RELATIVE INCIDENCE AND MOLECULAR ANALYSIS OF BREAST CANCER IN KASHMIRI POPULATION

DOCTORAL THESIS

By

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CERTIFICATE

This is to certify that Thesis entitled, **"*Relative Incidence and Molecular Analysis of Breast Cancer in Kashmiri population*"**, submitted by ***Mrs Shiekh Gazalla Ayub,*** has been evaluated by two external reviewers and recommended for the award of ***PhD Degree*** in the discipline of ***Biotechnology.*** The recommendation for the award of degree was further endorsed by the constituted viva-voce committee today _____ 2011.

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CERTIFICATE OF ORIGINAL AUTHORSHIP

The work contained in this thesis entitled, " *Relative Incidence and Molecular Analysis of Breast Cancer in Kashmiri population*", is the bonafide research work of Mrs. Shiekh Gazalla Ayub, and is worthy of consideration for the award of Doctorate of Philosophy in Biotechnology.

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DECLARATION

The research work entitled, "*Relative Incidence and Molecular Analysis of Breast Cancer in Kashmiri population*", presented in the thesis embodied results of the original work done by me for the PhD Degree. This work has not been submitted in part or in full for any other degree or diploma.

Shiekh Gazalla Ayub

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SHIEKH GAZALLA AYUB

Dedicated to

My

Family

CONTENTS

List of Abbreviations

ASR	Age Standardized Rate
ATM	Ataxia Telangiectasia Mutated
BAK	Bcl-2 Homologous Antagonist/Killer
BARD1	BRCA1-associated RING domain protein 1
BC	Breast Cancer
BCCIP	BRCA2 and CDKN1A Interacting Protein
BIC	Breast Cancer Information Core Database
BRCA1	Breast Cancer Type 1 Susceptibility Protein
BRCA2	Breast Cancer Type 2 Susceptibility Protein
BRCC3	BRCA1/BRCA2-Containing Complex, Subunit 3
BRE	Brain and Reproductive Organ-Expressed
BSA	Bovine Serum Albumin
CBE	Clinical Breast Exam
CDK	Cyclin-Dependent Kinase
CHEK2	CHK2 Checkpoint Homolog
CK1	Casein Kinase 1
CR	Crude Rate
CT	Carboxy Terminal
Cx	Connexin
DCIS	Ductal Carcinoma In Situ
DMBA	7,12-Dimethylbenz[α]anthracene
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSBs	DNA Double-Stranded Breaks
dsDNA	Doule Stranded DNA
DSS1	Deleted in Split-Hand/Split-Foot Syndrome 1
DTT	Dithiothritol
EDTA	Ethylenediaminetetraacetate
EMSY	Emma Hughes-Davies
ERT	Estrogen Therapy
FA	Fanconi – Anemia
FANC G	Fanconi Anemia Complementation Group G
FFTP	First Full-Term Pregnancy
FGFR	Fibroblastic Growth Factor Receptor

GADD	Growth Arrest and DNA Damage
GE	Gastro-Oesophageal
GJIC	Gap Junction Intercellular Communication
HER2	Human Epidermal Growth Factor Receptor 2
HDR	Homologous DNA Recombination
HRT	Hormone Replacement Therapy
IBC	Inflammatory Breast Carcinoma
IDC	Infiltrating ductal carcinoma
ILC	Infiltrating Lobular Carcinoma
IR	Incidence Rate
MAPK	Mitogen-Activated Protein Kinase
MDM2	Murine Double Minute 2
MHT	Menopausal Hormone Therapy
MR	Mortality Rate
NCRP	National Cancer Registry Project
NGO	Non-Government Organization
NLB	Nuclear Lysis Buffer
NLS	Nucleus Localization Signal
NT	Amino Terminal
PALB2	Partner and Localizer of BRCA2
PCBs	Polychlorinated Biphenyls
PHT	Post-Menopausal Hormone Therapy
PKA	Protein Kinase A
PKC	Protein Kinase C
PTEN	Phosphatase and Tensin Homolog
SDS	Sodium Dodecyl Suphate
Skp2	S-phase Kinase-Associated Protein 2
SNP	Single Nucleotide Polymorphism
TSP-1	Thrombospondin 1
UTR	U-Terminal Region
UV	Ultra-Voilet

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ABSTRACT

Section I

Abstract

Breast cancer is the third most common tumor in the world and represents 9% of global cancer burden. In India, breast cancer is the second common cancer in women after cervical cancer and has of late replaced cervical cancer as the leading site of cancer among women in Indian cities. Preliminary indications point towards an increasing trend in the occurrence of breast cancer amongst Kashmiri population. However, authentic data with regard to prevalence is almost non-existent in the state of Jammu and Kashmir. To our knowledge, this is the first attempt to examine the epidemiological distribution of different cancer types with particular emphasis on breast cancer in the whole valley. The source of our data include cancer registry in the Department of Radiation Oncology, Sheri-Kashmir Institute of Medical Sciences, Srinagar, and Department of Radiation Oncology, SMHS, Srinagar during Jan 2002 to Dec 2006. A total of 6943 cases registered between 1st January 2002 to 31st December 2006 comprised of 4345 males and 2598 females. The age standardized incidence rates were 34.9 per 100,000 for males and 24.8 per 100,000 for females. Oesophagus was the leading site of cancer in both the sexes (male ASR 11.2; female ASR 8.3) followed by lung (ASR 6.5), brain (ASR 2.2), head and neck (ASR 2.2) in males and breast (ASR 5.2), skin (ASR 1.6) and rectum (ASR 0.95) in females. The incidence of cervical cancer turned out to be surprisingly low in Kashmiri women as compared to other Indian Registries quite contrary to the pattern in rest of the country. Our studies imply that cancer incidence was significantly lower and cancer patterns were markedly different in Kashmir. The observed cancer pattern indicates that awareness campaigns, life style and dietary habit changes, tobacco-control measures and early detection of breast cancer are very important for cancer control in this cohort of population.

Section II

Abstract

Environmental and genetic factors are attributed to explain the spectrum of geographical and ethnic variations in the development and pathogenesis of disease. The genesis of disease has been further complexed by involvement of number of genes with small effects and above all by population heterogeneity. Accordingly variations in genes like Brca1/Brca2 that have been strongly associated with breast cancer phenotype present a scattered mutational pattern in different populations. This study attempts to analyze the frequently mutated exons of BRCA2 for sequence variations in surgically resected breast cancer tissue samples, from a high risk ethnic Kashmiri population. PCR followed by direct sequencing revealed presence of five variations, with four somatic mutations located on exon 11 and one germline variation located in UTR region of exon 2 at contig position 13870572 (rs1799943). All the four somatic mutations comprised of substitutions; two representing missense mutations leading to amino-acid substitution at codon positions 868 (novel) and 991 whereas other two were silent mutations at codon positions 846 and 1131. Codons for amino-acid positions 846 (TCC/TCA) and 868 (CCT/ACT) were seen to be present in heterozygous state in normal breast tissue samples and the heterozygous nature of both the codons was seen to be lost in associated tumor samples in 44 out of 50 patients. Incidentally these two mutations were always found to be linked. No sequence variations were observed in exons 9, 18, 20 and 25.

Gap-junction gene Connexin 43, which codes for a 43-kd gap-junction protein is a predominantly expressed gap junction protein in normal breast tissue and plays an important role in normal mammogenesis, lactogenesis and involution. Studies have shown down-regulation of connexin 43 gap-junction protein is involved in primary tumor formation as well as metastasis in breast cancer patients and restoration of gap-junction intercellular communication by up-regulation of

connexins has been shown to restore normal phenotypes in vitro and reduce tumor growth in vivo. However the molecular mechanisms behind these processes remain elusive and a better understanding of the key events is necessary to gain information relevant to the designing of anti-cancer treatment models against breast cancer. In this study, coding sequence of Connexin 43 was analyzed for polymorphic changes to establish its role (if any) in breast cancer in this cohort of population. After sequence analysis, none of the screened samples revealed any kind of variations in early or advanced stage of the disease.

The findings from this study add to the body of knowledge about mutation prevalence and nature of different breast cancer pre-disposing genes in ethnic Kashmiri population, and may inform strategies for genetic cancer risk assessment. Our studies also imply that mutational deactivation does not play any role in the down regulation of Connexin 43 expression in Kashmiri breast cancer patients. Instead, some other regulatory mechanism like hypermethylation or mutation of the promoter region of the gene may be involved. Additionally increased understanding of breast carcinoma pathways may enhance our ability to device targeted approaches to the prevention of disease.

SECTION I

CHAPTER 1

INTRODUCTION

CHAPTER ONE

INTRODUCTION

Cancer is characterized by abnormal or uncontrolled division of cells, dictated by pathological breakdown in the process of cell proliferation (Steward & Kliehues, 2003). The cells in our body normally grow and divide in a controlled way to keep the body functioning properly. When normal cells grow old or get damaged, they die, and new cells take their place. However, this process may go wrong to generate new cells even when the body doesn't need them, or the old damaged cells may not die as they should. The buildup of extra cells often forms a mass of tissue called a lump, growth, or tumor. Thus tumor is the outcome of an evolutionary process involving successive generations of cells, which progressively advance towards cancerous growth (1). The cancerous cells frequently break free from the tumor site and enter the bloodstream, spreading the disease to other organs and this process is called metastasis (2).

Cancer can develop from almost any type of cell in the body. So more than one type of cancer can develop in any one part of the body. Over all there are more than 200 types of cancers which develop in 60 different organs of the body. Accordingly, the cancers have been grouped into broader categories which include Carcinoma (cancer that begins in the skin or in tissues that line or cover internal organs), Sarcoma (cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue), Leukemia (cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood), Lymphoma and myeloma (cancers that begin in the cells of the immune system), Central nervous system cancers (cancers that begin in the tissues of the brain and spinal cord).

About 7.6 million people died of cancer in the year 2007 accounting for about 13% deaths the world over. Seventy percent of the total cancer related

mortality was faced by low and middle income countries. The global burden of cancer continues to rise with cases projected to jump from 12 million to 27 million and cancer related deaths projected to increase from 7.6 million to 17.5 million between 2007-2050 (3).

