sharan *et al.* (1997) performed a yeast two hybrid screen with a T-cell complementary DNA library. A C-terminal region of mouse BRCA2, coding for the amonoacids 3138-3232 was isolated. The results were confirmed in mammalian cell culture and the association appeared to be much stronger with MmRad51 as the prey and BRCA2 as the bait. This result lead the proposal of self transcriptional activity of the specific region of BRCA2, but it was shown to be not the case since without MmRad51, DNA binding domain fused

BRCA2 (residues3138-3232) had no transcription activation. This region was studied by Milner *et al.* (1997) and shown to produce no transcription activator activity alone. Sharan SK. *et al.* (1997) also performed a deletion analysis on BRCA2 as the bait, for determining the crucial aminoacids for interaction. The minimal region allowing a strong association with MmRad51 was turned out to be a 36 aminoacid fragment (residues 3196-3232). Deleting 13 aminoacids (3213-3226) of this fragment destroyed all the interaction. The 36 and 13 amino acids were 95% and 100% conserved between mouse and human suggesting an critical role. The same kind of deletion analysis performed on MmRad51 revealed the first 43 aminoacids to be critical for BRCA2-MmRad51 interaction.

Bacterial RecA protein is known to have role in homologous recombination and the generation of Holliday junctions. This protein has high affinity for binding single strand DNA (ssDNA). When it binds to ssDNA its affinity to double strand DNA increases and both ssDNA and dsDNA are accomodated in a 100 angstrom groove of the protein. The protein walks on the DNA, and reaching a homologous site, ATP catalyzed strand exchange begins (Alberts *et al.* 1994). Genetic recombination is essential to produce the genetic diversity and to repair DNA upon exposure to mutagens producing single strand and double strand DNA breaks. The murine homologue of bacterial recA protein (MmRAD51) was known to have functions like binding to single strand and double strand DNA and ATP catalyzed strand exchange (Radding *et al.* 1991 and Sung *et al.* 1994). Although, yeast mutated for RAD51 was shown to survive without double strand break repair and meiotic recombination (Shinohara *et al.* 1992), murine mutants of this gene are not viable (Lim *et al.* 1996). The hypersensitivity of MmRAD51 mutants to γ-radiation was also defined by Lim *et al.* (1996). Such a phenotype is expected in BRCA2

knock-out mice if MmRAD51-BRCA2 interaction is significant. Sharan *et al.* (1997) investigated the radiation sensetivity of BRCA2 null mice embryos at E3.5, since BRCA2 knock-out mice can not develop fully. Embryos were obtained from BRCA2-/+ intercrrosses and grown *in vitro* for seven days. The inner cell mass and the number of trophoblasts were assayed with exposure to 400 Rads of γ-radiation. Inner cell mass was marginally reduced in negative controls, but totally ablated in eight of the eight BRCA2-/- embryos. When the number of trophoblasts were concerned there was only a slight decrease in irradiated controls (average=31, n=8) when compared with non-irradiated ones (average=35, n=13). However in the case of the irradiated BRCA2 null embryos (n=8), the average number of trophoblasts were 15. The embryonic lethality, and sensitivity to γ-irrdiation proposes an interaction of BRCA2 and RAD51.

2 -AIM and STRATEGY

BRCA2 is an important gene whose germline mutations predispose to breast cancer. Most of the BRCA2 mutations give rise to truncated proteins lacking the C-terminal end of the protein. Although there is no clear evidence for the function of BRCA2, it was shown to interact with mammalian homologue of bacterial RecA protein which is involved in repair of DNA strand breaks and homologous recombination. Such large proteins may have multiple functions like being a matchmaker, transcription activator or functions concerning cell fate. For fully understanding the specific cellular functions of BRCA2, it is necessary to develop biochemical tools such as recombinant protein fragments and monoclonal antibodies directed against different regions of this large protein.

For this reason we decided to express human BRCA2 protein fragments in E. *coli*. and purify them in large quantities As a first step the extereme C-terminal portion of BRCA2 (E7) is expressed and purified.

First of all, we wanted the fragments to be modular and could be expressed either one by one or as combined proteins. This was achieved by identification of restriction enzymes that cut BRCA2 only once. Considering the fidelity and the capacity of PCR reaction, we divided BRCA2 (10485 bp) into 7 overlapping fragments which contain two single cutter sites, one at its 5 prime (5') and the other at its 3 prime (3') end. The fragments and the single cutters are defined in figure 5. Genomic DNA was an available source so, first the fragments corresponding the exon 11 (E2 and E3) were PCR amplified. The 3 prime (3') region of the gene was also

considered to be important since most of the mutations in BRCA2 are frameshift mutations leading to premature truncation of translation. With the available cDNA, E6 and E7 were also amplified to correspond to the exon 19 to exon 27, stop codon.

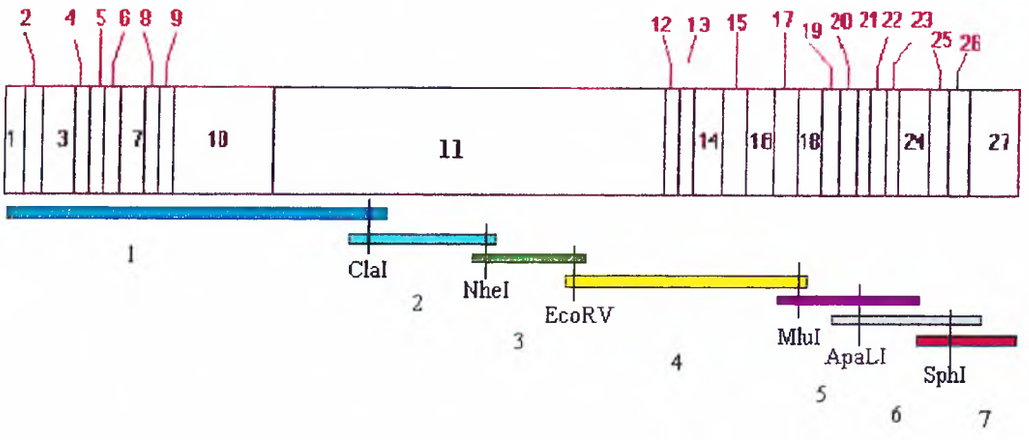


Figure 6: The PCR fragments of the BRCA2 gene: The fragments that were used for the cloning experiments are shown below the exon structure of BRCA2 gene with names of restriction endonucleases.

The forward primers were designed to contain a BamHI site and reverse primers were designed to contain a HindIII site. *Pfu* amplified blunt end PCR fragments were cloned into PCR-Script SK+ AMP cloning vector and then subcloned into pQE expression vector in correct frame. The advantages of the pQE vector are the high expression and easy purification of expressed proteins by the help of a 6XHis tag attached either the carboxyl or the amino terminus. 6XHis tag is a very uncommon protein motif in prokaryotes and it has a high affinity to bind Ni-NTA agarose columns. After binding to the column, 6XHis tag carrying proteins can be easily removed by antagonizing with imidazole (250 mM).

E7 was a trial for checking the feasibility of bacterial expression and purification of BRCA2 encoded proteins. The other domains of the protein will be cloned and expressed for defining the structural and functional aspects of BRCA2.

3- MATERIALS METHODS

Table-6: Primers used in this study.

Synthetic oligonucleotide primers used in this study

Name	Sequence 5'---------->3'	Location Strand position	Size	Tm ^{o}C
BRCA2F2	gggATCCACTTCTgAggAATgCAgA	sense 3691	25	61
BRCA2R2	gAAgCTTggCAgCTgTgATCTCAATgg	antisense 4992	27	65
BRCA2F3	CggATCCCAgTTggTACTggAAATC	sense 4708	25	61
BRCA2R3	CAAgCTTCAATACTggCTCAATACC	antisense 5548	25	55
BRCA2F6	AggATCCTCTgCCCTTATCATCgC	sense 8630	24	61
BRCA2R6	CAAgCTTCgTATTTggTgCCACAAC	antisense 10160	25	61
BRCA2F7	AggATCCAgAATCCAAATCAggCCTTC	sense 9614	27	63
BRCA2R7	AagCTTgTCgCCTTTgCAAATgCTTAg	antisense 10450	27	63

3.1 PCR

Polymerase chain reaction had been used during this study in order to amplify the human BRCA2 gene fragments. The requirements for PCR are listed below (Saiki *et al.* 1988). *Pfu* DNA polymerase was used to catalyze the reaction because *Pfu* DNA polymerase exhibits the lowest error rate of any thermostable DNA polymerase (1.3×10^{-6}) and the buffer conditions were described by the supplier.

1-Template DNA with known flanking sequences

2-Oligonucleotides with homologous sequences to that of the template flanking region (primers)

3-DNA polymerase and appropriate buffer

4-Deoxy-nucleotide triphosphates

5-Temperature cycling machine

A standard 100 μl reaction set up in a 0.2 ml PCR tube

primers	0.1-1 μM of each primer (reverse and forward)
dNTP	0.2 mM diluted from the 10 mM stock
buffer	1X diluted from the 10X stock
template	10 ng of genomic or plasmid DNA

Made up to 100 μl with MilliQ water

enzyme 2.5 unit of *Pfu* DNA polymerase

Each reagent and tube were placed on ice. To perform a hot-start PCR the thermal cycler was preheated to approximately 90°C before placing the tubes into it.

3.2 Purification of PCR products

PCR products were purified by precipitating the DNA with ammonium acetate. 1/10 volume of 10X STE buffer, equal volume of 4M ammonium acetate and 2.5 volume of 100% (v/v) ethanol were added to the PCR products sequentially. The reaction tube was centrifugated at 13000 rpm for 20 minutes at room temperature to pellet DNA. The supernatant was removed carefully and discarded. The pellet was washed with 200 μl of 70% (v/v) ice-cold ethanol and dried. DNA was resuspended by using TE buffer.

3.3 Horizantal electrophoresis of DNA

Agarose,which is extracted from seeweed is a linear polymer. Charged molecules like DNA or RNA became oriented in an electric field and migrate towards the attracting pole of the field. Such charged polymeric molecules are also capable to migrate within the porous structure of a linear polymer, like agarose. These properties of DNA and RNA were abused to qualitatively and quantitatively analyze nucleic acids. In the presence of a good electrolitic buffer and a electric field, nucleic acids migrate towords cathode through the linear pores of polymerized agarose. The seperation capacity of such gels depend on the agarose content which is summarized in table 7.

Table-7: The agarose content and the seperation range of DNA

Amount of agarose in the gel (%w/v)	**Efficient range of seperation of DNA (kb)**
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Generally TAE and TBE buffers were used to define a conductive medium. These buffers have their best electrolytic ability when used as 0.5-1X solutions. The contents of these buffers are summarized in table 8.

Table-8: TAE and TBE buffers

Buffer	**Working solution**	**Concentrated stock solution (liter)**
Tris-acetate (TAE)	1X: 0.04M Tris acetate 0.001M EDTA	**50X:** 242 g Tris base 57.1 ml Glial acetic acid 100 ml of 0.5 M EDTA
Tris-borate (TBE)	0.5X: 0.045M Tris borate 0.001M EDTA	**5X:** 54 g Tris base 27.5 g boric acid 20 ml of 0.5 M EDTA

Agarose is added at desired amount of 0.5-1X of electrophoresis buffer (volume adjusted with dH_2O) and the slurry mixture is heated until it gets transparent. The gel aparatus must be set before and the wells that DNA will be inserted must be defined with combs that were provided with the gel aparatus. After pouring and solidification, the gel is ready to be placed in a tank filled with the same running buffer and ready for DNA to be inserted to the holes provided by the comb. A steady voltage of 50-100V is applied to the buffer and the tank is frequently checked for overheating. It is of importance to consider the place of holes in the gel and the direction of the voltage since DNA will run from - to +.

Visualization of DNA in the gels could be achieved by soaking the gel in ethidium bromide solution (10 (g/ml) and staining for 10 minutes. The DNA could be viewed under UV light.

3.4 DNA ligation

DNA fragments, cut from agarose gel and recovered by Promega Wizard PCR Prep-kit were used for ligation. For directional cloning (where both vector and insert DNA has protruding ends) approximately 1:4 ratio of vector and insert DNA was mixed with the T4 DNA ligase buffer and 1 to 3 units of T4 DNA ligase. The reaction is carried out either 2 hours at room temperature or 16 hours at 12°C. For blunt end ligation, insert:vector ratio could be increased up to 40 and preferably the reactions are accomplished at 12°C. The reaction could be stopped directly by heating at 65°C for 10 minutes or directly used to transform competent bacterial cells.

3.5 Bacterial strains, media and storage

Table-9: E. *coli* strains used in this study.

Strains of E.coli :

Strain	Genotype	Usage	Source
DH5α	F^-, ϕ80d*lacZ* ΔM15,	Host for	Hanahan
	(Δ*lac*ZYA-*arg*F)U169,	recombinant	(1983)
	*rec*A1, *end*A1*hsd*R17 (rk^-, mk^+),	plasmid	
	*sup*E44, λ^-, *thi*-1, *gyr*A, *rel*A1		

Strain	Genotype	Usage	Source
M15 (pREP4)	Nal ^{s}, Str ^{s}, rif ^{s}, *lac* $^{-}$,	Host for	Villajero and
	ara $^{-}$, *gal* $^{-}$, *mtl* $^{-}$, F $^{-}$,	expression	Zabin
	uvr $^{+}$, *rec* A $^{+}$	studies	(1974)

Strain	Genotype	Usage	Source
JM 109	F', lac^{q}, *tra*D35 Δ (*lac* Z)*m*15	Propogation	Stratagene
	*pro*AB/*rec*A1 *e*14^{-}	of the	
	*end*A1 *gyr*A96 (*Nal^{r}*) thi	vector pQE	
	hsdR17 ($r^{-}_{K}m^{+}_{K}$) *sup*E44		
	(*mcr*A^{-}) *rel*A1Δ (*lac-pro*AB)		

Strain	Genotype	Usage	Source
Epicuran Coli	Δ (*mcr*A)183 Δ(*mcr*CB-	Host for	Stratagene
XL-1Blue MRF'	*hsd*SMR-*mrr*)173 *end*A1,	recombinant	#200248
Supercompetent	*sup*E44 thi-1 *rec*A1,	plasmid	
cells	*gyr*A96, *rel*A1, *lac*{F'proAB		
	*lac*I^{q}, *Z* (M15 Tn5 (Kan^{r})}		

Solid and liquid mediums :

LB medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl.

LA medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl and 1% agar.

Supplemented with appropriate antibiotics.

Antibiotics

Ampicilin and kanamycin were used in this study.

Table-10: Stock and working solutions of antibiotics.

	Stock solution		working concentration	
name of the antibiotic	**concentration**	**storage**	**stringent plasmids**	**relaxed plasmids**
Ampicilin	50 mg/ml in H_2O	- 20°C	20 µg/ml	60 µg/ml
Kanamycin	10 mg/ml in H_2O	- 20°C	10 µg/ml	50 µg/ml

3.6 Storage of *E.coli* strains and plasmid harboring bacteria

Strains of *E.coli* were stored in glycerol (long term storage). Glycerol cultures of *E.coli* were prepared by adding 0.81 ml of fresh culture to 0.91 ml of sterile 80% glycerol in a sterile screw capped tube. The tubes were vortexed until homogenous and were then frozen and stored at -70°C.

3.7 Preparation of competent cells

Cells were made competent using a modification of the $CaCl_2$ method described by Maniatis *et al.*, 1982.

5 ml LB was inoculated using a single colony from a freshly grown plate of the *E. coli* strain to be used, and was incubated for approximately 2 hours, until the culture was slightly turbid. The culture was then cooled on ice for 5 minutes, 1 ml

aliquots were added to microcentrifuge tubes and then the cells were pelleted by centrifugation (1 minute at 13,000 rpm). The cells were resuspended in 0.5 ml of 50 mM $CaCl_2$ by gentle vortexing, before being placed on ice for 30 minutes. The cells were pelleted by centrifugation (1 minute at 13,000 rpm) and the supernatant was discarded. The pellet was resuspended in 0.1 ml of $CaCl_2$ with gentle vortexing. The cells were then stored on ice until required for transformation. Alternatively competent cells were prepared and stored at -70°C until required. 500 ml of LB was seeded with a 10 ml of overnight culture and grown to an OD_{600}= 0.6. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C, before being incubated on ice for 20 minutes. The cells were harvested as before, resuspended in 25 ml of 50 mM $CaCl_2$/20% glycerol and aliquoted into microcentrifuge tubes before being frozen. Samples were stored at -70°C and were viable for at least 2 months. Cells were thawed on ice prior to the addition of DNA.

50 mM $CaCl_2$

0.735 g $CaCl_2.2H_2O$

Add H_2O to 100 ml

Filter sterilized using a disposable filter, or autoclaved

3.8 Transformation of bacterial cells

The DNA to be transformed (usually half of the ligation mixture or approximately 100 ng of plasmid DNA) was added to the 100 µl of competent cells, mixed gently and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 90 seconds and chilled by placing on ice for 2 minutes. 1 ml of prewarmed LB was then added and the suspension was incubated at 37°C for 1 hour.

Each sample was pelleted by centrifugation at 13,000 rpm for 2 minutes, resuspended in 100-200 μl of LB and plated onto selective medium and incubated overnight at 37°C to allow the growth of the transfomants.

If the vector-host system used featured α-complementation of β-galactosidase to enable for blue/white selection of recombinants, then 25 μl of a 20 mg/ml stock solution of X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethyl formamide and 20 μl of a 20 mg/ml stock solution of IPTG (Isopropylthio-β-D-galactoside) in dH_2O were added to the agar plates 1 hour prior to the spreading the cells. If the host was DH5αMCR then IPTG was omitted, since this strain lacks the *lac* repressor and hence expression from *Plac* promoter is constitutive.

3.9 Small scale preperation of plasmid DNA (Mini-preparation)

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979).

The transformed bacterial strain containing the plasmid of interest was grown at 37°C overnight in 5 ml of LB+antibiotic. 1.5 ml of the bacterial culture was pelleted by centrifugation for 1 minute (bench-top microfuge, 13,000 rpm) in a 1.5 ml mictofuge tube. After the discartion of the supernatant, the cells were resuspended in 0.1 ml of ice-cold solution I and stored for 5 minutes at room temperature. 0.2 ml of solution II was mixed by inversion, the tube was then stored on ice for 5 minutes. Bacterial chromosomal DNA and proteins were precipitated by the addition of 0.15 ml of ice-cold solution III. The mixture was left on ice for 5 minutes, then centrifugated in a bench-top centrifuge for 5 minutes to pellet the host DNA and proteins. The supernatant was mixed with an equal volume of phenol-chloroform

(1:1) and centrifugated in a bench-top microfuge for 3 minutes to seperate the two phases. The top phase was removed and plasmid DNA precipitated by mixing it with 2.5 volumes of 95% ethanol, and pelleted by centrifugation for 10 minutes (bench-top microfuge, 13,000 rpm) after keeping the mixture at 4°C for 15 minutes. The supernatant was discarded and the pellet was left for 15-20 minutes at room temperature to dry and then resuspended in 20-30 μl of TE buffer containing 10 μg/ml RNase A. Samples were stored at 4°C.

This procedure yields approximately 3 μg of DNA.

Solution I

50 mM glucose

25 mM Tris·Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/sq. in. on liquid cycle, and stored at 4°C.

Solution II

0.2 NaOH (freshly diluted from 10 N stock)

1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

3.10 Restriction enzyme digestion of DNA

DNA restriction reactions were carried out in a total volume of 20-100 µl with 5-10 units of restriction enzyme. 1 µl of boiled Rnase A (1mg/ml) was used where appropriate. The volume of the reaction buffers were arranged to be 1X and the enzyme volume was never greater than $1/10^{th}$ of the reaction volume so as to prevent star activity due to the high glycerol concentration.

3.11 Midiprep of plasmid preparation

Midi-preparations were carried out by using the kit supplied by Promega (Catalog number:A7510)

3.12 Maxiprep of plasmid preparation

Preparation of large scale bacterial culture

The bacterial strain containing the plasmid of interest is first grown in a 30 ml culture with necessery antibiotic until late log phase (OD_{600} of 0.6) then inoculted in 500 ml LB or Terrific Browth medium for 10-12 hours (2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol) could be added when OD_{600} of 0.4 is reached so as to increase the yield from low copy number plasmids.). The cells are harvested by centrifugation at 4000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor (or equivalent). The supernatant will be discarded and allow to drain away in a upside down position. Resuspend the cells in 100 ml of ice cold STE.

STE **:**

0.1 M NaCl

10 mM Tris·Cl (pH:8.0)

1 mM EDTA (pH:8.0)

Collect the cells again by centrifugation like described above.

Lysis by Alkali

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979) and the solutions were described above in the miniprep preparation of plasmid section.

The bacterial pellet is resuspend and washed in 18 ml solution I (in the presence of chloramphenicol, resuspend in 10 ml and follow the instructions in parentheses).

2 ml (1 ml) of freshly prepared solution of lysozyme is added (10 mg/ml in 10 mM Tris·Cl (pH:8.0).

40 ml (20 ml) of freshly prepared solution II is added, the top of centrifuge tube is closed and the bottle is inversed several times. The bottle must be stored at room temperature for 5-10 minutes.

20 ml (15 ml) of ice cold solution III is added, the top of the bottle is closed and shaked several times. The bottle must be stored on ice for 10 minutes.

The cell lysate iscentrifuged at 4000 rpm for 15 minutes at 4°C. The rotor must stop without braking.

The suprenatant is filtered through 4 layers of cheesecloth into a 250 ml centrifuge bottle. Adding 0.6 volume of isopropanol and mixing well, the nucleic acids are ready to precipitate. The bottle must be store at room temperature for 10 minutes.

Nucleic acids are recovered by centrifugation at 5000 rpm for 15 minutes at room temperature in a Sorvall GS3 rotor (or equivalent). Salt may precipitate if the centrifugation is performed at 4°C. The supernantant is poured off gently and the

bottle is inverted so as to allow all fluid to drain away. The pellet and the walls of the bottle is washed with 70% ethanol at room temperature. After draining off ethanol, by the help of a pasteur pipette attached to vacuum, remove any beads of liquid attached to the walls of the bottle. The unaccesable ethanol is left to evaporate at room temperature. The pellet is dissolved in 3 ml of TE (pH 8.0) and ready for centrifugation in CsCl-ethidium bromide gradients.

Purification of closed circular DNA by equilibrium centrifugation in CsCl-ethidium bromide gradients

The volume of the DNA solution must be measured exactly and for every mililiter, 1 g of solid CsCl must be added to the solution. It is feasable to warm the solution to 30°C to allow easy dissolution of the salt. The mixture is centrifuged at 8000 rpm for 5 minutes so as to separate the proteins complexed with ethidium brromide and pure nucleic acids. The clear red solution at lower phase contains nucleic acids and must be transfered to a Beckman Quick seal tube or equivalent. For this process, a disposable syringe could be used. It is very important to seal the centrifuge tube correctly and not to overload the tube. The density gradients are centrifuged at 80 000 rpm for 16 hours at 20°C. Two bands appear after centrifugation in the center of the gradient and upper band contains the linear bacterial chromosome and nicked plasmid DNA, whereas the lower and thicker band corresponds to the circular plasmid DNA. The deep red pellet on the bottom of the tube consists of ethidium bromide-RNA complexes. For collection of DNA bands, first a 21-gauge hypodermic needle is inserted into the top of the tube to allow air to enter. For minimizing the possibility of contamination, first the upper band is collected. The surface of the tube is cleaned

with ethanol and a piece of Scotch Tape is attached to the outside of the tube. An 18-gauge hypodermic needle is inserted just below the upper band and the upper band is collected. As collection is finished, the needle is plugged and a second needle is inserted just below the lower band of DNA and the viscous solution is collected. The ethidium bromide must be removed from the DNA solution by extracting at least 6 times an equal volume of isoamyl alcohol. The DNA must also be cleared away from CsCl so the final solution of DNA is diluted with 3 volumes of water and 2 volumes of ethanol (total volume is 6X of the original) and precipitaed by centrifugation at 10000g for 15 minutes at 4°C. This method yields 1-5mg of plasmid DNA.

3.13 Quantification of double strand DNA

The amount of DNA in samples were determined by by reading the absorbance of the samples at 260 nm. An OD_{260} of 1 corresponds to a concentration of 50 μg/ml for double stranded DNA, 40 μg/ml for single stranded DNA and 20 μg/ml for oligonucleotides.

3.14 Expression studies and induction of bacteria

Freshly transformed bacteria harboring the expression plasmid were inoculated from a single colony from LB agar plate to 5-10 ml LB medium with selective antibiotics. The bacteria were allowed to grow to reach a OD. of 0.7-0.9 then seperated into two tubes, one being the uninduced control. IPTG is added to be 0.4-2.0 mM in the final concentration to one of the tubes and inoculation proceeds for another 5 hours.

3.15 Purification of recombinant protein

Protein purification is done under denaturing and non-denaturing conditions.

3.15-1 Small scale protein purification under denaturing conditions :

Bacteria harboring expression plasmid was cultured overnight in 5-10 ml LB with adequate sellection of antibiotic. The concentration of antibiotic could be inreased so as to minimize the plasmid loss. Inducution of the cells were done with 0.4-2 mM IPTG for 5 hours. Cells were harvested by centrifugation at 20000 rpm for 2 minutes. The cell pellet was dissolved in 2 ml of buffer B.

Buffer A :

8 M Urea

300 mM NaCl

50 mM NaH_2PO_4

Buffer B : same as buffer A but the overall pH of this buffer is adjusted to 8.0 with HCl.

The suspension was vortexed so as to lyse the cells avoiding frothing. The suspension gets clear when lysis is complete. The sample must be qickly loaded to the column prepared and equilibrated before.

3.15-2 Preparation and equilibaration of the column

The column can be prepared from a 10 ml syringe. The opening of the syringe was plugged with cotton glass and wetted with dH_2O. 200-400 µl 50% Ni-NTA agarose was placed into the column. It is very important not to plug very much glass cotton that could prevent flow of the fluids by gravitational force. After allowing the Ni-NTA agarose to settle, 2 ml of buffer B is added and allowed to flow by

gravity. It is very important not to allow the column dry, so as the flow ends, 200 µl of buffer B is placed into column and the opening of the column is plugged. The column must equilibrate in this position for 30 min. at room temperature before loading.

3.15-3 Loading and purification of the sample

100 µl aliquot is taken from total lysate (fraction 1) and kept at -20°C for SDS-PAGE analysis of proteins. The rest of the toyal lysate is loaded on the pre-equilibrated column. The fluid must flow by gravity. 6XHis tagged proteins were supposed to bind the column and the rest of the proteins were supposed to flow down and produce the second fraction namely; flowthrough. Washing the column 3 times with 2 ml of buffer C is essential to remove any unspecific bound proteins from the column.

Buffer C : Same as buffer A but pH adjusted to 6.3 with HCl.

These elutions are called fractions 3,4 and 5. Finally the 6XHis tagged proteins were removed from the column by 1 ml of buffer D (fraction 6).

Buffer D : Same as buffer C but 400 mM imidazole is added.

If wanted all the proteins that bind the column could be eluted with strip buffer (fraction 7), which removes the Ni^{++} ions from the column so the proteins bound the Ni^{++} ions. It is of imporance that the stripped columns can not be used again.

Strip buffer : 0.1-0.5 M EDTA pH : 8.0

The fractions can be analyzed by SDS-PAGE protein electrophoresis.

3.15-4 Large scale protein purification under non-denaturing conditions

100 µl of bacteria which had been inoculated from a colony on agar plate harboring the expression vector was inoculated into 20 ml of LB media including the

appropriate antibiotic and incubated overnight at 37°C. 10 ml of this culture was then used to inoculate 500 ml of fresh LB media+antibiotic. Bacteria were grown until the absorbtion at 600 nm reach 0.7-0.9. IPTG is added to a final concentration of 2 mM and incubation was further continued for 5 hours.