The most common types of cancers with highest rate of mortality are the cancers of lung, stomach, liver, colorectal and eosophagus among men and the breast, lung, stomach, colorectal and cervical cancers among women. Breast cancer, one of the common malignancies affecting women worldwide (Parkin, 2004), account for 25% of all new cases of cancer. However, it ranks as the fifth most common cause of death, because of the relatively more favorable prognosis (mortality to incidence ratio, 0.35) making it the most prevalent cancer in the world today. Breast cancer incidence rate varies widely worldwide (Parkin et al., 2001) primarily due to range of socio-economic, reproductive, hormonal, nutritional and genetic factors (Mcpherson et al., 2000). People in less-developed countries surprisingly report low incidence rate than in developed countries. More than half of the cases are reported from countries like North America and Europe (Parkin et al., 2001) whereas South-east Asia contributes over 1/5th of the worldwide prevalence of breast cancer.

The wide differences in global incidence rates and resources have led to diverse approaches to breast cancer control. In the West, high rates and abundant resources have resulted in intense laboratory and clinical research as well as aggressive screening programs and abundant treatment trials. In poor-resource countries with low rates, the ability and incentive to mount large efforts to address breast cancer have been lacking. However, the perspective on breast cancer control in lower income countries is changing because of the clear trend of steeply increasing incidence largely affecting countries with historically low rates of breast cancer (Parkin et al., 2001). In Asia; Japan, Singapore, and Korea have reported doubled or tripled incidence over the past 40 years and China's urban registries have documented a 50% increase in the years between 1972 and 1994 (Jin et al., 1999).

In India, Breast cancer is the second most common cancer among women (19%) after cervical cancer (30%), however in the metropolitan cities like Delhi and Mumbai, breast cancer has overtaken cervical cancer in frequency (ICMR, 2001). An average of 80,000 women are diagnosed with carcinoma of the breast, and 40,000 die of the disease every year in India (ICMR, 2001). In India, the major burden of breast cancer is due to early onset breast cancer cases while it is mainly a postmenopausal disease in western population. Therefore, although the incidence of breast cancer is relatively very high in the west (90.7 versus 23.5), there is not much difference in the incidence of early onset breast cancer cases which varies between 12.33 cases per 100,000 women (Parkin & Iskovich, 1997).

Kashmir valley (India) which is located at an altitude of 1800-2400 m from the sea level and borders the low-incidence country and the high-incidence area of Pakistan, is distinct from other areas in terms of its unique geographical locale, intra-community marriages, tradition, culture, food habits and ethnicity. Over the past few years, the valley has witnessed a tremendous increase in the incidence of breast cancer in its unexplored ethnic population. However, we don't have much and recent population based data available from the valley. Hence in this study, we have attempted to examine the epidemiological distribution of different cancer types in Kashmir with special emphasis on breast cancer. This is the first kind of study which represents the trend in cancer pattern throughout the valley.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER TWO

REVIEW OF LITERATURE

2.1 DEFINING CANCER

Cancer is a term used for a group of diseases that cause cells in the body to change and grow out of control. In normal situations, the cells in our body grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. However, sometimes the genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens cells do not die when they need to and the new cells form when they are not required in the body resulting in lump or mass called a tumor (1).

Cancers are capable of spreading throughout the body by two mechanisms: invasion and metastasis. Invasion refers to the direct migration and penetration by cancer cells into neighboring tissues. Metastasis refers to the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade normal tissues elsewhere in the body. Depending on whether or not they can spread by invasion and metastasis, tumors are classified as being either benign or malignant. Benign tumors are tumors that cannot spread by invasion or metastasis; hence, they only grow locally. Malignant tumors are tumors that are capable of spreading by invasion and metastasis (2).

There are more than 200 different types of cancers which may arise in over 60 different organs in the body. Each organ is made up of several different types of cells. For example, there is usually a surface covering of skin or epithelial tissue. Underneath that, there is often a layer of muscle tissue and so on. Each type of tissue is made up of specific types of cells. Cancer can develop

from almost any cell type in the body, although one type of cancer will be much more common than the others. Broadly, cancer can be grouped into following categories:

- **Carcinoma** - cancer that begins in the skin or in tissues that line or cover internal organs.
- **Sarcoma** - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- **Lymphoma and myeloma** - cancers that begin in the cells of the immune system.
- **Central nervous system cancers** - cancers that begin in the tissues of the brain and spinal cord.

Cancer is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposure to external factors and detectable cancer. Knowledge about the causes of cancer, and interventions to prevent and manage the disease is extensive. Cancer can be reduced and controlled by implementing evidence-based strategies for cancer prevention, early detection and management. One-third of cancers could be cured if detected early and treated adequately, based on the observation that treatment is more effective at early stages. In addition, more than 30% of cancers could be prevented simply by behavioral changes that include abstinence from using tobacco, use of healthy diet, maintaining a healthy weight, being physically active and preventing infections that may cause cancer (Danaei et al., 2005).

2.2 OCCURRENCE

In 2007, 7.6 million people died of cancer, around 13% of all deaths worldwide, with more than 70% of deaths occurring in low and middle income countries. The global burden of cancer continues to rise wherein the new global cases are estimated to jump from 12 million to 27 million and global cancer deaths are projected to increase from 7.6 million to 17.5 million deaths in 2007-2050 (3). Lung cancer is the main cancer in the world today, whether considered in terms of numbers of cases (1.35 million) or deaths (1.18 million), because of the high case fatality (ratio of mortality to incidence, 0.87). Breast cancer, the second most common cancer overall (1.15 million new cases), ranks less highly (fifth) as a cause of death because of the relatively favorable prognosis (mortality to incidence ratio, 0.35), and cancers of the stomach (934,000 cases, 700,000 deaths), liver (626,000 cases, 598,000 deaths), and colon and rectum (1.02 million cases, 529,000 deaths) rank more highly (Parkin et al., 2002).

2.3 BREAST CANCER INCIDENCE WORLDWIDE

Globally, breast cancer is the second most common tumor in the world and represents 9% of global cancer burden (Parkin et al., 2000). It is the most frequent cancer of women, account for 25% of all cancers. More than half of the cases are in industrialized countries—about 361,000 in Europe (27.3% of cancers in women) and 230,000 in North America (31.3%). Incidence rates are high in most of the developed areas (except for Japan, where it is third after colorectal and stomach cancers), with the highest age-standardized incidence in North America (99.4 per 100,000) (Jemal et al., 2004). In part, the high incidence in the more affluent world areas is likely because of the presence of screening programs that detect early invasive cancers, some of which would otherwise have been diagnosed later or not at all (IARC, 2002). The incidence is more modest in Eastern Europe, South America, Southern Africa, and Western Asia, but it is still

the most common cancer of women in these geographic regions. The rates are low (30 per 100,000) in most of Africa (with the exception of South Africa) and Asia. The Globocan database indicates that the age-standardized rate (ASR) of breast cancer incidence is 66.4 per 100000 in more-developed regions (Europe, Australia, New Zealand, North America and Japan) compared with 27.3 per 100000 in less-developed regions (Africa, Central America, South America, all regions of Asia except Japan, the Caribbean, Melanesia, Micronesia and Polynesia) (3).

Breast cancer incidence varies widely within regions and countries, likely due to differences in racial and ethnic make-up, health resources, and lifestyle patterns (Mcpherson et al., 2000; Parkin et al., 2001). Incidence in largely rural and black sub-Saharan Africa (16.5 per 100000), for instance, is half that of South Africa (33.5 per 100000), which has a higher proportion of urban white women (Ferlay et al., 2002). In China, the 1993-1997 incidence of breast cancer in urban Shanghai was 27.2 per 100000 compared with 11.2 per 100000 in the more rural Qidong country (Chen et al., 2006; Parkin et al., 2005). In Korea there was an almost two-fold difference in incidence between Seoul (20.8 per 100000) and more rural Kanghwa county (12.7 per 100000) (Parkin et al., 2005).

The wide difference in global incidence rates and resources has led to diverse approaches to breast cancer control. In the West, high rates and abundant resources have resulted in intense laboratory and clinical research as well as aggressive screening programs and abundant treatment trials. In resource-poor countries with low rates, the ability and incentive to mount large efforts to address breast cancer have been lacking. However, the perspective on breast cancer control in lower income countries is changing because of the clear trend of steeply increasing incidence largely affecting countries with historically low rates of breast cancer (Parkin et al., 1992). In Asia; Japan, Singapore, and Korea have reported doubled or tripled incidence over the past 40 years and China's urban registries documented a 50% increase in the years between 1972 and 1994 (Jin et al., 1999). India similarly reported increased incidence in urban Bombay

(Mumbai) from 1978 to 1997 (20.5 to 31.5 per 100000) (Parkin et al., 2005). In Africa, trends in breast cancer incidence are difficult to evaluate, given the general lack of large registries and accurate population data. However, local registry efforts in Uganda, reported increased incidence from 11.7 in 1960 to 23.4 in 2002 (Parkin et al., 2008). Some Latin American countries have also reported increasing incidence: in Cali, Colombia rates increased from 32.2 in 1983 to 44.4 in 1997. However, in Costa Rica, which has one of Latin America's only national registries, the rate has remained fairly constant at around 30 per 100000 (Parkin et al., 2005).

2.4 BREAST CANCER INCIDENCE IN INDIA

Breast cancer shows uneven geographical distribution in its occurrence reflecting the influence of local environmental conditions, lifestyle, hormonal/reproductive pattern and genetic predisposition in the development of the disease (McPherson et al., 2000; Parkin et al., 2001). People in less-developed countries report low incidence rate than in developed countries. The high incidence areas include North America and European countries (Parkin et al., 2001). South-East Asia contributes over $1/5^{th}$ of the worldwide prevalence of breast cancer with Pakistan having highest incidence among Asian population after Israel (Bhurgri et al., 2000). Breast cancer is the second most common cancer among Indian women (19%) after cervical cancer (30%), however in the metropolitan cities like Delhi and Mumbai, breast cancer has overtaken cervical cancer in frequency (ICMR, 2001). In India an average of 1,15000 women are diagnosed with carcinoma of the breast, and 50,000 women die of the disease every year (5). Interestingly, there is also a significant difference in the incidence of breast cancer between urban and rural population of India and it also varies among various religious groups, the highest (1.5–2.1 times) being in Parsi population as compared to either Hindu, Muslim or Christian population (Jussawalla & Jain, 1977). Regionally, incidence rates ranges from 18.8 per 105

in Trivandrum to 28.2 per 105 in Mumbai. These differences in breast cancer incidence clearly reflect the diversity of ethnic and religious groups in India. Kashmir valley located at an altitude of 2000m above sea level is bordering the low incidence country India and the high-incidence area of Pakistan. Breast cancer in Kashmiri women is the 2nd common cancer after oesophageal cancer.