Cells were harvested by spinning at 5000 rpm for 10 minutes at 4°C using a Sorvall GS3 rotor (or equivalent), supernatant was discarded. The pellet was resuspended in 5-volumes of solubilization buffer and homogenized in the homogenizer for 5 times of 90 second periods. It is very imprtant to keep the samples on ice between each homogenization to aviod protein degredation. Samples were left on a rotative shaker in a refrigerated room for 20 minutes. An aliquot of 0.5 ml was taken, and soluble and insoluble fractions were seperated by centrifugation at 15,000 rpm for 5 minutes at 4°C. Both fractions were kept at -70°C. The remaining suspension was treated with liquid nitrogen and 37°C iteratively (freeze-thaw) 5 times. An aliquot of 0.5 ml was taken, and soluble and insoluble fractions were seperated by centrifugation at 15,000 rpm for 5 minutes at 4°C. Both fractions were kept at -70°C. The remaining suspension was sonicated in ice cold water for 2 minutes. An aliquot of 0.5 ml was taken, and soluble and insoluble fractions were seperated by centrifugation at 15,000 rpm for 5 minutes at 4°C. Both fractions were kept at -70°C. The remaining suspension was centrifugated at 4°C for 10 minutes at 13,000 rpm. The supernatant and the pellet was freezed at -70°C.

Solubilization Buffer

50 mM Sodium phosphate (NaH_2PO_4) pH:8

300 mM Sodium Chloride (NaCl)

1mg/ml lysozyme (freshly added)

Preparation of the column was described above in 'small scale purification of proteins under denaturing conditions' section, but since this is a large scale purification, 1 ml of 50% Ni-NTA agarose is placed in the column and equilibrated with 2 ml of buffer A.

3.15-5 Loading and purification of the sample

2.5 ml of supernatant from the supernatant was applied to the column and allowed to flow with gravity. This fraction was flowthrough and the 6XHis tagged proteins were supposed to bind the column. The column was washed 2 times with buffer A and 1 times with buffer B. The wash steps producing fractions W1, W2 and W3 supposed to clear all the non-specific bindings, whereas the 6XHis tagged proteins were supposed to retain. The elution of tagged proteins were done in 2 steps, eluting with 300 µl of buffer C and 300 µl of buffer D. The fractions obtained as total lysate, flowthrough, W1, W2, W3 and buffer C and buffer D could be run in a SDS-PAGE gel and stained with coomassie blue.

Buffer A

50 mM Sodium phosphate (NaH_2PO_4) pH:8

300 mM Sodium Chloride (NaCl)

Buffer B

Buffer A with 20mM imidazole

Buffer C

Buffer A with 250mM imidazole

Buffer D

Buffer A with 500mM imidazole

3.16 Analysis of proteins by SDS-PAGE electrophoresis

The glass plates were assembled according to the manufacturer's instructions (BioRad). The volume of the gel mold was determined according to the information provided by the manufacturer (BioRad). In an Erlenmeyer flask, the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel was prepared. Without delay, the mixture was swirled rapidly and the acrylamide solution was poured into the gap between the glass plates. Sufficient space (the length of the teeth of the comb plus 1 cm.) for the stacking gel was left. The acrylamide solution was overlayed by using a pasteur pipette with 0.1 % SDS (for gels containing <8% acrylamide) or isobutanol (for gels containing >10% acrylamide). The gel was placed in a vertical position at room temperature. After polymerization was complete, the overlay was poured off and the top of the gel was washed several times with deionised water to remove any unpolymerized acrylamide. As much fluid as possible was drained from the top of the gel and then any remaining water was removed with the edge of a paper towel.

Stacking gel was prepared in a disposable plastic tube at an appropriate volume and at desired acrylamide concentration. Without delay, the mixture was swirled rapidly and the stacking gel solution was poured directly onto the surface of the polymerized resolving gel. The comb was immediately inserted into the stacking gel, being careful to avoid trapping air bubbles. The gel was placed in a vertical

position at room temperature. While the stacking gel was polymerizing, the samples to be loaded were prepared by heating them to 100°C for 3 minutes in 1X SDS gel-loading buffer to denaturate the proteins. After polymerization was complete, the comb was removed carefully. By using a squirt bottle, the wells were washed with deionized water to remove any unpolymerized acrylamide.

The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. The bubbles that were trapped at the bottom of the gel between the glass plates were removed by a bent hypodermic needle attached to a syringe. 100-200 μg of protein was loaded in a predetermined order into the wells. The electrophoresis apparatus was attached to an electric power supply and the gel was run at a voltage that is approximately 8V/cm, After the dye front has moved to the resolving gel, the voltage was increased to 15 V/cm, until the bromophenol blue reaches the bottom of the resolving gel. Then the power supply was turned off.

The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. By using a spatula, the plates were pried apart. Orientation of the gel was marked by cutting a corner from the bottom. The effective range of seperation of SDS-PAGE gels are illustrated in table 11 and the contents of the running and stacking gels are illustrated in tables 12 and 13.

Table-11: Effective range of separation of SDS-PAGE gels.

Acrylamide concentration (%)	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

1X SDS gel-loading buffer

50 mM Tric·Cl (pH 6.8)

100mM dithiothreitol (DTT) [61]

2% SDS (electrophoresis grade)

0.1% bromophenol blue

10% glycerol

1X SDS gel-loading buffer lacking DTT can be stored at room temperature. DTT should be added, just prior to use, from 1 M stock.

Tris-glycine electrophoresis buffer

25 mM Tris

250 mM glycine (electrophoresis grade)

0.1% SDS

Table 12: Solution for preparing resolving gels in Tris-glycine SDS-PAGE.

Solution components	**Component Volumes (ml)**							
8%	**5 ml**	**10 ml**	**15 ml**	**20 ml**	**25 ml**	**30 ml**	**40 ml**	**50 ml**
dH_2O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.030
10%								
dH_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

12%								
dH_2O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020
15%								
dH_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
10% APS	0.05	0.1	0.15	0.20	0.25	0.30	0.40	0.50
TEMED	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.020

Table 13: Solution for preparing 5% stacking gels

Solution components	Component Volumes (ml)							
5% gel	**1 ml**	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**	**8 ml**	**10 ml**
dH_2O	0.68	1.4	2.1	2,7	3.4	4.1	5.5	6.8
30% mix	0.17	0.33	0.50	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0,25	0.38	0.50	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

30% mix (Acrylamide and bis-acrylamide solution)

A stock solution of 29% (w/v) acrylamide and 1% (w/v) bis-acrylamide. Solution was stored in dark bottles at 4°C.

10% SDS

A 10% (w/v) stock solution was prepared in deionized water

APS

A small amount of 10% stock solution was prepared in deionized water and stored at 4°C.

3.17 Staning of proteins with Coomassie blue

Polypeptides separated by SDS-polyacrylamide gels were fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250.

0.25 g of Coomassie Brilliant Blue R250 was dissolved in 90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid. In order to remove any particulate material, the solution was filtered through a Whatman No. 1 filter. The gel was immersed in at least 5 volumes of staining solution and was placed on a slowly rotating platform for a minimum of 4 hours at room temperature.

At the end of staining, the stain was removed and saved for future use. The gel was destained by soaking in the methanol-acetic acid solution without the dye (90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid), on a slowly rotating platform for 4-8 hours at room temperature, changing the destaining solution three or four times destaining for 24 hours allows as little as 0.1 μg of protein to be detected as a single band.

After destaining, gels can be stored indefinitely in water or can be dried.

4- RESULTS

The aim of this project is bacterial cloning and expression of BRCA2 encoded proteins. The achievements and the results obatained in this study are summarized in a chronogical order. In figure 7 the structure of BRCA2 as 7 overlapping fragments is shown.

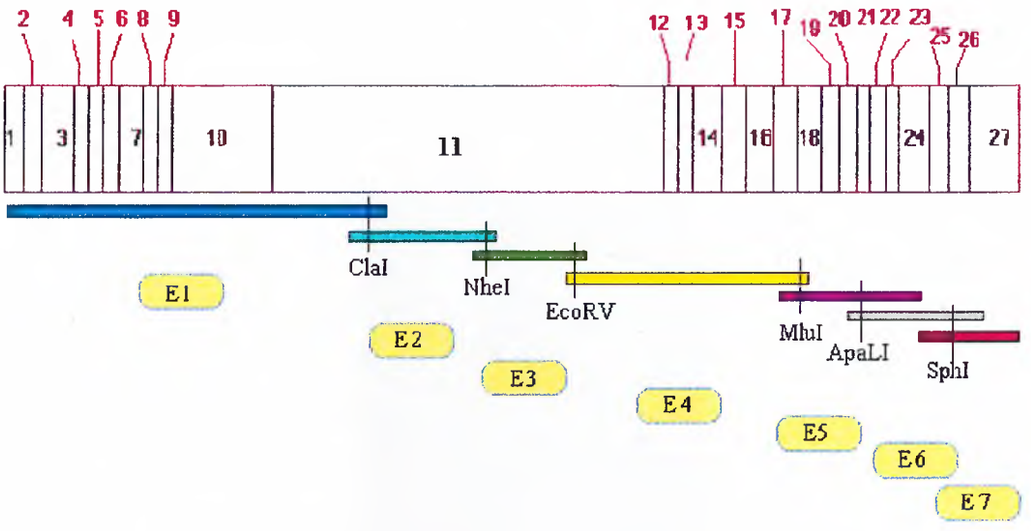


Figure-7: Fragments of BRCA2 gene.

- PCR amplification of E2, E3, E6 and E7 was accomplished
- E3, E6 and E7 were cloned into PCR-Script cloning vector
- Positive clones were selected and confirmed by restriction enzyme digestion
- Maxiprep plasmid preparation of pQE expression vectors were done
- pQE expression vectors were digested for subcloning and gel isolated
- Inserts in PCR-Script cloning vectors were digested and gel isolated
- Expression vectors and inserts were ligated and transformed into bacteria

- Positive clones were selected
- E7 was further confirmed for expression studies
- Induction of bacteria harboring E7 was achieved and the recombinant protein was purified.

4.1 Polymerase chain reaction (PCR)

For cloning of BRCA2 fragments into pCR-Script SK (+) cloning vector, first of all 4 of the BRCA2 fragments were amplified (Figure 8, 9 and 10).

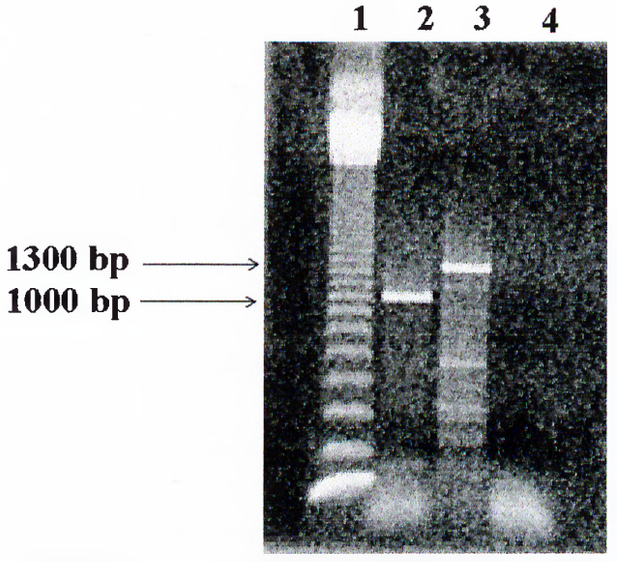


Figure-8: PCR amplification of E2. **Lane 1**: 123 bp DNA ladder (Sigma), **lane 2**: Control PCR (1008 bp), **lane 3**: BRCA2 F2R2 (1301 bp), **lane 4** : negative control.

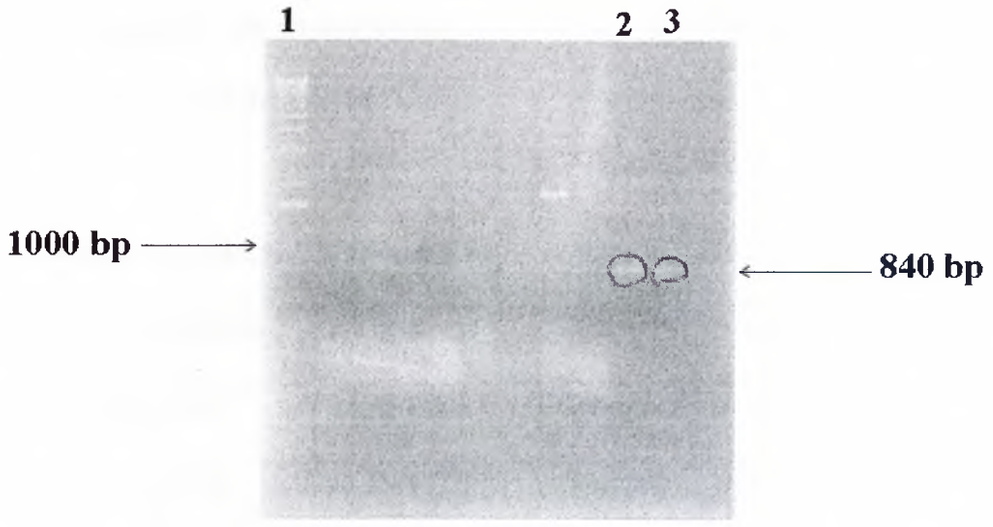


Figure 9: PCR amplification of E3. **Lane 1** : 1 kb ladder (HT Ltd.), **lane 2**: BRCA2 F3R3 (840 bp), **lane 3**: BRCA2 F3R3 (840 bp)

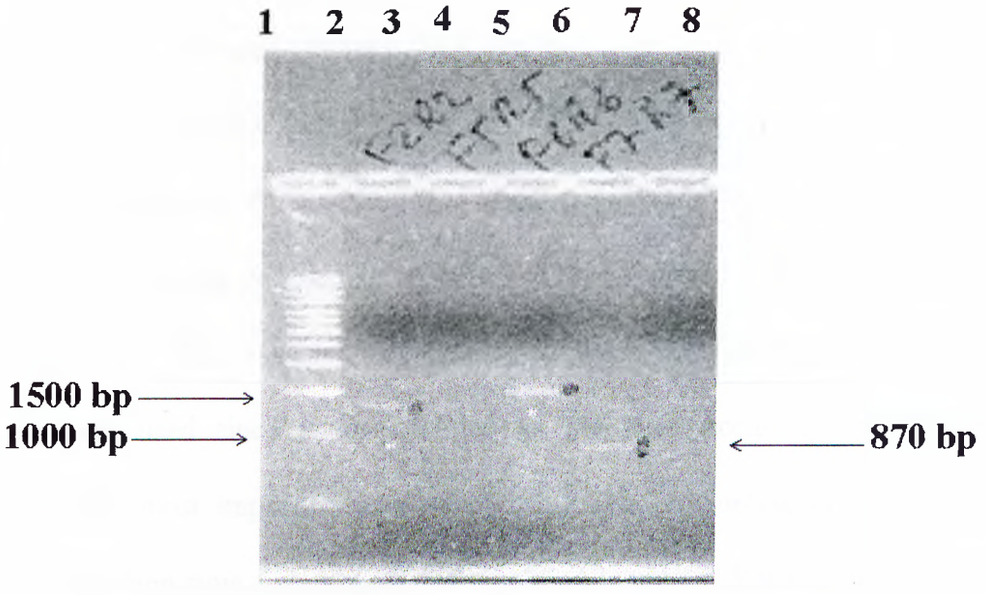


Figure 10: PCR amplification of E2, E6 and E7. **Lane 1** :1 kb DNA ladder (MBI), **lane 2**: BRCA2 F2R2 (1301 bp), **lane 3**: BRCA2 F5R5 (-), **lane 4**: BCA2 F6R6 (1530 bp), **lane 5**: BRCA2 F7R7 (870 bp)

These fragments are named as E2, E3, E6 and E7. The features of these fragments are summarized in table 14.

Table-14: The features of E2, E3, E6 and E7.

Primer Set	Base numbers	a.a numbers	Template	size	T (A)
F2R2 (E2)	3691-4992	1154-1588	Genomic	1301	56
F3R3 (E3)	4708-5548	1495-1773	Genomic	840	52
F6R6 (E6)	8630-10160	2800-3311	cDNA	1530	57
F7R7 (E7)	9614-10485	3128-3418	cDNA	871	58

E2 and E3 were designed to amplify 1301 and 840 basepairs of DNA from exon 11 of BRCA2, so I used genomic DNA obtained from blood as the template. E6 and E7 correspond to the C-terminal part of BRCA2 covering exons 19 to 27, so cDNA must be used for PCR reactions. I used cDNA that was obtained from a hepatocellular carcinoma cell line (Hep3BTR). These fragments were designed to amplify 1530 and 871 basepairs of DNA. In these amplifications *Pfu* DNA ploymerase was used since it was defined as the most accurate thermostable polymerase. The most important aspects of using *Pfu* polymerase is to allow 2 minutes of extension time for every kb of amplified template. *Pfu* polymerase also lacks the nonspecific terminal transferase activity so produce blunt end fragments. Addition of non-template directed nucleotides is a common property of all thermostable polymerases except *Pfu*. Therefore, this high fidelity enzyme is a good choice for DNA amplifications in expression studies as well as the usage of amplified

fragments with pCR-Script Amp SK (+) cloning kit where blunt end ligation is recomended.

In amplifications from genomic DNA, I tried the essence of Dimethylsulfoxide (DMSO) and saw that adding 5% DMSO of to the final volume of PCR reaction increases efficiency. In figure 11 the increased efficiency of PCR amplification by DMSO is shown.

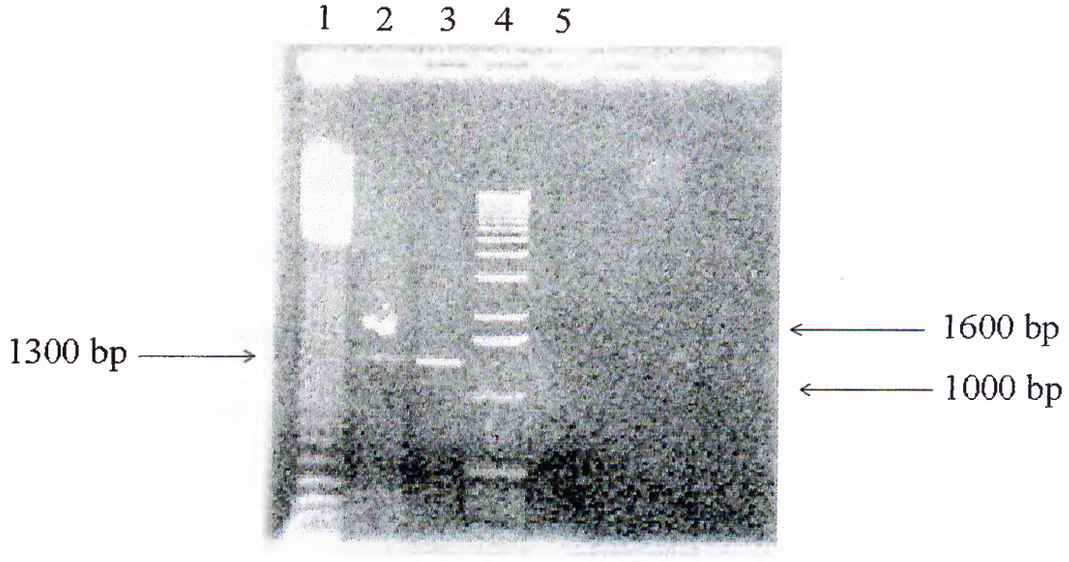


Figure 11: PCR amplification of E2 with and without DMSO. **Lane 1**: 123bp DNA ladder, **lane 2**: BRCA2 F2R2 (without DMSO), **lane 3** : BRCA2 F2R2 (with 5% DMSO)

After each PCR reaction I run 2-5% of the reaction volume on an agarose gel to qualitatively and quantitatively analyze the products. For direct cloning procedures, viewing the PCR result and cutting and purifying the specific band from agarose gels is essential, especially if multiple products were observed in the primary analysis.

The amplifications of BRCA2 fragments were succesful with little or no non-specific bands, at the expected sizes.

Purification of the PCR results were done either like described in the materials methods section or by using the MBI DNA extraction kit (#K0513), which utilizes the midified glass beads protocol of Vogelstein and Gillespie (1979) for recovering DNA from agarose gels. After purifying the specific band from agarose gel, 1 or 2μl of the recovered DNA must be run on an agarose gel to define the exact concentration of DNA since defining the concentration of DNA is essential for ligation reactions.

4.2 Cloning of the three different PCR fragments of BRCA2 into the pCR-Script cloning vector.

The PCR-Script cloning kit allows the easy ligation of blunt ended PCR products to PCR-Script cloning vector. The map and multiple cloning site of this vector is illustrated in figure 12.

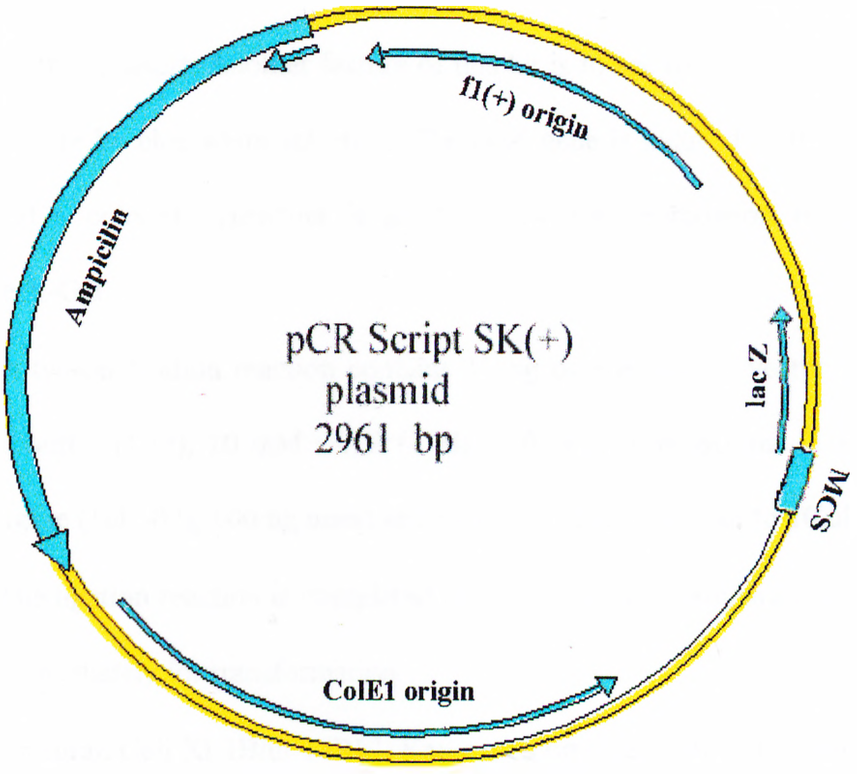


Figure 12: PCR-Script cloning vector and multiple cloning site (MCS).

Multiple Cloning Site can be cleaved with following restriction endonucleases at the sites shown in paranthesis: *Kpn*I (657), *Apa*I (663), *Dra*II (660), *Sal*I (674), *Cla*I (684), *Hind*III (689), *EcoR*V (697), *EcoR*I (701), *BamH*I (719), *Srf*I (728), *Not*I (738), *Sac*I (759).

pCR-Script AMP (SK+) cloning vector (Stratagene #211190) is supplied with a kit for allowing the direct cloning of *Pfu* polymerase amplified fragments. A novel *Srf*I site was generated in the muliple cloning site of pBlueScript AMP (SK+) vector to produce PCR-Script AMP (SK+) cloning vector. *Srf*I is rare cutter which recognizes GCCCGGGC and cuts after GCCC, producing a blunt end. A more

important feature of *Srf*I is the ability to work in the ligation mixture. The novel restriction site is cleaved by *Srf*I during ligation, so the ligated vectors are freed for ligation with the insert. Another feature of this kit is to see the clones directly on the LB agar plate by blue/white selection. The lacZ gene is induced in the presence of IPTG and a colored byproduct is produced by the degradation of a galactose analogue X-Gal.

A typical ligation reaction contains 10 ng of predigested vector (1 ul), 10X ligation buffer (1 ul), 10 mM rATP (1 ul), *Srf*I restriction enzyme (1 ul, 5U), T4 DNA ligase (1ul, 4U), 100 ng insert and enough dH2O to add up to 10 ul.

The ligation reaction is completed in 1 hour in room temparature and it must be used immediately for transformation.

Epicuran Coli Xl-1Blue MRF' Kan supercompetent cells, that were provided with the PCR-Script kit were used for transformations. Spreading 25 ul of 0.1M IPTG and 40 ul of 2% X-Gal (in dimethylformamide) onto LBA (LB agar) plate is essential for color selection. Since IPTG is toxic, spreading must be done 1 hour prior to inoculation of bacteria. It is also essential to incubate the transformants in SOC (or LB) medium without any selection for 45 minutes for the pre-induction of ampicilin resistance gene (β-lactamase).

4.3 Minipreps of plasmid DNA

The transformed supercompetent cells produced lots of colonies on the plate of each transformation for BRCA2 fragments but the efficiency of transformation turned out to be not as much as stated in the manuel of the kit. Out of 87 white colonies investigated for the presence of vector+insert, only 6 were right. Conformation of the colonies were done by miniprep isolation of plasmid technique,

which was previously defined in materials-methods section and illustrated in figures 13, 14 and 15.

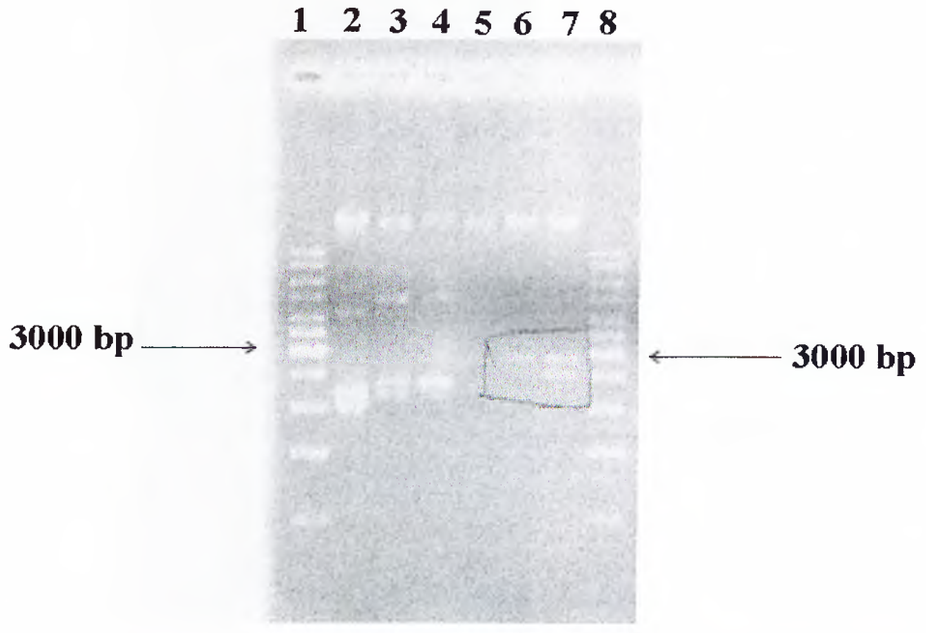


Figure 13: Clones from E3. **Lane 1**: 1 kb ladder (MBI), **lane 2, lane3, lane 4, lane 5, lane 6, lane 7**: white colonies selected from the plate, **lane 8**: 1 kb ladder (MBI)