2.5 MORTALITY FROM BREAST CANCER

Although breast cancer is the second common cancer in the world, but it ranks as the fifth cause of death from cancer overall, because of its good prognosis which is illustrated by the survival rates, the average in developed countries is 73% and 57% in developing countries. Because of its high incidence and relatively good prognosis, breast cancer is the most prevalent cancer in the world today. There are an estimated 4.4 million women alive who have had breast cancer diagnosed within the last 5 years (compared with just 1.4 million survivors—male or female—from lung cancer). The worldwide ASR for mortality from breast cancer between 1993 to 2001 was 13.2 per 100000, ranging from 8.8 in Asia to 19.7 in Europe (Kamangar et al., 2006). The rate in 'more developed' countries was 18.1 compared with 10.4 in 'less developed' countries. The higher overall mortality rate in high resource countries reflects the high incidence of the disease in many of those countries. However, the burden of deaths due to breast cancer in lower resource countries is disproportionately high. Although the ratio of the mortality rate to incidence rate (MR:IR) world-wide is 0.35, it varies widely and ranges from a high of 0.69 in Africa to a low of 0.19 in North America.

High mortality rates in low resource countries are primarily due to late-stage disease presentation. For example, the high MR:IR ratio in Africa to a large degree reflects the high proportion of women in many African countries who present with late-stage cancer. In sub-Saharan African countries, small studies indicate that up to 90% of women present with stage III or VI disease, many with

large tumors (median of 10 cm) and clinically apparent lymph node metastasis (Fregene & Newman, 2005; Parkin et al., 2008). These late-stage tumors cannot be treated successfully in the most optimal settings. Inadequate health care systems also contribute to high mortality rates. There are no examples of universal breast cancer screening in low resource countries that could down-stage breast cancer diagnoses. In addition, there are typically only a few hospitals that can administer radiotherapy and chemotherapy and very few trained oncologists (Nigeria, for instance, has approximately 100 oncologists for a population of 140 million) (Igene 2008). National expenditure on health care overall inversely correlates with mortality from breast cancer, but there are exceptions (Igene, 2008). In Cuba, health expenditure is only moderate but mortality remains lower than that in the US (2004 ASR 14.2 and 15.6 respectively) where per capita health care spending is the highest in the world (Igene, 2008). Sweden also spends less on health care than the US, but maintains a similar mortality rate (ASR 15.6). Given these kinds of exceptions, it is important that not only the total health expenditure but the distribution of that expenditure into effective private or public systems be considered when judging approaches to reducing the burden of breast cancer.

2.6 APPROACHES TO REDUCING BREAST CANCER INCIDENCE AND MORTALITY

Although progress in slowing the rate of increasing incidence would be desirable, the increasing prevalence of lifestyle habits that accompany improving economic conditions –delayed childbearing, lower parity, reduced breast-feeding, and a sedentary workplace– are expected to continue to drive up breast cancer incidence rates in lower and middle resource countries in the coming decades. The most important task will be to alter the trend of increasing mortality that will accompany the increasing rate. The strategies that have been most effective in predominantly Caucasian, affluent populations have been early detection through

mammography, targeted hormonal and anti-HER2 therapies, and improvements in chemotherapy. The common presentation of breast cancer at advanced stage in lower income countries is undoubtedly one of the main reasons for high mortality rates; metastatic disease is not treated successfully in countries with the highest resources. Down staging of breast cancer by early detection is seen as one of the most promising long-term strategies for preventing disease-related deaths but it is difficult for many countries to make the economic investment required to carry out broad screening programs. The lowest cost screening modality, breast self exam (BSE), can find individual tumors and increase awareness and acceptance of breast health in the population but does not appear to lower mortality from the disease (Thomas et al., 2002). Clinical breast exam (CBE) can improve detection of early stage cancers and clinical follow-up within the health care system (Okonkwo et al., 2008) and efforts are being made in many countries to train medical personnel to conduct high quality CBE. Full-scale mammography screening has been successful in reducing mortality in developed countries (Cronin et al., 2006), but this requires considerable human, health system and technical resources. It is likely that a combined introduction of BSE, CBE and mammographic screening as is possible for an increasing proportion of the population, will be the most feasible course of action for lower resource countries. The multinational groups involved in the Breast Health Global Initiative have incorporated economic and cultural information into guidelines to help plan just such country-specific breast cancer early detection strategies in lower-resource countries with different racial, ethnic, and cultural makeup (Anderson, 2008).

It is clear that breast cancer will be an increasing burden in many countries and there will have to be a redirection of health resources to diagnose, treat and monitor the growing numbers of women who are affected by the disease. This will take political will, reliable data and cancer registries, public and medical community awareness, and partnerships between advocates, government, foundations and NGOs, and biotechnology companies. These partnerships can

provide the incentives and the means to make crucial advances in lowering the unacceptable burden of mortality in lower resource countries.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

2.1 DATA SOURCE

This study is based on the records of cancer patients registered in the Department of Radiation Oncology, Sheri-Kashmir Institute of Medical Sciences, Srinagar, and Department of Radiation Oncology, SMHS, Srinagar. All the patients who are diagnosed of cancer and come to these hospitals for further treatment are registered in the registration section of these departments. These are leading medical centers in the valley and draw almost all of cancer patients from all over Kashmir for treatment. Although some of the patients report to district hospitals, but they are also finally referred to these specialist hospitals for treatment.

The patient's records were screened for information on the nature and distribution of all forms of cancer reported to the department between January 2002 and December 2006. Records of patients from outside the valley of Kashmir were excluded from the study. The mean annual frequencies of all cancer types were worked out and tabulated by sex. The main forms of cancer were identified separately for men and women as well as collectively.

2.2 STATISTICAL METHODS

The results are presented as incidence rates of cases by age, sex, site, crude rate, age specific incidence rates and age standardized incidence rates. The data on annual population of Kashmir region by 5-year age group and sex for the period 2002–2006 was provided by Population Research Centre, University of Kashmir. The annual populations during the study period of 2002–2006 were

cumulated for calculation of measures of incidence. All incidence rates were expressed as average annual ones per 100,000 persons. The total population during 2002-06 was 28672263. The sex ratio in Kashmir is 918 females per 1,000 males. Table 3.1 gives the population at risk by sex and 5 year age groups.

Age group (in years)	Males	Females
<1	270362	245739
1 to 4	976306	873740
5 to 9	1607149	1447131
10 to 14	1635688	1625975
15-19	1785887	1544061
20-24	1515527	1421192
25-29	1365326	1325627
30-34	1034884	957018
35-39	974804	902409
40-44	751004	697626
45-49	732980	547452
50-54	539221	615713
55-59	479140	479192
60-64	495663	354956
65-69	300401	245739
70-74	270361	163826
75-79	120161	95565
80+	165220	109217
Total	**15020084**	**13652178**

Table 3.1: Population at risk by five year age group and sex, Kashmir, 2002-06.

CHAPTER 4

RESULTS

CHAPTER FOUR

RESULTS

During the period under review that is from 2002 to 2006, 6943 new cases were registered in Kashmir valley, which gives an average of 1389 new cases per annum. Of these new cases, 4345 were males and 2598 were females indicating a male female ratio of 1.67: 1.0. The number of cases by site, age group, percentage, crude rates and Age-standardized rates for males and females are given in Tables 4.1a and 4.1b and Age-specific incidence rates are given in Tables 4.2a and 4.2b respectively.

In males, Oesophageal cancer (Oesophagus and GE junction) was the most commonly reported malignancy (30.9% of all cases, ASR 11.2) followed by lung (17.9%% of all cases, ASR 6.5), brain (7.0% of all cases, ASR 2.2), head and neck (6.4% of all cases, ASR 2.2), skin (5.4% of all cases, ASR 1.9) and stomach (5.2% of all cases, ASR 1.8). The crude rate for all sites combined was 29 per 100,000 and 34.9 ASR per 100,000 for males.

Oesophageal cancer (32.8%, ASR 8.3) was the most frequently reported malignancy in females followed by breast (22.8%, ASR 5.2), skin (6.2%, ASR 1.6), rectum (4.2% ASR 0.95), brain (4.2%, ASR 0.9) and lung (4.1% ASR 1.1). The crude and age adjusted (world population) incidence rates for females were 19.2 per 100,000 and 24.8 per 100,000 respectively. Oesophageal and lung cancers alone contributes about 45% of total cases in males whereas in females esophageal and breast cancers contribute more than 50% of the cases. Overall in both the sexes, oesophagus is the leading cancer site followed by breast and lung in the valley. Our results have shown that breast cancer occurs with highest incidence in the age group of 20-50 whereas above 50, Breast cancer is the 2nd leading cancer after oesophageal cancer. In males, the occurrence of Breast cancer is very less in all age groups.

In cancer types common to both the sexes, the proportion in the men exceeds that in women in almost all types of cancer Tobacco-related cancers, i.e., Head & Neck (oral cavity, pharynx and larynx) and lung constituted 24% of the total male cancers, while in females they accounted for only 7.6% of the cases. Our results clearly indicate that cancer occurs mostly at older ages as only 3.4% of the total number of cancer cases registered, were falling in the age group of less than 20 years, although this age group accounts for a good percentage of the total population. The overall percentage of new cancer cases was more in the age group of 50-70 years as compared to other age groups in both sexes. In male children, the most prominent cancer was brain followed by lymphomas and bone & soft tissue; whereas in female children, bone & soft tissue is the leading site followed by brain and eye cancers.

Figure 4.1 shows the comparison of total number of incident cases of cancer (from year 2002-2006) at different cancer sites in males & females. Figure 4.2 show the age-specific incidence rate curves for all sites in males and females. In males there is a steady increase up to 50 years age group and then sharp increase in the age group of 50-70 which is followed by progressive decrease in the older age groups (70+). Whereas in females, the rates increase steadily up to the age of 70 years and then falls in the older age groups. Figure 4.3a shows the age-specific curves for esophagus, lung, brain, head and neck, stomach and skin in males and Figure 4.3b the equivalent curves for oesophagus, breast, skin, rectum, brain and lungs in females.

Tables 4.3a and 4.3b compare the age-standardized incidence rates for common sites in Kashmir with the registries reported from India in males and females respectively (Curado et al., 2007; Manoharan et al., 2009, 2010; Sen et al., 2002). Among males as well as females, Kashmir reports the highest incidence of oesophageal cancer cases in India. Compared to other Indian cancer registries, the incidence of cervical cancer in females is lower in Kashmir.