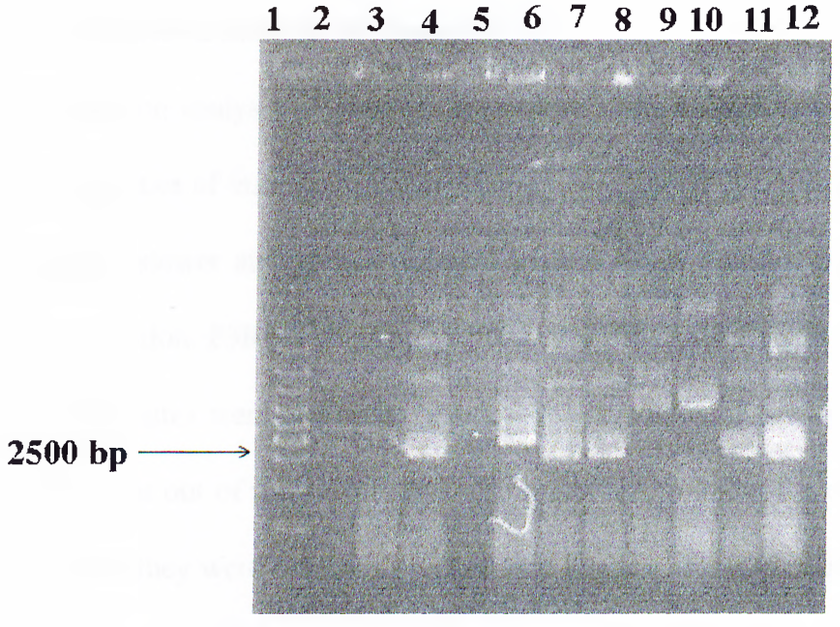


Figure 14: Clones from E6. **Lane 1**: 1 kb ladder (MBI), **lane 2, lane3, lane 4, lane 5, lane 6, lane 7, lane 8, lane 9, lane 10, lane11, lane 12**: white colonies selected from the plate

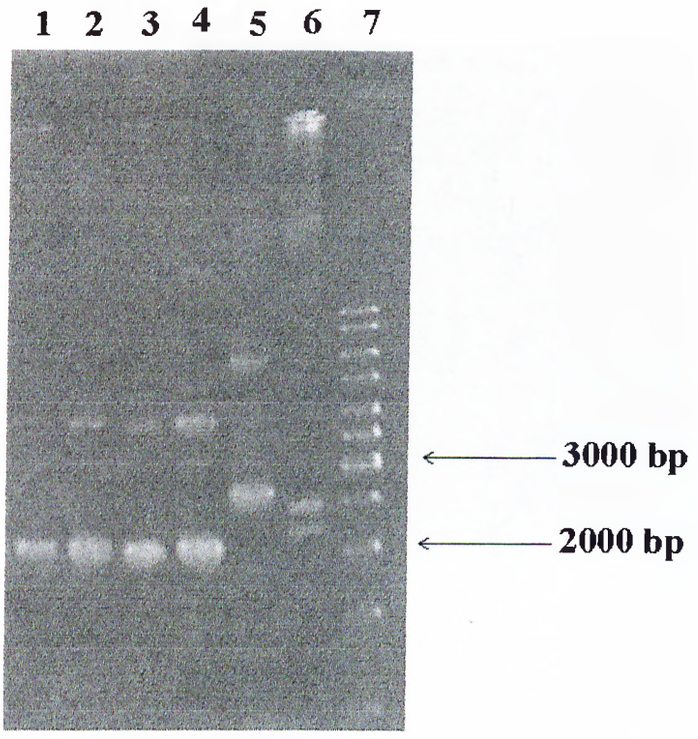


Figure 15: Clones from E7. **Lane 1, lane 2, lane3, lane 4, lane 5, lane 6**: white colonies selected from the plate, **lane 7**: 1 kb ladder (MBI)

4.4 Restriction analysis of plasmid DNA

Restriction analysis of plasmid DNA were done according to the specific restriction properties of inserts. Probable plasmids carrying an insert, unlike religated vectors, migrate slower and gave a different pattern. Such retarded plasmids were chosen for restriction. F3R3 containing vector is digested with BamHI and HindIII. These restriction sites were artifically introduced to the ends of each primer so E3 could be easily cut out of the vector. It is also important to know the orientation of the inserts, since they were obtained by blunt end ligation. The restriction results of plasmids are illustrated in figures 16, 17, 18, 19.

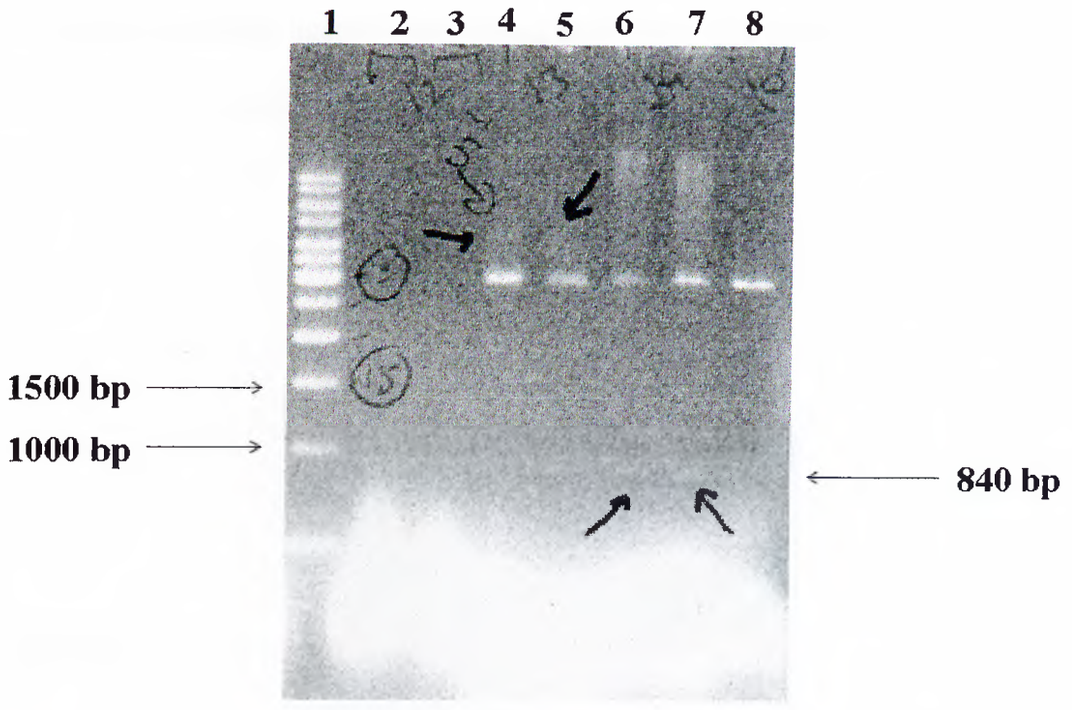
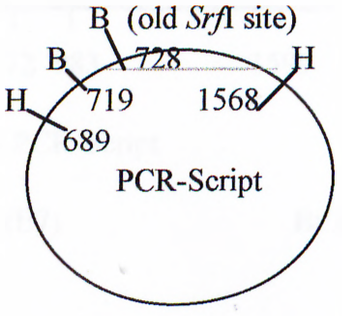


Figure 16: HindIII, BamHI/HindIII digestion of probable clones of E3. **Lane 1**: 1 kb ladder (MBI), **lane 2**: HindIII cut of proable clone 1, **lane3**: BamHI and HindIII cut of proable clone 1, **lane 4**: HindIII cut of proable clone 2, **lane 5**: BamHI and HindIII cut of proable clone 2, **lane 6**: HindIII cut of proable clone 3, **lane 7**: BamHI and HindIII cut of proable clone 3, **lane 8**: HindIII cut of a plasmid obtained from a blue colony.

The result, although not very clear due to high RNA contamination, is good and the expected size of insert (840 bp) could be visualized. It is also probable to deduce the orientation of the clone since HindIII digestion yield a bigger DNA fragment than the BamHI/HindIII digestion. This size difference is due to the internal BamHI and HindIII sites of PCR-Script (see MCS).



B: BamHI, H: HindIII

The insert had been ligated in the orientation above and HindIII digestion yields a 921 bp fragment, whereas BamHI and HindIII digestion yields only 840 bp (exact size of insert).

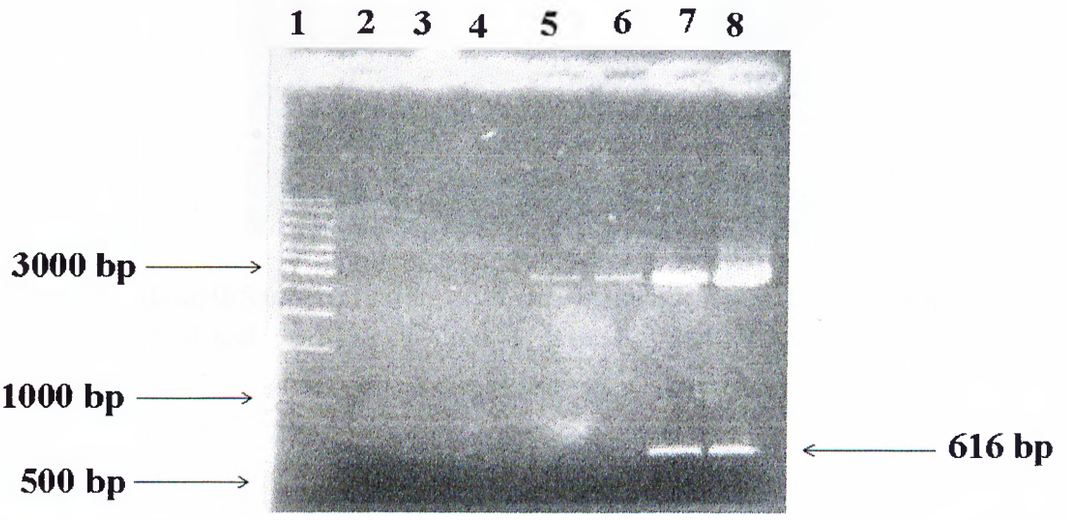
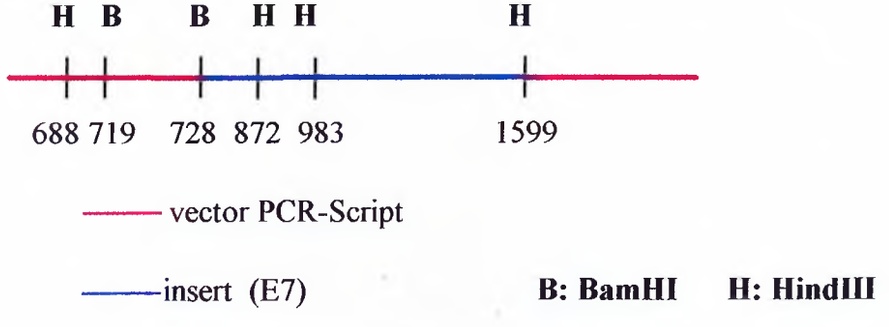


Figure 17: HindIII restriction of probable clones from E7. **Lane 1**: 1 kb ladder (MBI), **lane 5**: BamHI and HindIII cut of a plasmid obtained from a blue colony, **lane 6**: HindIII cut of a plasmid obtained from a blue colony, **lane 7**: HindIII cut of probable clone 1 (colony 5), **lane 8**: HindIII cut of probable clone 2 (colony 6).

The results indicate that these clones have the same orientation and the expected size of HindIII cut E7 is 616 bp (since there are internal HindIII sites in E7).



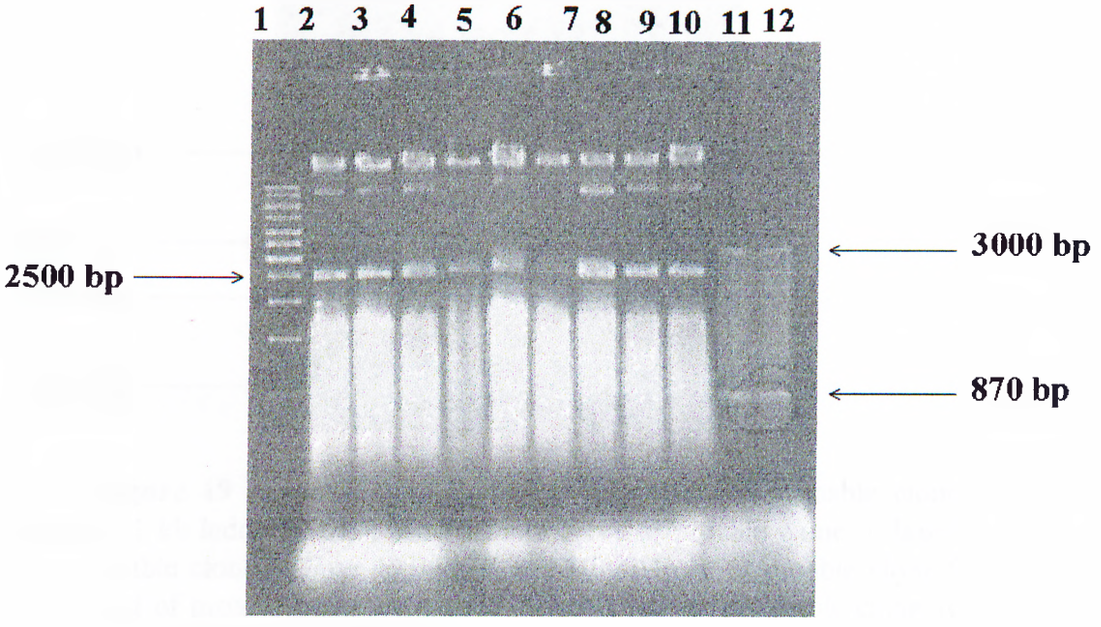


Figure 17: BamHI/SacI restriction of clones from E7. **Lane 1**: 1 kb ladder (MBI), **lane 11**: BamHI and SacI cut of clone 1, **lane 12**: BamHI and SacI cut of clone 2.