Site	0-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90+	Total	%age Distribution	Crude rate	ASR
Oesophagus	x	x	32	126	302	448	199	44	7	1158	26.7	7.7	9.7
Lung	x	4	15	123	210	267	127	29	2	777	17.9	5.2	6.5
GE Junction	x	x	4	16	50	71	38	3	x	182	4.2	1.2	1.5
Skin	x	4	7	29	61	71	39	20	2	233	5.4	1.6	1.9
Prostrate	x	x	X	2	5	27	19	6	1	60	1.38	0.4	0.5
Rectum	1	31	20	31	42	57	12	3	x	197	4.5	1.3	1.5
Stomach	x	5	17	35	69	67	25	7	x	225	5.2	1.5	1.8
Colon	x	6	14	17	31	29	16	x	x	113	2.6	0.8	0.9
Testis	4	7	22	27	10	6	5	x	x	81	1.9	0.5	0.6
Urinary Bladder	x	x	3	9	12	21	11	3	2	61	1.4	0.4	0.5
Thyroid	1	2	5	7	11	19	2	x	x	47	1.1	0.3	0.4
Bone & Soft tissue	18	16	16	13	13	9	4	x	x	89	2.0	0.6	0.6
Eye	10	3	3	x	7	3	x	x	x	26	0.6	0.2	0.2
Brain	53	49	57	55	51	34	6	x	x	305	7.0	2.0	2.2
Breast	x	x	5	9	13	11	7	4	x	49	1.1	0.3	0.4
Multiple myeloma	x	x	5	7	20	21	4	x	x	57	1.3	0.4	0.5
Gall bladder	2	1	1	4	12	3	x	x	x	23	0.53	0.2	0.2
Renal	7	2	X	12	14	13	4	x	x	52	1.2	0.4	0.5
Muscle	5	1	5	9	7	2	3	x	x	32	0.74	0.2	0.2
Head & Neck	10	6	29	57	57	69	25	15	9	277	6.4	1.8	2.2
Lymphomas	49	42	30	49	27	36	7	4	x	244	5.6	1.6	1.7
Others & Unspecified	2	4	6	14	21	7	3	x	x	54	1.2	0.4	0.4
Total	162	183	296	651	1045	1291	556	138	23	4345	100	29	34.9

Table 4.1a: Incidence Cases, Rates by site and age group, relative frequencies (%), Average annual Crude Incidence rates (CR) and Age-Standardized Incidence rates (ASR) in men in Kashmir, 2002-2006.

Cancer Type	0-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90+	Total	%age Distribution	Crude rate	ASR
Oesophagus	1	6	33	141	273	223	97	25	2	801	30.8	5.9	7.8
Breast	x	22	125	192	152	73	25	4	x	593	22.8	4.3	5.2
Lung	x	x	7	22	24	33	15	5	x	106	4.1	0.8	1.1
Skin	x	8	7	23	44	53	17	6	2	160	6.2	1.2	1.6
GE Junction	1	3	4	9	18	9	7	1	x	52	2.0	0.4	0.5
Rectum	x	20	19	27	24	17	3	x	x	110	4.2	0.8	0.95
Stomach	x	x	6	5	22	14	11	3	x	61	2.3	0.5	1.0
Colon	1	4	7	13	17	16	3	x	x	61	2.3	0.5	0.6
Cervix	x	x	12	26	15	33	2	6	x	94	3.6	0.7	0.9
Urinary Bladder	x	x	3	4	8	5	x	x	x	20	0.8	0.2	0.2
Thyroid	4	4	8	5	1	3	4	x	x	29	1.1	0.2	0.2
bone & Soft tissue	16	12	6	6	8	5	3	x	x	56	2.2	0.4	0.5
Eye	11	x	x	2	3	x	x	x	x	16	0.6	0.1	0.1
Brain	13	20	24	11	28	9	3	x	x	108	4.2	0.8	0.9
Multiple Myeloma	x	x	3	7	11	19	x	x	x	40	1.5	0.3	0.4
Gall Bladder	1	1	4	14	14	4	2	x	x	40	1.5	0.3	0.5
Renal	4	1	3	4	11	8	2	x	x	33	1.3	0.2	0.5
Muscle	8	x	2	3	5	2	x	x	x	20	0.8	0.1	0.2
Head & Neck	5	4	4	21	22	25	6	3	x	90	3.5	0.7	0.8
Lymphomas	6	19	12	9	10	9	3	x	x	68	2.6	0.5	0.6
Others & unspecified	2	5	6	8	12	4	2	1	x	40	1.5	0.3	0.3
Total	73	129	295	552	722	564	205	54	4	2598	100	19.2	24.8

Table 4.1b: Incidence Cases, Rates by site and age group, relative frequencies (%), Average annual Crude Incidence rates (CR) and Age-Standardized Incidence rates (ASR) in women in Kashmir, 2002-2006.

Site	0-19	20-29	30-39	40-49	50-59	60-69	70-79	80+
Oesophagus	x	x	1.59	8.49	29.65	56.27	50.95	30.86
Lung	x	0.13	0.75	8.28	20.62	33.54	32.52	18.76
GE Junction	x	x	0.2	1.1	4.9	8.92	9.73	1.82
Skin	x	0.14	0.35	1.95	6	8.92	9.98	13.3
Prostrate	x	x	x	0.13	0.49	3.4	4.86	4.23
Rectum	0.016	1.08	0.99	2.08	4.12	7.16	3.07	1.81
Stomach	x	0.17	0.85	2.35	6.77	8.41	6.4	4.23
Colon	x	0.2	0.69	1.15	3	3.64	4.1	x
Testis	0.063	0.24	1.09	1.81	0.98	0.75	1.28	x
Urinary Bladder	x	x	0.15	0.6	1.18	2.63	2.81	3
Thyroid	0.016	0.069	0.25	0.47	1.08	2.4	0.51	x
Bone & Soft tissue	0.29	0.21	0.79	0.88	1.27	1.13	1.02	x
Eye	0.16	0.1	0.15	x	0.69	0.38	x	x
Brain	0.84	1.7	2.83	3.7	5	4.27	1.53	x
Breast	x	x	0.25	0.6	1.27	1.38	1.8	2.4
Multiple myeloma	x	x	0.25	0.47	1.96	2.63	1.02	x
Gall bladder	0.032	0.035	0.049	0.27	1.18	0.38	x	x
Renal	0.11	0.07	x	0.8	1.37	1.63	1.02	x
Muscle	0.08	0.035	0.25	0.6	0.68	0.25	0.77	x
Head & Neck	0.16	0.2	1.44	3.84	5.59	8.66	6.4	14.5
Lymphomas	0.78	1.45	1.5	3.3	2.65	4.52	1.78	2.4
Others & Unspecified	0.03	0.14	0.29	0.94	2.1	0.88	0.77	x
Total	2.6	5.97	14.7	43.8	102.6	162.2	142.3	97.3

Table 4.2a: Age-Specific Incidence rates (ASR) by site and age group in men in Kashmir, 2002-2006.

Cancer Type	0-19	20-29	30-39	40-49	50-59	60-69	70-79	80+
Breast	x	0.8	6.7	15.4	13.9	12.2	9.64	3.66
Lung	x	x	0.38	1.77	2.2	5.5	5.8	4.6
Skin	x	0.3	0.38	1.8	4	8.8	6.6	7.3
GE Junction	0.017	0.11	0.22	0.72	1.64	1.5	2.7	0.92
Rectum	x	0.73	1	2.2	2.2	2.83	1.6	x
Stomach	x	x	0.32	0.4	2	2.33	4.2	2.75
Colon	0.017	0.15	0.38	1	1.55	2.66	1.6	x
Cervix	x	x	0.65	2	1.37	5.5	0.77	5.5
Urinary Bladder	x	x	0.16	0.32	0.73	0.83	x	x
Thyroid	0.07	0.15	0.43	0.4	0.09	0.5	1.54	x
bone	0.28	0.44	0.32	0.48	0.73	0.83	1.6	x
Eye	0.19	x	x	0.16	0.27	x	x	x
Brain	0.23	0.73	1.3	0.88	2.56	1.5	1.6	x
Multiple Myeloma	x	x	0.16	0.56	1	3.1	x	x
Gall Bladder	0.017	1	0.22	1.1	1.3	0.67	0.77	x
Renal	0.07	1	0.16	0.32	1	1.33	0.77	x
Muscle	0.14	x	0.11	0.24	0.46	0.33	x	x
Head & Neck	0.09	0.036	0.22	1.7	2	4.2	2.3	2.75
Lymphomas	0.1	0.69	0.65	0.72	0.91	1.5	1.6	x
Others & unspecified	0.035	0.18	0.32	0.64	1.1	0.67	0.77	0.92
Total	1.27	6.54	15.9	44.1	65.9	93.9	81.3	53.1

Table 4.2b: Age-Specific Incidence rates (ASR) by site and age group in women in Kashmir, 2002-2006.

Site	Kashmir	Chennai#	Kolkata**	Rural Delhi##	Urban Delhi*	Mumbai#	Poona#	Nagpur#	Trivandrum#	Karunag apally#
Oesophagus	11.2	9.1	4.5	2.7	4.9	6.7	6.1	8.7	3.5	8.3
Lung	6.5	10.8	18.7	13.8	10.7	9.7	6.2	7.5	9.9	21.3
Brain	2.2	3	2.0	1.7	4	3.7	3.6	3.0	2.9	3.7
Head & Neck	2.2	21.9	20.3	16	25	22.2	19.5	23.5	23.3	22.3

*Data from Curado et al, 2007; **Sen et al., 2002; ## Manoharan et al., 2009; * Manoharan et al., 2010

Table 4.3a: Age Standardized Incidence Rates (per 100,000) for Common Sites in Kashmir and other Indian Registries- Males.

Site	Kashmir	Chennai#	Kolkata**	Rural Delhi##	Urban Delhi*	Mumbai#	Poona#	Nagpur#	Trivandrum#	Karunag apally#
Oesophagus	8.3	5.4	3.5	2.6	3.1	3.4	5.0	5.7	0.9	2.0
Breast	5.2	26.5	25.1	7.8	30.0	26.9	24.4	27.4	24.6	16.0
Rectum	0.95	1.4	2.0	0.6	1.8	1.4	2.4	1.8	2.2	1.4
Brain	0.9	2.1	1.5	1.5	2.4	2.8	1.9	2.6	2.2	1.9
Lung	1.1	2.6	4.9	1.0	3.4	3.1	3.0	2.4	1.7	2.3
Cervix	0.9	28.0	9.4	10.3	17.5	14.5	17.3	18.4	9.4	10.6

*Data from Curado et al, 2007; **Sen et al., 2002; ## Manoharan et al., 2009; * Manoharan et al., 2010

Table 4.3b: Age Standardized Incidence Rates (per 100,000) for Common Sites in Kashmir and other Indian Registries- Females.