Since there are internal HindIII sites in E7, it is convinient to cut with BamHI and another enzyme in the MCS of PCR-Script. It is also important to find the other enzyme in the MCS of pQE, so subcloning could be achieved. The other enzyme was identified as SacI which allowed the isolation of total E7 as 880 bp.

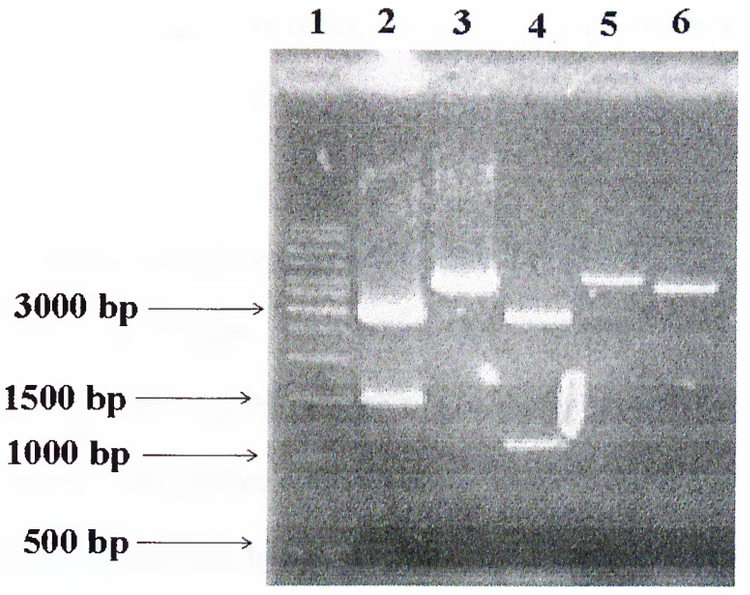


Figure 19 : BamHI, BamHI/HindIII restriction of probable clones from E6. **Lane 1**: 1 kb ladder (MBI), **lane 2**:BamHI cut of proable clone 9, **lane3**: HindIII cut of proable clone 9, **lane 4**: BamHI and HindIII cut of proable clone 9, **lane 5**: BamHI cut of proable clone 10, **lane 6**: HindIII cut of proabable clone 10, **lane 7**: BamHI and HindIII cut of PCR-Script, **lane 8**: HindIII cut plasmid obtained from a blue colony.

The results indicate that clone 9 has the right inset of size 1530 whereas clone 10 has lost its BamHI site. The orientation and the expected sizes of restrictions can be understood from the figure below.

——— vector PCR-Script

———insert (E7)

B: BamHI H: HindIII

BamHI restriction of the plasmid must yield a 1539 bp fragment whereas HindIII digestion will produce multiple bands but the visible one will be 1130 bp.

Since the clones were ready, I began small scale and large scale preparation of the expression vector, pQE

4.5 Transormation and miniprep isolation of pQE

pQE is an expression vector which also allows the purification the expressed proteins by the 6XHis tag. Purification of mammalian proteins with the 6XHis tag is easy in bacterial sytems since 6XHis is not a common epitope of bacterial proteins. The purification is achieved by applying the cellular lysate into a Ni-NTA column and allowing the 6XHis tagged proteins to bind the column material. Nickel-6XHis interaction is very strong and the recombinat proteins are held in the column even under very stringent washing conditions (1M NaCl). Such an interaction could only be diminished by antagonizing the 6XHis tag, washing the column with imidazole. The plasmid map of pQE and the detailed multiple cloning site of pQE are illustrated in figures 20 and 21.

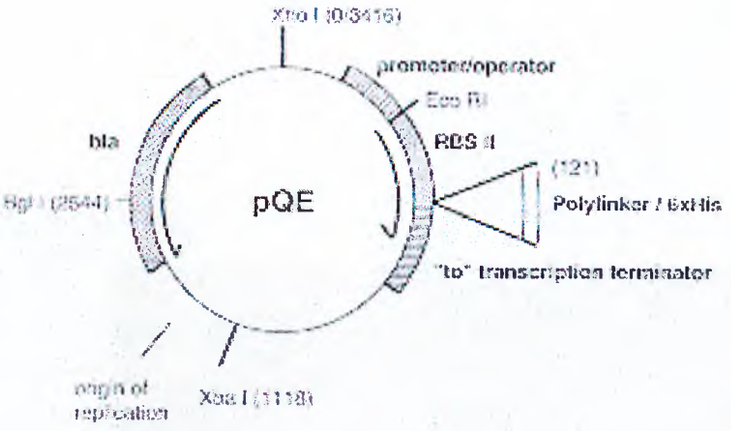


Figure 20: The pQE expression vector

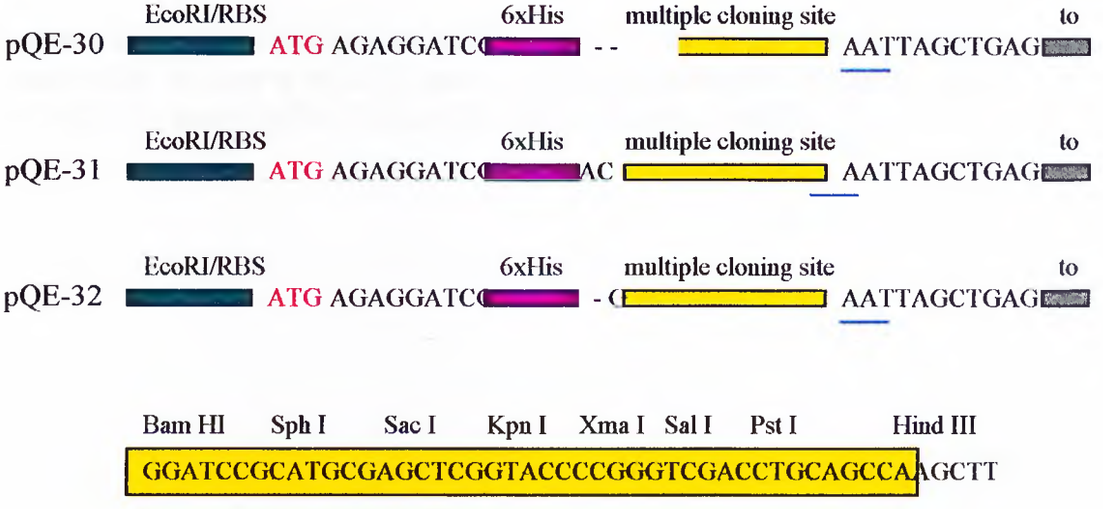


Figure 21: The 6XHis tag and the MCS of pQE

We had the DNA of pQE 30,31 and 32 and transformed them into DH5α so as to have large quantity of vector plasmid. The miniprep and the BamHI restriction results were summarized in figures 22 and 23.

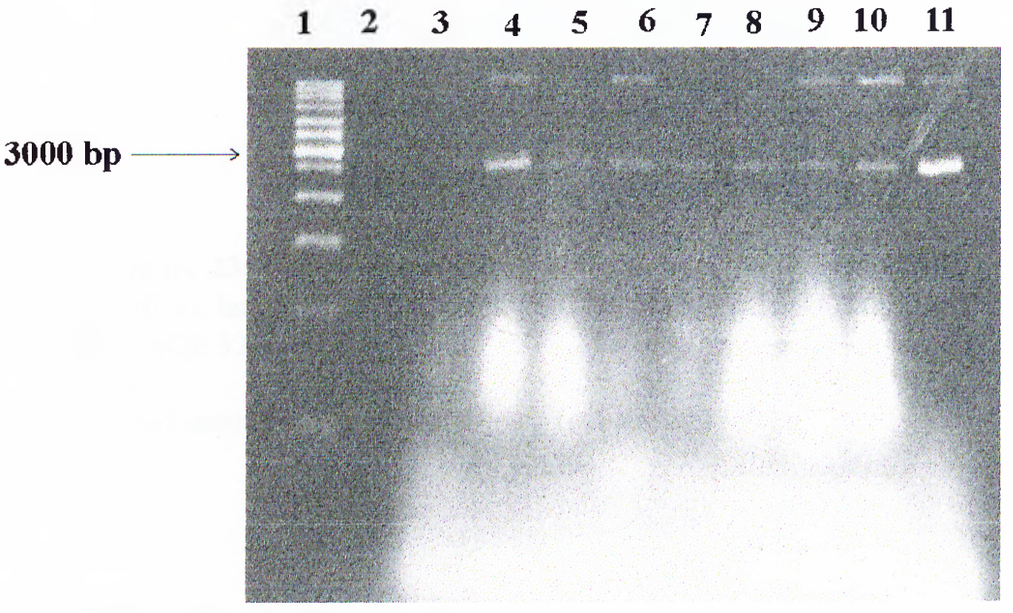


Figure 22: Miniprep isolation of pQE. **Lane 1**: 1 kb ladder (MBI), **lane 2**: empty, **lane3**: pQE 30, **lane 4**: pQE 30, **lane 5**: pQE 30, **lane 6** pQE 31, **lane 7**: pQE 31, **lane 8**: pQE 31, **lane 9**: pQE 32, **lane 10**: pQE 32, **lane11**: pQE 32

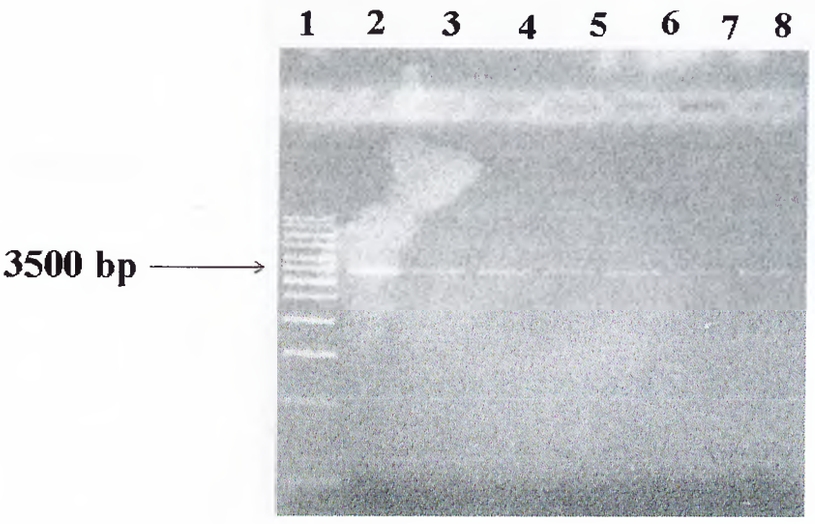


Figure 23: BamHI restriction of pQE. **Lane 1**: 1 kb ladder (MBI), **lane 2**: pQE 30, **lane3**: pQE 30, **lane 4**: pQE 30, **lane 5**: pQE 31, **lane 6** pQE 31, **lane 7**: pQE 32, **lane 8**: pQE 32

The expected uncut size of pQE is 2500 bp (cut size is 3460 bp)

4.6 Purification of plasmid DNA by equilibrium centrifugation in caesium chloride-ethidium bromide gradients (Maxiprep)

After CsCl gradient centrifugation of plasmid DNA, we obtained about 2 mg of plasmid DNA dissolved in 1 ml of TE buffer. Applying 1ul of the results and 1ul of a previous miniprep, we saw the results in picture 24.

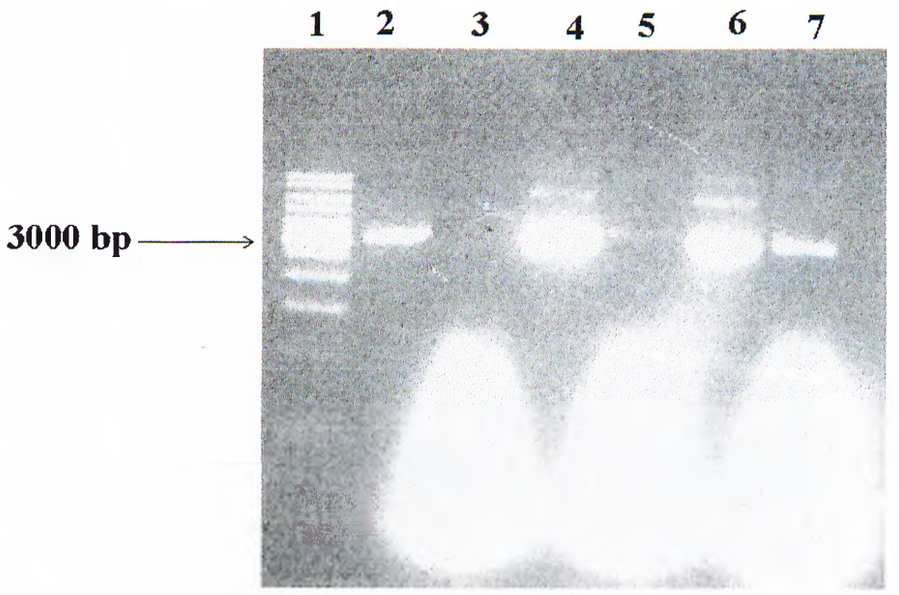


Figure 24: Maxiprep isolation of pQE. **Lane 1**: 1 kb ladder (MBI), **lane 2**: pQE 30 maxiprep, **lane3**: pQE 30 miniprep, **lane 4**: pQE 31 maxiprep, **lane 5**: pQE 31 miniprep, **lane 6** pQE 32 maxiprep, **lane 7**: pQE 32 miniprep

4.7 Subcloning of the BRCA2 fragments into the expression vector pQE

For subcloning BRCA2 fragments into pQE expression vector I made midipreps of the E3, E6 and E7 PCR-Script constructs and cut them with appropriate restriction enzymes. It is also very important to put these fragments in frame for expression so right pQEs were identified to be pQE32 for E3 and pQE31 for E6 and E7. These fragments are gel purified and the restriction results of pQE 31 and 32 and E3, E6 and E7 are shown in figures 25, 26 and 27.

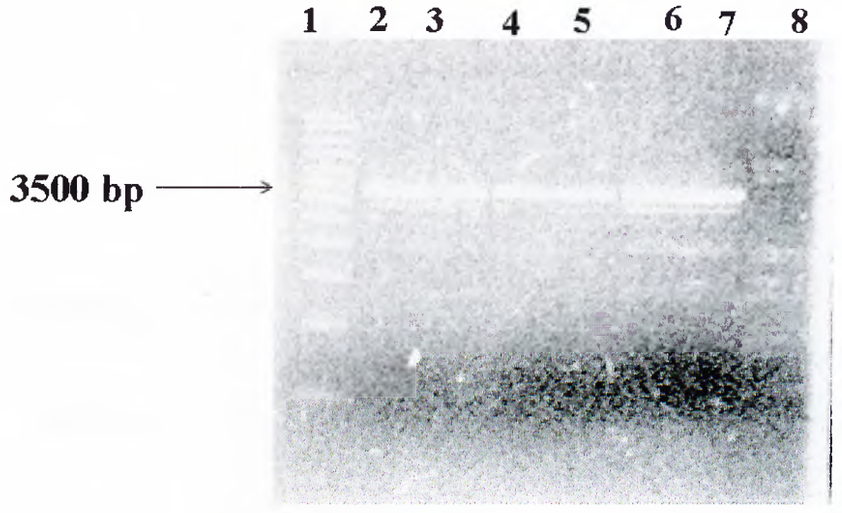


Figure 25: Restriction results of pQE31 and PQE32. **Lane 1**: 1 kb ladder (MBI), **lane 2** and **lane3**: pQE 31 BamHI/HindIII cut, **lane 4** and **lane 5**: pQE 32 BamHI/HindIII cut, **lane 6** and **lane 7**: pQE 31 BamHI/SacI cut, **lane 8**: λHindIII/EcoRI digest (MBI).

These fragments were cut out of the gel and purified from agarose.

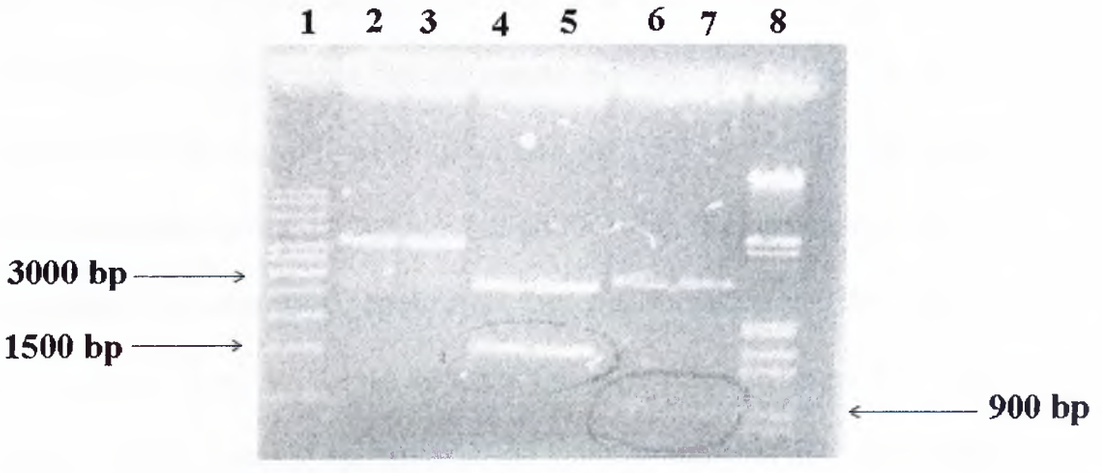


Figure 26: Restriction results of E6 and E7. **Lane 1**: 1 kb ladder (MBI), **lane 2** and **lane3**: E6 clone 10 BamHI/SacI cut, **lane 4** and **lane 5**: E6 clone 9 BamHI/SacI cut, **lane 6** and **lane 7**: E7 clone 1 BamHI/SacI cut, **lane 8**: λHindIII/EcoRI digest (MBI)

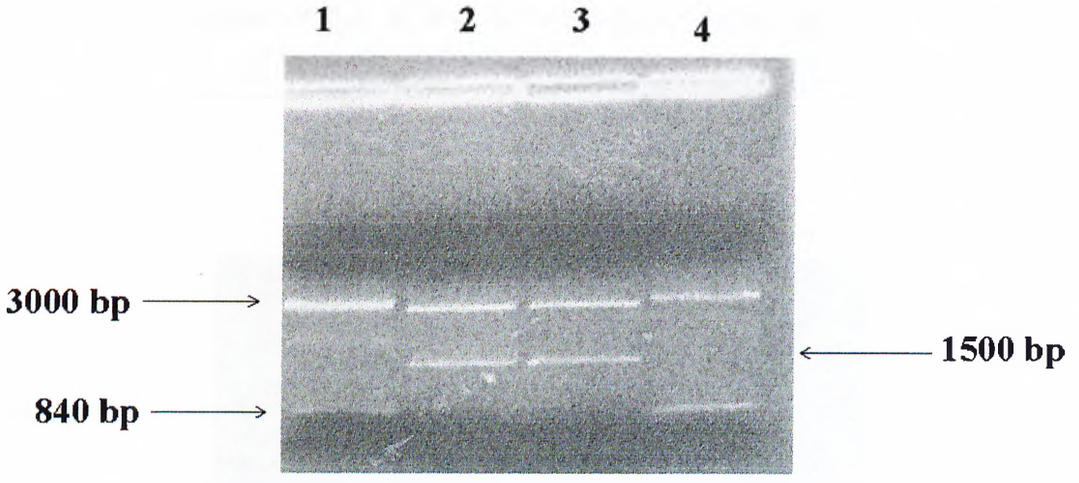


Figure 27: BamHI/HindIII digestion of E3 and BamHI/SacI digestion of E6. **Lane 1**: E3 clone 1 Bam HI/HindIII cut, **lane 2**: E6 clone 9 BamHI/SacI cut, **lane3**: E6 clone 9 BamHI/SacI cut, **lane 4**: E3 clone 1 Bam HI/HindIII cut

I had high amount, pure DNA for transformation after the gel purifications of these products. Concerning 1:10 vector:insert ratio, ligation reactions were set as described in materials-methods section. Our final aim is to express and purify the proteins encoded by this fragments. For this reason the choice of the host bacteria is very important. JM109 was offered to propogate the pQE vectors and M15 was offered for expression studies. JM109 was transformed with the ligation mixtures so as to propogate the expression constructs. The transformation or the ligation efficiency was very low, so we could obtained only a few colonies for each transfromation. Only 1 colony appeared in the transformation of E7, 3 colonies appeared in the trasformation of E6 and 13 colonies appeared in the transformation of E3. All of the colonies in E7 and E6 were conformed to be positive whereas 2 of the 11 colonies in E3 were positive.

4.8 Miniprep results of E3, E6 and E7

The miniprep results of E7, E6 and E3 are summarized in the figures 28,29 and 30.

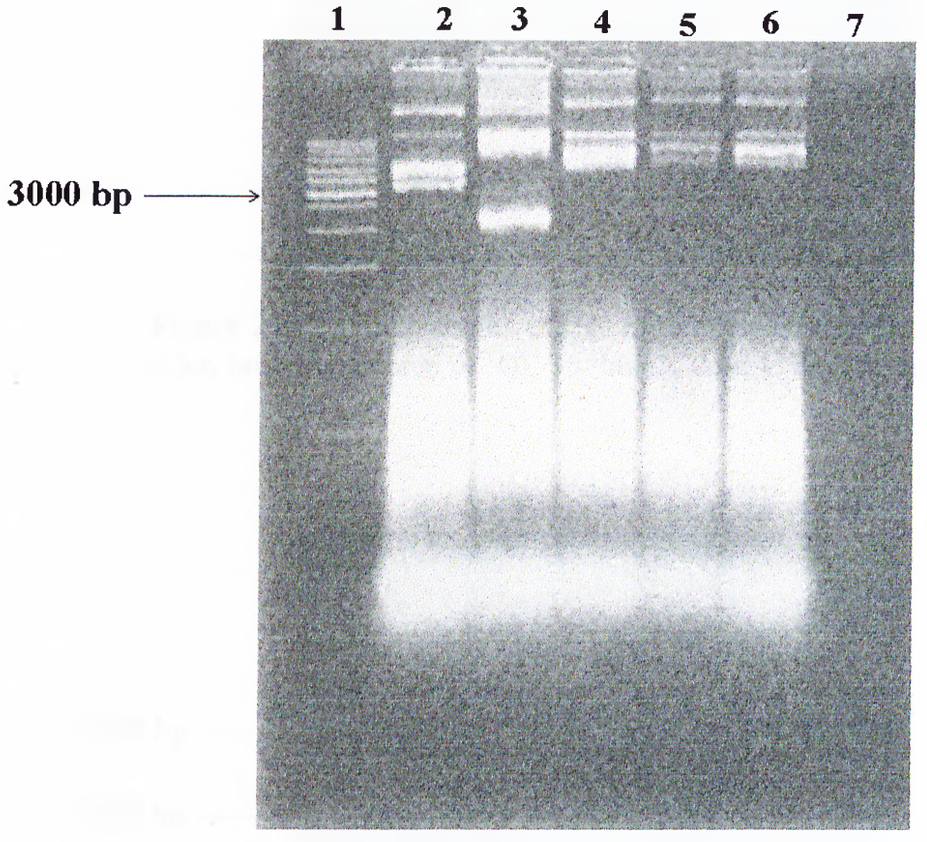


Figure 28: Transformation result of E7 and miniprep. **Lane 1**: 1 kb DNA ladder (MBI), **lane 2**: miniprep of the E7 in pQE, **lane3**: uncut empty pQE vector

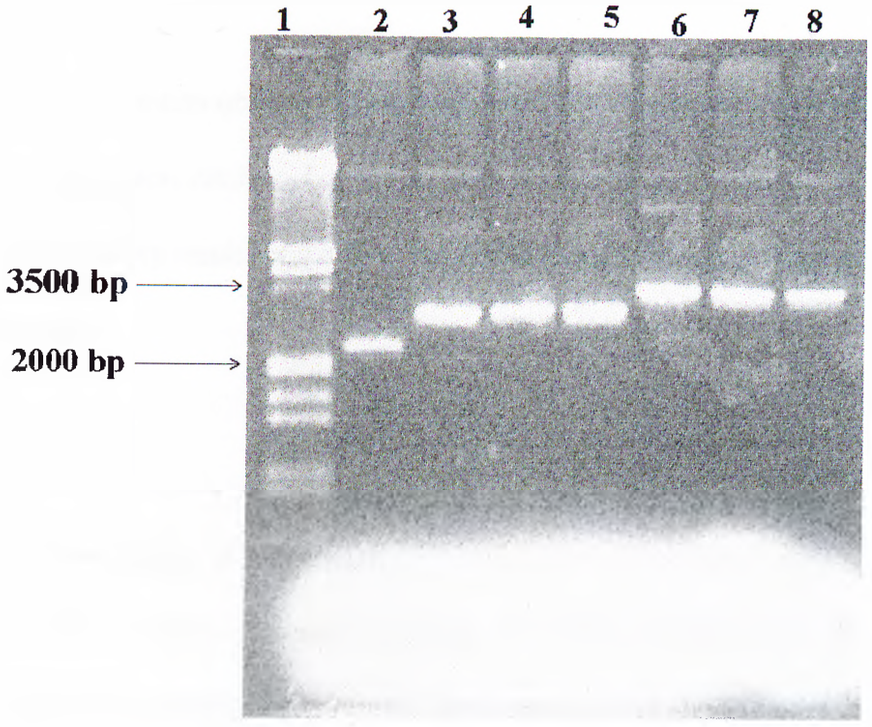


Figure 29: Miniprep results of E6. **Lane 1**: λHindIII/EcoRI digest DNA ladder, **lane 2**: miniprep of QE 30, **lane3, lane 4, lane 5**: miniprep of E6

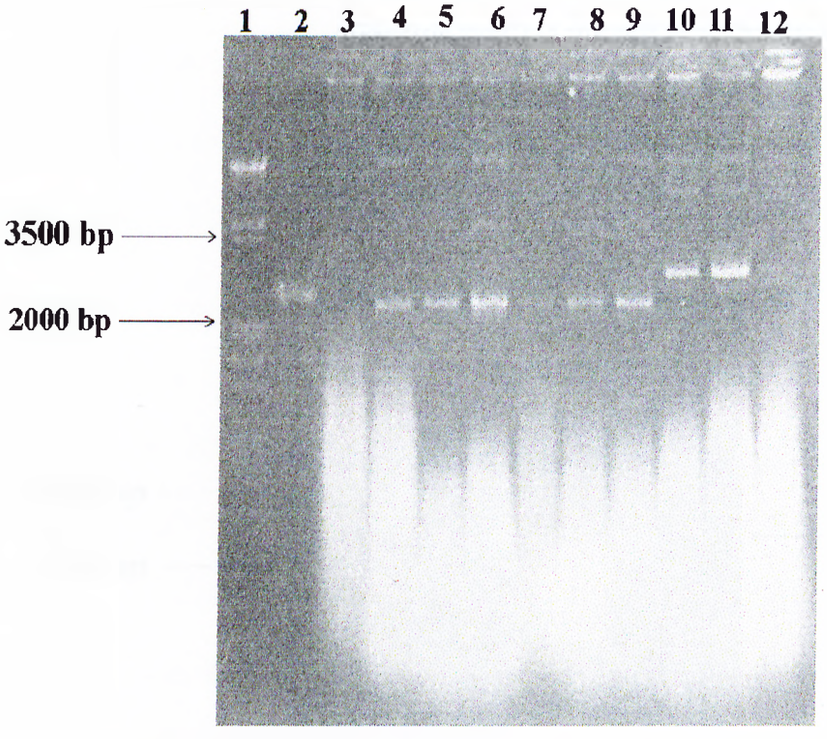


Figure 30: Miniprep results of E3. **Lane 1**: λHindIII/EcoRI digest DNA ladder (MBI), **lane 2, lane3, lane 4, lane 5, lane 6, lane 7, lane 8, lane 9, lane 10, lane11**: miniprep of all the colonies of the plate.