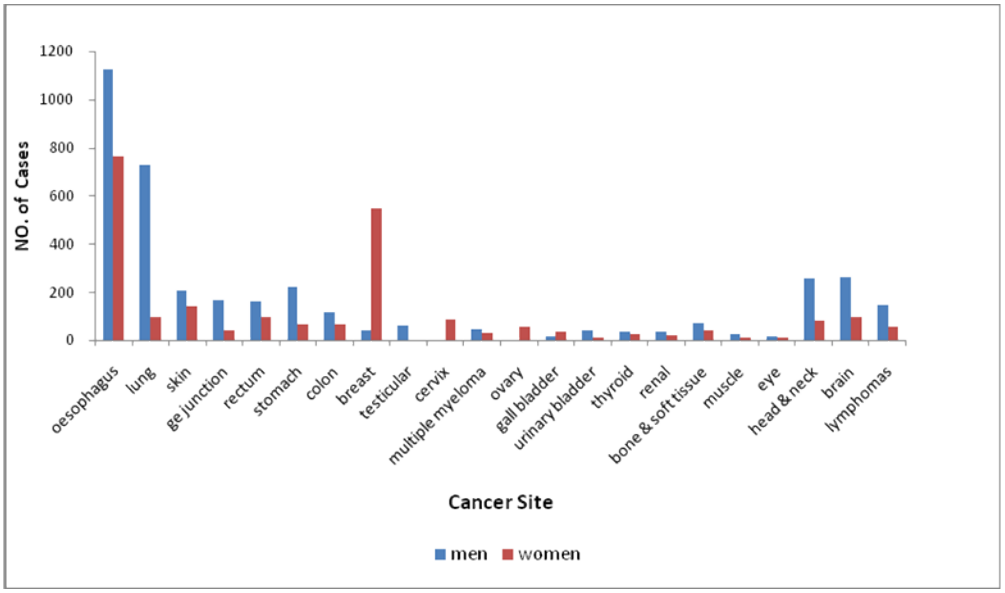


Fig 4.1: Comparison of total number of incident cases of cancer (from year 2002-2006) at different cancer sites in males & females.

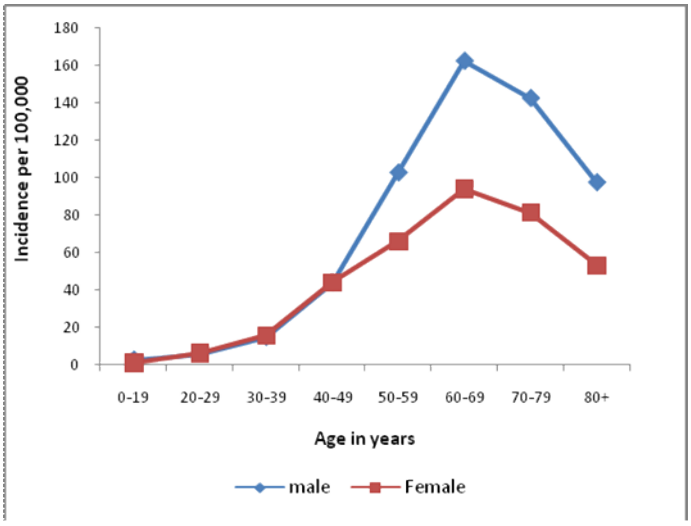


Figure 4.2: Age-specific Incidence Rate Curves.

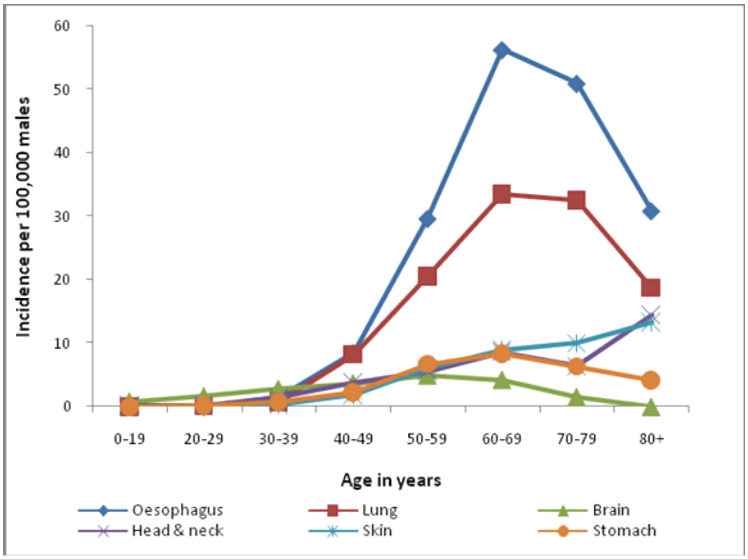


Figure 4.3a: Age-specific Incidence Rate Curves for Oesophagus, Lung, Brain, Head & Neck, Skin and Stomach cancers in Males.

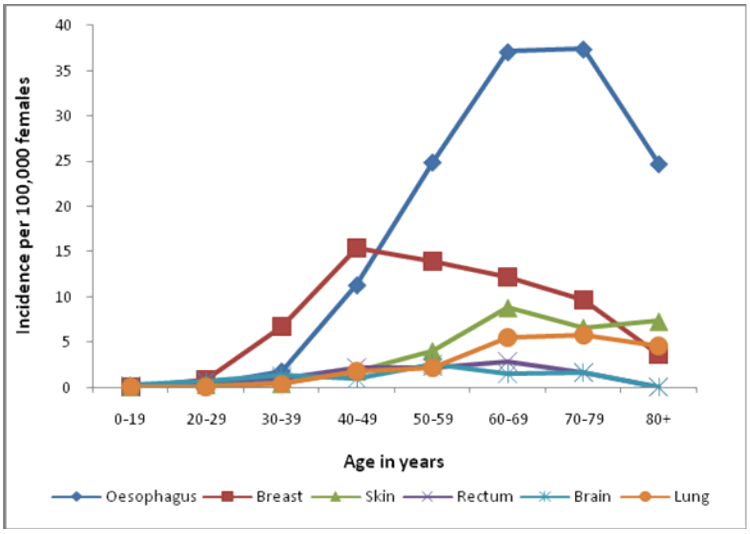


Figure 4.3b: Age-specific Incidence Rate Curves for Oesophagus, Breast, Skin, Rectum, Brain and Lung cancers in Females.

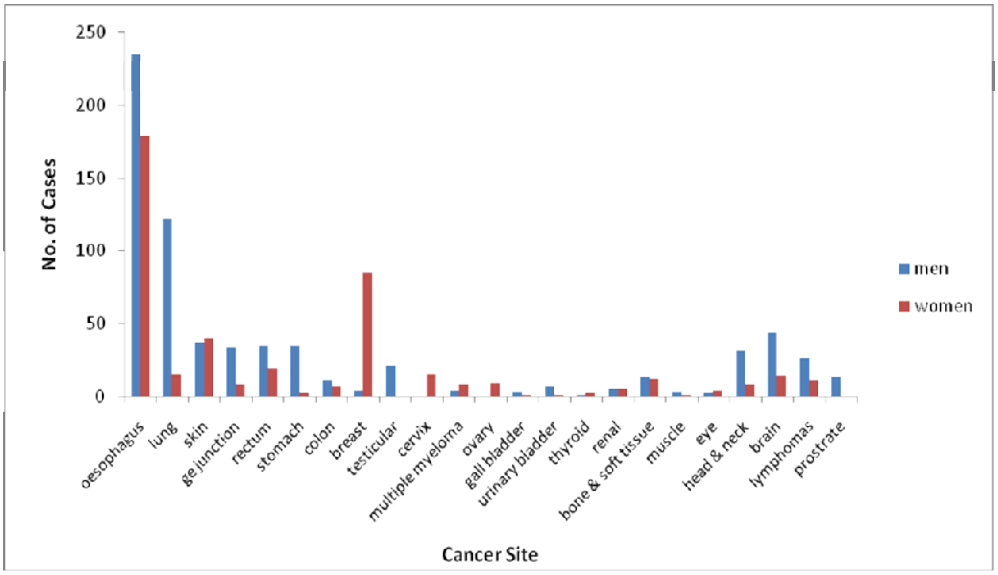


Figure 4.4a: Comparison of new incident cases at different sites in males & females (Year 2002).

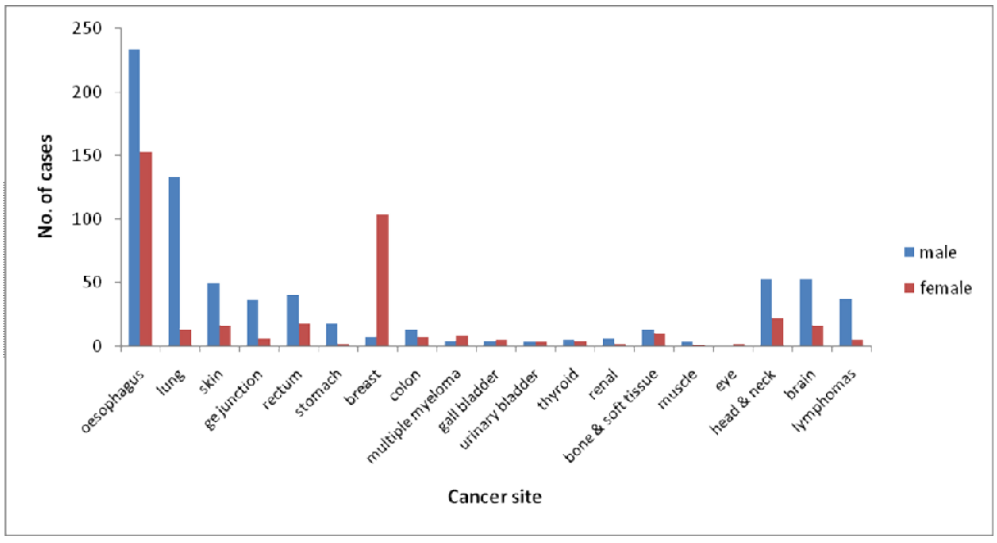


Figure 4.4b: Comparison of new incident cases at different sites in males & females (Year 2003).

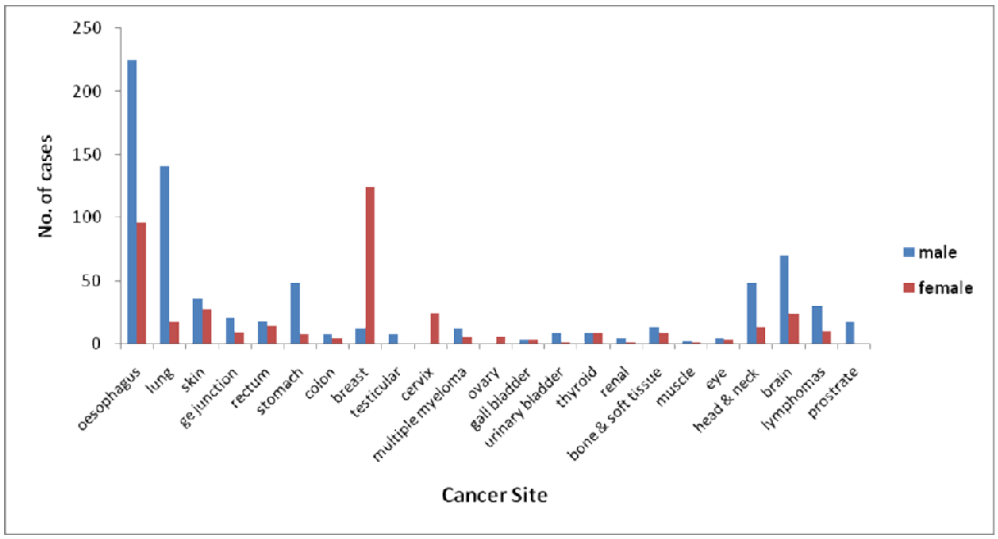


Figure 4.4c: Comparison of new incident cases at different sites in males & females (Year 2004).

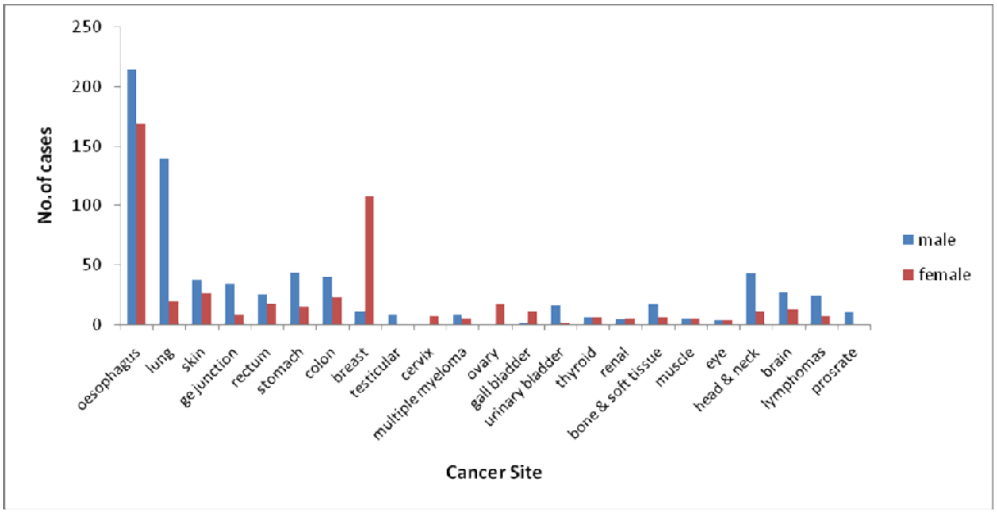


Figure 4.4d: Comparison of new incident cases at different sites in males & females (Year 2005).

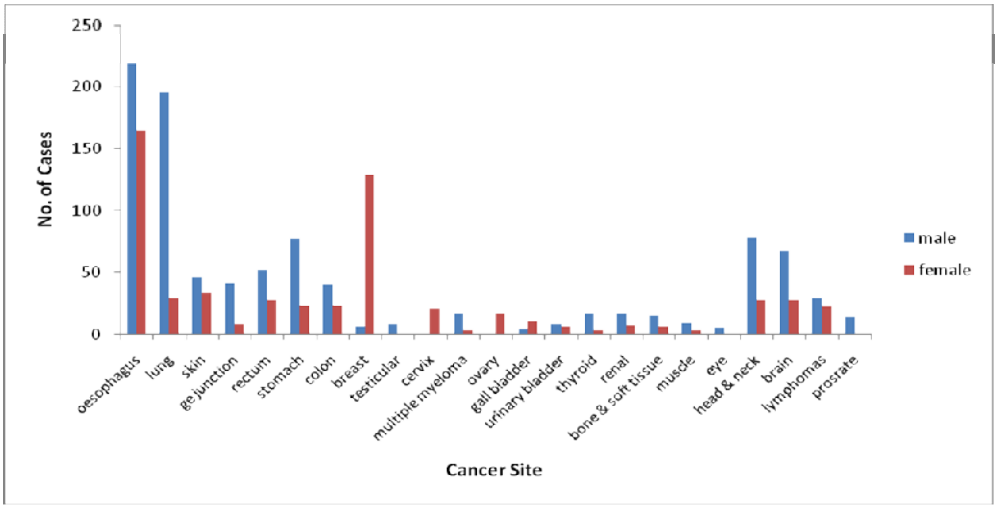


Figure 4.4e: Comparison of new incident cases at different sites in males & females (Year 2006).

CHAPTER 5

DISCUSSION

CHAPTER FIVE

DISCUSSION

This study on the incidence pattern of different types of cancer was conducted on an ethnic population and almost all of the patients report either to the SMHS Hospital or Sher-i-Kashmir Institute of Medical Sciences, except the few numbers that go outside valley for treatment. The public acceptance of allopathic treatment is high and no alternative therapies such as homeopathy and ayurvedic exist in the valley. A well-flourished network of investigative laboratories at district and sub-district level is present and a good referral system between these health centers and the specialist institutions primarily involved in treating this disorder exists.

In Kashmir, oesophageal cancer is the most predominant cancer present in males as well as females. This cancer by far exceeds the frequency of other cancers in both the sexes contributing about 30% to the total number of cancer cases in Kashmir. The preponderance of oesophageal cancer can be ascribed to the life style and local dietary habits among Kashmiris as various epidemiological studies in regions of the world with high incidences of oesophageal cancer have substantiated the important role of these two in the incidence pattern of oesophago-gastric cancer (Kmet & Mahboubi, 1972; Malhotra, 1967; Siddiqi & Preussmann, 1989; Warwick, 1973; Yang, 1980). The most important specific food habit in Kashmir is the frequent consumption of hot salt tea by local inhabitants, more so during the winter months when the temperature falls to subzero levels. The salt tea is prepared traditionally in a specially designed copper vessel called a 'samawar'. The samawar consists of a central smoke stack with a perforated base (fed with live charcoal) mounted on a stand and a surrounding jacket for boiling the salt tea infusion. The tea is prepared by brewing green tea leaves (not grown in Kashmir) in sodium bicarbonate until a

thick red brown-colored extract is obtained. The extract is diluted with water and then milk and salt are added. The tea is customarily served boiling hot and is consumed with or without snacks. The components of salted tea like sodium bicarbonate and common salt, a well known irritant of gastric epithelium (Correa, 1987) are believed to be the chief suspects causing oesophageal cancer.

Another specific dietary habit which is very particular to Kashmiri population is the practice of drying raw food stuffs in open sun and this dried food is stored and later on consumed in the winter months (Siddiqi & Preussmann, 1989). Studies have shown that these foods contain significant amounts of N-nitroso compounds (Tricker et al., 1988). In addition, other Kashmiri food items contributing substantial amount of N-nitroso compounds include salt tea, dried fish, vegetables especially Brassica olerecea (haak), red chillies, and spice cake (wur) (Siddiqui et al., 1988, 1990). In addition, salt tea showed the formation of high amounts of N-nitrosopipe-colic acid with several unidentified non-volatile N-nitroso compounds on nitrosation of tea extracts under conditions simulating the fasting human stomach (Siddiqui et al., 1988(2)).

Lung cancer ranks next to esophageal cancer in males in the valley. Its association with smoking has been substantiated by innumerable studies, some of which have also established a dose response relationship, in terms of the number of cigarettes smoked per day and the risk of developing lung cancer (Arya et al., 1991; Charles, 1991; Peter, 1993).

Case control studies conducted in places like Kerala (Sankarnarayanan et al., 1994), Bhopal (Dikshit & Kanhere, 2000) and Chandigarh (Gupta et al., 2001) reported a high risk of lung cancer among bidi and cigarette smokers showing a direct link between the two. A case control study from Chandigarh also suggested that environmental smoke exposure may be a strong risk factor for lung cancer in India (Rapiti et al., 1999). Smoking is popular throughout India, where besides cigarettes, pipes and cigars; it takes various indigenous forms such as bidi, hookah, and chilam. In Kashmir, besides cigarette smoking, which is mainly practised in urban and semi-urban localities, the most popular form is

hubble bubble (hukkah) whereby the smoke is inhaled after it is cooled and filtered by passing through water placed in an earthen container and is largely responsible for passive smoking of other family members, especially during winter months. Smoking in the valley is a male pastime; the women do not generally smoke. That is the why we can clearly see the preponderance of lung cancer in males as compared to the females. The relatively higher prevalence of oesophageal cancer in the men may also result from the additional influence of smoking. While in developed countries, such as the USA, lung cancer is a leading cause of death in both sexes (Jemal et al., 2002), it is mostly a disease of men in India, as is also shown in this study.

Brain cancer is the third common site of cancer in males in Kashmir accounting for 7% of all cancer cases. This cancer was found to be more common in the male children accounting for 17.4% of total cases in the age group of 0-20 and more than 50% of the cases in the age-group of 0-40 years.

Breast cancer, the most frequent cancer of women in the world is the second leading site of cancer in females in Kashmir. It constituted about 23% of all cancers in women whereas in males, it is hardly found to occur and just contributes 1% of all cancers. Overall in both males and females, it is the second most common cancer present in the valley. On comparing the incident cases of breast cancer in different age groups, it is clearly seen that the early onset cases (0-40 age group) contribute about 25% of the total number of breast cancer cases in females. Although the disease is mainly postmenopausal in western population, but the picture in Kashmir is no different than the rest of the country where the burden of breast cancer due to early onset cases is increasing at an alarming rate. Though the etiology of breast cancer in India has not been widely studied, case-control studies from Mumbai (Rao et al., 1994) and Chennai (Gajalakshmi & Shanta, 1991) have identified null parity, early menarche, late age at marriage and late age at pregnancy as risk factors.

The higher frequency of skin cancer in men as well as women is perhaps caused by the greater exposure to the sun that they receive especially while

farming. Additionally, studies have shown that the use of firepot, (kangri) an ingenious mode of providing warmth during extreme cold conditions in the valley also contribute towards skin cancer in the valley (Wani, 2010).