With the results obtained I have concluded the cloning of fragments into pQE and start expression studies. I made a fresh transformation with E7 in pQE and restrict the miniprep result for assuring the correct insert and for having fresh colonies for expression..

4.9 Restriction of E7 in pQE

The probable plasmid containing the E7 is digested with BamHI and HindIII for conformation. Since there are internal HindIII sites in E7, it is expected to give a 616 bp fragment. The restriction of E7 in pQE is shown in figure 31.

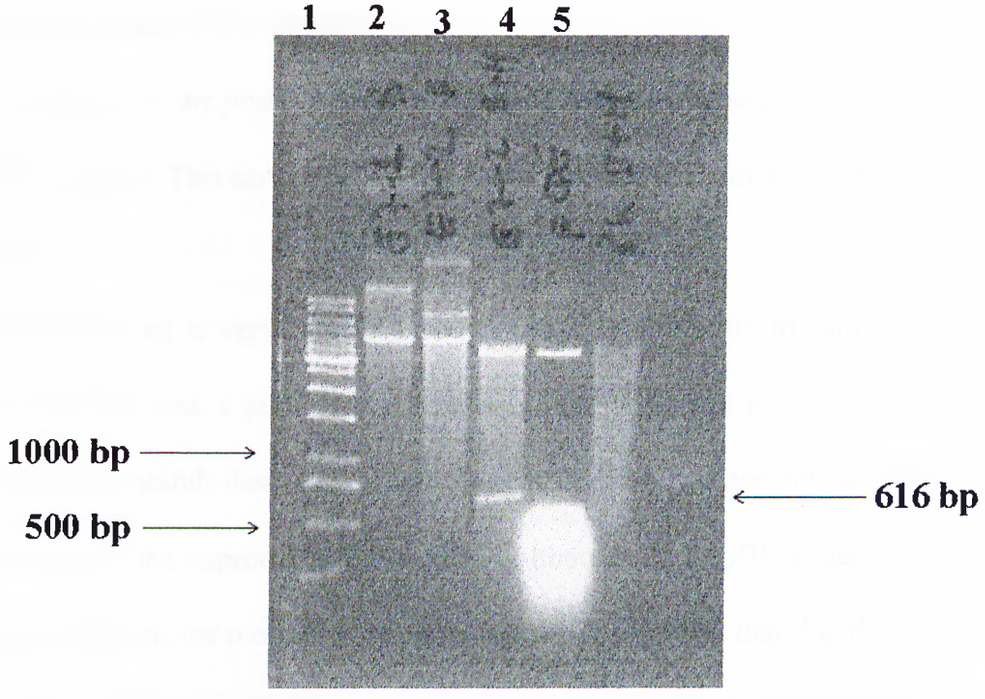


Figure 31: Restriction of E7 in pQE. **Lane 1**:Modified 1 kb DNA ladder (MBI), **lane 2**: E7-1 restricted with BamHI, **lane3** E7-2 restricted with BamHI, **lane 4** E7-2 restricted with BamHI and HindIII, **lane 5**: BamHI cut pQE 31

The expected size of BamHI/HindIII digest of the insert is 616 bp.

4.10 Induction of C-terminal of BRCA2 expression by IPTG

After being sure about the insert, I transformed E7 in pQE into M15. Picking fresh colonies from the plate, I have incubated them in LB amp, kan. As the OD. of inoculate reaches 0.7-0.9, I induced them with 0.4-2 mM IPTG. The induction results in 5 hours. Then the cells were ready to lyse and isolate proteins.

The expression of a recombinant protein depends on some factors, including the size, solubility and the toxicity of the protein. Even the expression pattern of different parts of a same protein may differ due to the aminoacid content, size and solubility. Insoluble proteins can form inclusion bodies if expressed in high amounts. Foreign and highly expressed proteins are also targets of intracellular proteases and degraded either in the cell or during the lysis of the cell.

I have expressed E7 in bacterial system. The trials to view the expression SDS-PAGE didn't gave any positive result and I could not see any band getting dark or thick after induction. This can be due to the degradation of the protein or the high basal expression.

Since 6XHis tag is very rare in bacteria, it is also plausible to purify the protein and check the size. I purified the protein in denaturing and non-denaturing conditions with the methods described in materials-methods section and able to see a very specific band at the expected size (39 kDa). Although E7 in pQE codes for a 301 aminoacid fragment, the molecular weight calculations showed that it codes for a 39 kDa. protein. The SDS-PAGE results of protein purification is illustrated in figure 32.

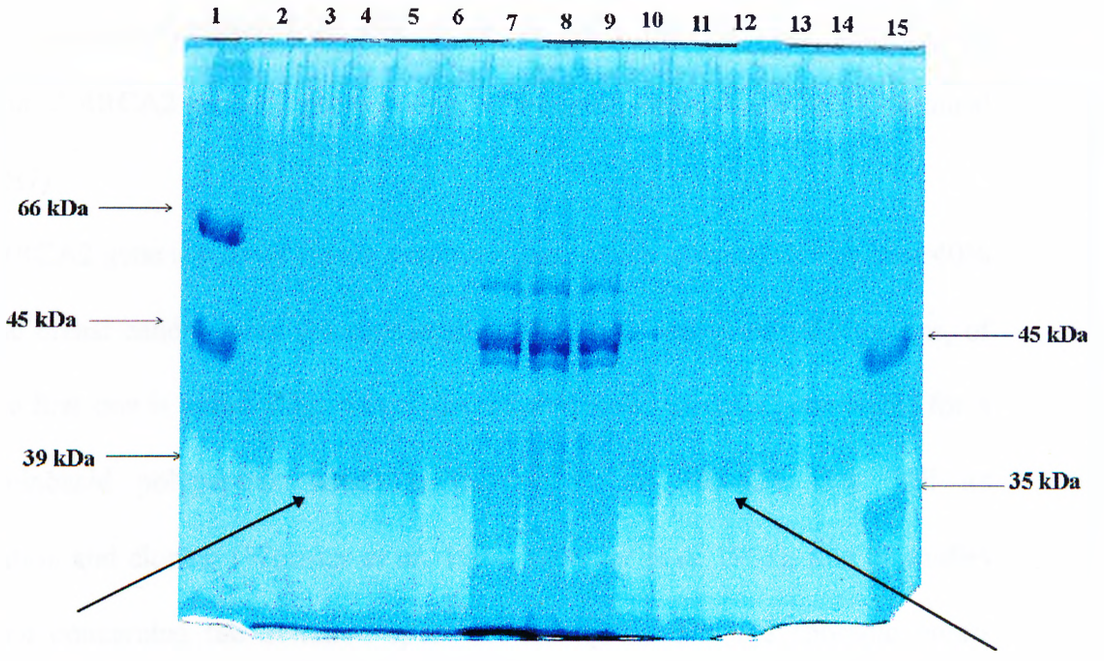


Figure 32: SDS-PAGE results of purified protein fragments. **Lane 1**: 66 kDa and 45 kDa protein size marker, **lane 2**: 500 mM imidazole fraction of purified E7 (clone 1), **lane3**: 250 mM imidazole fraction of purified E7 (clone 1), **lane 4**: 250 mM imidazole fraction of purified E7 (clone 2), **lane 5**: 250 mM fraction of purified E7 (see below), **lane 6**: 500 mM imidazole fraction of purified E7 (see below), **lane 7**: Flowthrough, **lane 8**: total lysate, **lane 9**: Flowtrough , **lane 10**: Wash 1, **lane11**: Wash 2, **lane 12**: 20 mM imidazole wash, **lane 13**: 250 mM imidazole fraction of purified E7, **lane 14**: 500 mM imidazole fraction of purified E7, **lane 15**: 45 kDa and 34 kDa protein size marker.

The fractions at lane 5 and 6 were purified under non denaturing conditions.

5- DISCUSSION and FUTURE PERSPECTIVES

The studies presented in this thesis describe the bacterial cloning and expression of BRCA2 encoded proteins and subsequent purification of the C-terminal region (E7).

BRCA2 gene is a novel tumor supressor gene which is mutated in at least 40% of familial breast cancer cases (Marcus *et al.* 1996). It is composed of 27 exons, of which the first one is non-coding (Tavtigian *et al.* 1996). BRCA2 gene codes for a 3418 aminoacid polypeptide which is thought to be 390-424 kDa. Till its identification and cloning (Wooster *et al.* 1994, Wooster *et al* 1995), lots of studies were done concerning the mutation spectrum among familial and sporadic breast cancers. There are more than 70 mutations identified in BRCA2, mostly destrupting the reading frame and producing immature stop of translation. There are recurrent mutations defined in closed populations like 999 del 5 in Iceland (Thorlacius *et al.* 1996) and 6147 del T in Ashkenazi Jews (Couch FJ. *et al.* 1996), but the other mutations found in random high risk breast/ovarian cancer families are targeting all 27 exons except the extereme 3 prime (3') 26^{th} and the 27^{th} exons. Due to the constant loss of a minimal 200-250 amino acid fragment of BRCA2, the C-terminal region was assumed to carry out some important functions. The identification of a granin domain in the minimal loss region led proposals that BRCA2 protein could be secreted and exhibits properties of granin (Jensen *et al.* 1996). However this proposal was ruled out by the identification of a polymorphic stop codon leading the production of functional BRCA2 without the granin domain (Mazoyer *et al. 1996)*. The significance

of the putative granin domain is not determined for now. There are two more reports defining the domains of this novel protein. These include the definition of a jun homology region at exon 3 (Milner *et al.* 1997) and identification of 8 conserved repeats in exon 11 with unknown function (Bork *et al.* 1996).

There are no proteins defined to confer a significant homology to BRCA2 but the number of exons, distribution through genomic DNA, having a huge exon 11 and the high incidence of mutations in familial breast cancer presumes a similarity with BRCA1. The similarities with BRCA1 also includes a common protein-protein interaction. Both BRCA1 and BRCA2 were shown to bind the bacterial RecA homologue of eukaryotic Rad51 protein (Sharan *et al.* 1997). It is of importance to figure out that the C-terminal region (aminoacids 3196-3232) of BRCA2 is essential for this interaction. The similarities between BRCA1 and BRCA2 suggested these proteins to be redundant, but they were associated with different phenotypes of high risk breast cancer families. Families with male breast cancer are extremely linked to BRCA2 whereas families with multiple breast and ovarian cancer are more linked to BRCA1.

Important proteins, such as DNA repair proteins are evolutionary conserved. The embrionic lethality of BRCA2 defines an important role for BRCA2 but BRCA2 is not a well conserved protein. It confers a 59% homology with mice and the percentage gets lower with pig, dog and swine. The identification of the 8 conserved repeats in BRCA2 and the same conserved repeat in C. *elegans* may show the importance of BRCA2 throuhout the evolution (Bignell *et al.* 1997). It could be intersting to study a breast cancer predisposing gene in marsupials, birds, reptiles, fish and yeast.

All of the tumor supressors identified for which are taking a role in cell cycle, like p53 and Rb, negatively regulate cell proliferation. In knock out and *in vitro* studies BRCA2 was mostly associated with cell proliferation. In the absence of BRCA2, cell proliferation is mostly inhibited and BRCA2 knock out mouse embryos can not develop fully to born. BRCA2 may define a novel kind of tumor supression action which was seen in other tumor supressor genes (Sharan *et al.* 1997).

Protein-protein interactions and post-tanslational modifications define another aspect of proteins which give clues for the function. Only MmRad51 protein was cofirmed to bind BRCA2 (Sharan *et al.* 1997) and a phosphorylation at tyrosine 42 was proposed (Milner *et al.* 1997). If BRCA2 is identified to interact with proteins of known functions, its cellular roles could be more precisely predicted.

For identification of the unknowns that I summarized above purified BRCA2 protein fragments and antibodies against specific regions of BRCA2 are essential. As a optimization step of this study I cloned 3 BRCA2 fragments into an expression vector and expressed and purified a 39 kDa protein corresponding to the extereme C-terminus of BRCA2 protein.

The pQE expression system allows high expression and easy purification with the 6XHis tag attached to the N-terminal of the peptide and the Ni-NTA based affinity chromatography. Although 6XHis-Ni-NTA binding is very strong and specific, there are some limitations in protein purification studies. Such difficulties include the limited expression capacity of bacterial system (maximum 100-120 kDa), the solubility and the toxicity of the expressed protein and preserving the 3 dimensional shape and activity of purified proteins. Purifying under denaturing conditions is better since the 6XHis tag is more exposed and more proteins could be purified with this system if enzymatic activity is not essential. Proteins purified under denaturing

conditions could be used in antibody production. It is of essense to purify in non-denaturing conditions if enzymatic activity and 3 dimensional shape is important. Protein-protein interactions and kinetic assays could be accomplished only with proteins purified under non-denaturing conditions. I must also note that even the fragments of a large protein can confer different chracteristics and expression and purification strategies must be developed for each polypeptide.

The number of antibodies against BRCA2 (which are polyclonal) is very limited for now and their specificity is a question to be determined. The expression of BRCA2 as 7 overlapping and combinable fragments will allow the generation of antibodies against specific regions of protein. The antibodies, especially against the C-termial (E7) of the protein could also be used in identification of the mutant BRCA2, which lacks a minimal 250 aminoacid fragment in mutant form.

The 6XHis tag also provides an experimental advantage in *in vitro* protein interaction assays. Proteins bound to BRCA2 could be co-purified with the help of the 6XHis tag and Ni-NTA affinity chromatography. Most of the proteins have known protein-protein interactions at their C-terminal (dimerization, tetramerization) and transcription activation domains at their N-terminal region. Proteins binding to the last 290 aminoacid portion of BRCA2 could be identified by the help of 6XHis tagged E7.

As a future perspective I want to optimize and express whole BRCA2 as fragments and produce specific antibodies against them as well as study the proteins interacting with BRCA2.

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