Interestingly, our resulting are showing a different pattern of cancer in Kashmiri women population. In our women population, the frequency of cervical cancer is very low which is quite contrary to the pattern in rest of the country where cervical cancer is the second leading cancer in females after breast cancer (ICMR, 2001). This change in pattern and the lower rate of cervical cancer which is just 3.6% can be attributed to the practice of universal circumcision, prevalent in the majority community of Kashmir.

The sex differentiation was exceedingly significant in breast and tobacco associated cancers. The former was excessively present in females whereas latter was present in males. Though, this difference in occurrence of tobacco associated cancers can be attributed to smoking habit in men, however in case of breast cancer, its distribution can be attributed to anatomical and physiological differences.

The pattern observed from the analysis of the available data provides comprehensive information on cancer occurrence in the valley and these results will help to evaluate a cancer control program in this region. In conclusion, our study has shown that in Kashmir valley oesophageal cancer is the leading cancer in both males as well as females, followed by lung cancer in males and breast cancer in females. So, counseling, awareness campaigns, change of dietary habits and life style are the primary measures to be taken to prevent this oesophageal cancer epidemic in the valley. Additionally, the primary prevention of lung and other tobacco related cancers by implementation of strict tobacco control measures are important. Early detection of breast cancer should be encouraged in women through health education and prompting early diagnosis. Procedures like physical examination of the breast may prove to be useful early diagnostic tool in this context.

SECTION II

CHAPTER 1

INTRODUCTION

CHAPTER ONE

INTRODUCTION

Breast cancer is the most common malignancy affecting women worldwide (Parkin, 2004), accounting for 25% of all new cases of cancer. One in eight to one in twelve women is likely to suffer from Breast cancer during her life-time in the developed countries and one in twenty two is likely to have the disease in developing countries. Breast cancer incidence rate varies atleast tenfold worldwide (Parkin et al., 2001) largely because of range of socio-economic, reproductive, hormonal, nutritional and genetic factors (Mcpherson et al., 2000). Areas of high-incidence include North America and Europe (Parkin et al., 2001). South-east Asia contributes over 1/5th of the worldwide prevalence of the disease with Pakistan having highest incidence among Asian population after Israel (Bhurgri et al., 2000). In India, breast cancer is the second common cancer in women after cervical cancer; however breast cancer has replaced cervical cancer as the leading site of cancer among women in Indian cities (ICMR, 2001). Kashmir, which lies at an altitude of 1800-2400 km from the sea-level and borders the low-incidence country, India and the high-incidence area of Pakistan, is distinct from other areas in term of its unique geographical locale, intra-community marriages, tradition, culture, food habits and ethnicity. Over the past few years, the valley has witnessed an increase in the incidence of breast cancer and threatens to overtake oesophageal cancer as the most common cancer among Kashmiri women.

Breast Cancer refers to cancers originating from breast tissue, most commonly from the epithelial cells that line the milk ducts or the lobules that supply the ducts with milk (Jensen, 1976). Depending on wherein the glandular or ductal unit of the breast the cancer arises, it develops certain characteristics that are used to sub-classify breast cancer into types. Cancers originating from

ducts are known as ductal carcinomas and those originating from lobules are known as lobular carcinomas. The primary tumor begins in the breast itself but once it becomes invasive, it may progress beyond the breast to the regional lymph nodes or travel (metastasize) to other organ systems in the body and become systemic in nature. Cancer cells that remain confined to the boundaries of the lobular unit or the draining duct are classified as in situ or non-invasive. An invasive breast cancer is one in which there is dissemination of cancer cells outside the basement membrane of the ducts and lobules into the surrounding adjacent normal tissue. There are many different types of breast cancer, with different stages (spread), aggressiveness, and genetic makeup and the survival of the patient varies greatly depending on those factors (6).

Although many risk factors of the disease like ageing (Moolgavkar et al., 1979), early menopause (Magnusson et al., 1998), alcohol consumption (Hamajima et al., 2002), radiation exposure (Grevias-Faqnou et al., 1999), tobacco use (Hamajima et al., 2002), obesity or over weight (Calle et al., 2003), physical activity (Bray et al., 2004), urbanization (McMichael et al., 1998), sedentary life style, dietary fat (Schulz et al., 2008), changes in reproductive patterns (Lord et al., 2008), such as delayed childbearing and having fewer children, nulliparity (Huo et al., 2008; Winer et al., 2000), no breast-feeding (Huo et al., 2008; Lord et al., 2008; Zeng et al., 2010), multiple abortions (Zeng et al., 2010) and post-menopausal hormone replacement therapy (Beral, 2004), stand identified but a higher proportion of breast cancer is associated with a strong family history, with first-degree relatives of patients having an approximately two fold elevated risk (Colditz et al., 1993; Madigan et al., 1993).

Genetic susceptibility as a result of highly penetrant germ-line inactivation in cancer predisposing genes characterizes approximately 5-10% of breast cancer and 25% of the early onset of breast cancer (Palma et al, 2006; Wooster et al., 1994). Several genes have been identified that are associated with inherited susceptibility to breast cancer and have provided means to begin identifying individuals and families with an increased risk of cancer. One of the

most exciting and highly anticipated break through in cancer genetics was the cloning of BRCA1 and BRCA2 in early nineties (Wooster et al., 1994; Miki et al., 1994). BRCA1 and BRCA2 genes appear to account for the majority of hereditary breast cancer cases via autosomal dominant inheritance mechanism. Tumorigenesis in women with BRCA1 or BRCA2 mutations requires the loss or inactivation of the remaining wild-type allele, resulting in expression of a nonfunctional protein and a loss of cell cycle control and DNA repair mechanisms (Welcsh & King, 2001). BRCA1 and BRCA2 perform multiple discrete functions like regulates DNA repair, gene transcription and maintain genome integrity and tumor is initiated when genetic instabilities lead to increased mutations in these genes (Hall et al., 1990). In addition, many other genes like p53, ATM, CHEK2, MDM2, PTEN, the GADD repair group, the HER2/neu oncogene (Campeau et al., 2008; Lavin, 1998; Nechushtan et al., 2009; Walsh et al., 2006) have also been implicated in the genesis of the disease. However, their involvement account for only a minor fraction of breast cancers. Some of these genes directly influence BC risk, whereas others are involved in the general processes of cancer growth and metastasis.

In families with breast cancer consistent with hereditary breast cancer, it has been reported that BRCA1 mutations account for approximately 45% of families with significantly high breast cancer incidence and at least 80% of families with increased risk of both early-onset breast and ovarian cancer (Ford et al., 1994; Thompson & Easton, 2002). Whereas germ-line mutations of BRCA2 are predicted to account for approximately 35% of families with multiple cases, early onset female breast cancer, and they are also associated with an increased risk of male breast cancer and ovarian cancer (Gayther et al., 1997; Thorlacius et al., 1996). In women the overall risk of breast cancer associated with mutations in the BRCA1 or BRCA2 gene is from 40% - 85% over a lifetime, whereas the lifetime risk in the general population is approximately 12.5%, which differ in populations (Japan (2%) and USA (14%)) (Antoniou et al., 2002; Begg, 2002). BRCA1 or BRCA2 mutation carriers (women) with a history of breast cancer

exhibit an elevated risk of contralateral breast cancer, at 40% to 60% (Begg, 2002). BRCA1 and BRCA2 mutation carriers also have an elevated risk of ovarian cancer, ranging from 15% to 40%, compared with an approximate risk of 2% in the general population (Ford et al., 1998). It is generally accepted, that carriers of mutations in BRCA1 or BRCA2 have an excessive risk for both breast and ovarian cancer, thus screening for and detection of BRCA1/BRCA2 mutations may be helpful in determining the overall risk for the development of breast carcinoma, especially in families with hereditary cases. Individuals who are mutation carriers can undertake different surveillance strategies, chemoprevention interventions, or surgical prophylaxis for carcinomas of the breast and ovary.

BRCA2 was the second breast and/ovarian cancer susceptibility gene to be discovered, mutated form of which when inherited strongly predispose to breast or breast and ovarian cancers (Sowter & Ashworth 2005; Wooster et al., 1995)**.** BRCA2 plays an important role in the error-free repair of DNA double strand breaks as well as transcriptional regulation (Davies et al., 2001; Moynahan et al., 2001; Pellegrini et al., 2002). In normal cells, BRCA2 ensures the stability of the cell's genetic material (DNA) and helps to prevent uncontrolled cell growth. The spectrum of BRCA2 mutations has been characterized in different populations worldwide, with significant variation of the relative contribution of these genes to hereditary cancer between populations. Various population-based studies have shown population specific BRCA2 founder mutations and also variable number of novel mutations in different populations, and thus have defined high and low risk subsets for developing breast cancer based on ethnic origin (Oddoux et al., 1996; Thorlacius et al., 1997). Since Kashmiri women represent a cohort of genetically pure population, the mutational pattern of BRCA2 can serve as genetic marker for the identification of women who are at high risk of breast cancer.

Among the other genes likely to be involved in breast cancer is the gap-junction gene Connexin43, which codes for a 43-kd gap-junction protein.

Connexin forms membrane spanning hexameric hemichannels called as connexons that, when in register in apposed cell membranes, couple to form a continuous hydrophilic channel through which ions, metabolites, and molecules involved in signal transduction can move from cell to cell. An array of many such channels constitutes a gap junction. Gap junctions initially identified to serve as passageways for the cell-to-cell exchange of low molecular weight growth regulatory molecules (Flagg-Newton et al., 1997; Loewenstein, 1981; Loewenstein & Kanno, 1966), have now been proposed to play an important regulatory role in cell growth, cell differentiation, cell apoptosis and tissue development (El Sabban et al., 2003; Gramsch et al., 2001; Ma & Dahl, 2006; Vinken et al., 2006; Wiszniewski et al., 2000). Down regulation of intercellular communication via gap junctions, either by down regulation or mistargeting of connexins (due to post-translational processing, germline or somatic mutations), appears to play a role in many types of pathologies, including cancer (Laird et al., 2006; Mesnil et al., 2005; Severs et al., 2004). A large number of studies have indicated that connexins like Cx43, Cx32, Cx26 have tumor suppressing effects due to maintenance of cellular homeostasis via gap junctional intercellular communication and thus are players in the arena of growth regulation and in many types of cancer. (King et al., 2005; Naus et al., 1992). Studies on neoplastic cells using ultrastructural, biochemical and immunological means and by introduction of fluorescent or radio-active tracers have shown that the majority of neoplastic cells have fewer and smaller gap junctions, express less connexins and have reduced GJIC compared to their non-neoplastic counter-parts and up-regulation of Cxs has been shown to restore normal phenotypes and retard tumor cell growth (Qin et al., 2002).

Connexin 43 is the predominantly expressed gap junction protein in normal breast tissue and plays an important role in normal mammogenesis, lactogenesis and involution (Cai et al., 1998; Monaghan et al., 1996). Studies have shown down-regulation of connexin 43 gap-junction protein in human breast cancer tissues or a relocalization of the connexin to intracellular

compartments, resulting in a predicted loss of GJIC compared to matched normal or benign breast tissue (Kanczuga- Koda et al., 2003, 2005; Laird et al., 1999; Lee et al., 1991; Wilgenbus et al., 1992). Cx43 gap junction down-regulation has been observed in breast tissue at various stages of tumorigenesis, including Ductal carcinoma in situ (DCIS), invasive Infiltrating ductal carcinoma (IDC), and infiltrating lobular carcinoma (ILC) and restoration of gap-junction intercellular communication by up-regulation of connexins has been shown to restore normal phenotypes in vitro and reduce tumor growth in vivo (Hirschi et al., 1996; Mehta et al., 1991). Studies have also shown that down-regulation of endogenous Connexin 43 expression by small interfering RNA promoted a more aggressive phenotype in human breast cancer cell lines (Shao et al., 2005). Further more, data obtained with rat mammary carcinoma induced by DMBA also demonstrates that the loss of Connexin 43 gap junction is a common feature of mammary neoplastic transformed. Studies have also shown that Cx43 expression in cancer cells leads to decrease expression of proteins involved in increased motility, invasion and metastases clearly indicating that it contributes to decreased metastasis in vivo. However the molecular mechanisms behind the down-regulation of Connexin 43 and contribution to development of primary tumor and its metastasis in breast remain elusive. A better understanding of the key events that lead to the down-regulation of connexins in breast cancer is necessary to gain information relevant to the designing of anti-cancer treatment models against breast cancer. Multiple mechanisms appear to be responsible for the down regulation of Connexin43 in breast neoplastic tissue, and one of the potent mechanisms can be mutations in gap-junctions genes.

We propose to study the sequence variations in breast cancer patients of the Kashmir region, and workout association of such variations (if any) with the disease phenotype. Despite the existence of a large population of breast cancer patients, there is a paucity of such kind of data from the state of the Jammu and Kashmir. The present study is therefore aimed to investigate the mutation status

of BRCA2 and Connexin 43 in Kashmiri breast cancer patients to verify its possible role in breast cancer incidence and development in Kashmiri population.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER TWO

REVIEW OF LITERATURE

2.1 BREAST CANCER

2.1.1 DEFINITION

Breast cancer is the formation of a malignant tumor that has developed from cells in the breast. Each breast has 15 to 20 sections called lobes, which have many smaller sections called lobules and these lobes and lobules are connected by thin tubes, called ducts. The malignant cells either originate in the lining of the milk glands or ducts of the breast (Jensen, 1976) and depending on where in breast the cancer arises, it will develop certain characteristics that are used to sub-classify breast cancer into types. The primary tumor begins in the breast itself but once it becomes invasive, it may progress beyond the breast to the regional lymph nodes or metastasize to other organ systems in the body and become systemic in nature (Cady, 1984; Jatoi, 1987). Breast cancer follows this classic progression though it often becomes systemic or widespread early in the course of the disease. When primary breast cancer spreads, it may first go to the axillary nodes leading to regional metastasis (Cady, 1984). Or it may proceed elsewhere either by lymphatic or blood-borne spread and the patient develops systemic metastasis that may involve a number of other organs in the body (Jatoi, 1987). Favorite sites of systemic involvement for breast cancer are the lung, bones (Rose & Siegel, 2006), liver, skin and soft tissue (7). As it turns out, the presence of, and the actual number of, regional lymph nodes containing cancer remains the single best indicator of whether or not the cancer has become metastatic. Because tests to discover metastasis in other organs may not be sensitive enough to reveal minute deposits, the evaluation of the axilla for

regional metastasis becomes very important in making treatment decisions for widely this disease. If breast cancer spreads to other major organs of the body, its presence will compromise the function of those organs.

2.1.2 TYPES OF BREAST CANCER

Breast cancer is considered as highly heterogeneous group of cancers arising from different cell types and each having its own clinical implications. Breast cancer can begin in different areas of the breast – the ducts, the lobules, or in some cases, the tissue in between. Depending on from where the cancer arises, breast cancer is divided into different types.

2.1.2.1 Non-invasive breast carcinoma

When breast tumors are discovered at an early stage, they are still small and confined. In such cases, cancer cells have not grown into the surrounding tissues and remain within the borders of a duct or lobule. These tumors are known as non-invasive or in situ tumors. In situ tumors are too tiny to form a 'lump,' and so they usually are not felt or detected during a physical exam and are diagnosed by mammography. Non-invasive carcinomas include

2.1.2.1.1 Ductal carcinoma in situ (DCIS): DCIS also known as intraductal carcinoma or non-invasive ductal carcinoma starts inside the milk ducts and is the most common type of non-invasive breast cancer. DCIS isn't life-threatening, but having DCIS can increase the risk of developing an invasive breast cancer later on. (Bellamy et al., 1993; Page et al., 1982). Each year, about 1.9% of women with high-grade DCIS develop invasive breast cancer after lumpectomy (Page et al., 1982). Thus, DCIS is a potential marker for invasive carcinoma.

2.1.2.1.2 Lobular carcinoma in situ (LCIS): LCIS also known as non-invasive lobular carcinoma usually occurs in women who have not undergone menopause.

Lobular means that the abnormal cells start growing in the lobules, the milk-producing glands at the end of breast ducts. LCIS is a multifocal (located in more than one area) disease that typically affects both breasts in contrast to DCIS, which generally is unifocal or at least limited to one region of the breast. Because of the multifocal character of LCIS, women with this disease should receive careful examinations of both breasts. However, over 99% people with LCIS do not develop invasive breast cancer. (Frykberg et al., 1987; Singletary et al., 1994).

2.1.2.2 Invasive Carcinomas

If breast cancer penetrates the membrane that surrounds the lobules or ducts, it is called an infiltrating or invasive carcinoma. Invasive carcinoma can grow into the supporting tissue between the ducts, blood vessels, lymph nodes, and other structures within the breasts. Therefore, there is a greater chance that invasive carcinoma will metastasize, spreading throughout the body. Invasive carcinomas include

2.1.2.2.1 Ductal Invasive carcinomas: About 75% percent of all invasive breast cancers are ductal carcinomas (Li et al., 2003). Under the microscope, ductal carcinoma looks like a mass with poorly defined edges that have begun to extend into the surrounding tissue (Mai et al., 2000). As the cancer invades the fatty tissue around a duct, it causes the formation of fibrous, scar-like tissue (Harris et al., 1984). Such scar formation makes ductal carcinoma appear larger than it actually is. Depending upon the location of the tumor, the symptoms of invasive ductal carcinoma may include retraction (drawing inward) of the nipple or nipple discharge and skin changes such as wrinkling or dimpling.

2.1.2.2.2 Lobular Invasive carcinomas: These cancers make up approximately 5% to 10% of all invasive breast cancers (Borst & Ingold, 1993; Li et al., 2003; Martinez & Azzopardi, 1979). ILC is characterized by small round cells that are

bland in appearance and have scant cytoplasm, which infiltrate the stroma in single file and surround benign breast tissues in a targeted manner (Fisher 1975; Martinez & Azzopardi, 1979). Lobular breast cancer is more difficult to detect by mammography because it may not occur as a distinct lump (Krecke & Gisvold 1993; Yeatman et al., 1995). Instead, lobular carcinoma may appear as an irregular thickening in the breast. A small proportion of women (~5%) may develop lobular carcinoma in both breasts (Dixon et al., 1983; Du toit et al., 1989; Lesser et al., 1982).

In addition to ductal and lobular carcinoma, three well recognized types of invasive breast cancer are: tubular cancers (slow-growing, tube-shaped cancers), medullary cancers (cancers that look like the medulla [gray matter] of the brain), and mucinous cancers (cancers that contain a mucous protein) (Diab et al., 1999; Li et al., 2003).

2.1.2.2 Inflammatory carcinoma

It is a very serious, rapidly spreading type of tumor that accounts for about 1% of all breast cancers (Anderson et al., 2004; Wingo et al., 2004). It produces symptoms like swelling, redness, and skin warmth, which result from blockage of the skin's lymphatic vessels by cancer cells (Green et al., 2002; Taylor et al., 1938). Because of such symptoms, inflammatory carcinoma sometimes is confused with mastitis - a breast infection that may or may not be associated with breastfeeding and can be cured by antibiotics.

2.1.2.3 Paget's disease

Paget's disease of the nipple is a rare form of breast cancer in which cancer cells collect in or around the nipple. The cancer usually affects the ducts of the nipple first (small milk-carrying tubes), then spreads to the nipple surface and the areola (the dark circle of skin around the nipple). The nipple and areola often become scaly, red, itchy, and irritated (Kaelin, 2004). It usually strikes

middle-aged women and may occur in association with an underlying in situ or invasive ductal carcinoma of the breast (Kaelin, 2004).

2.1.3 SIGNS AND SYMPTOMS

The following are the symptoms associated with breast cancer:

- The first noticeable symptom of breast cancer is typically a lump that feels different from the rest of the breast tissue. More than 80% of breast cancer cases are discovered when the woman feels a lump (6). The earliest breast cancers are detected by a mammogram (3). Lumps found in lymph nodes located in the armpits (6) can also indicate breast cancer.
- Indications of breast cancer other than a lump may include changes in breast size or shape, skin dimpling, nipple inversion, or spontaneous single-nipple discharge. Pain (mastodynia) is an unreliable tool in determining the presence or absence of breast cancer, but may be indicative of other breast health issues (3, 6).
- Unexplained weight loss can occasionally herald an occult breast cancer, as can symptoms of fevers or chills.
- Symptoms of inflammatory breast cancer include pain, swelling, warmth and redness throughout the breast, as well as an orange-peel texture to the skin referred to as peau d'orange (Green et al., 2002; Taylor & Meltzer, 1938).
- In Paget's disease of the breast, the syndrome presents as eczematoid skin changes such as redness and mild flaking of the nipple skin (Kaelin, 2004). As Paget's advances, symptoms may include tingling, itching, increased sensitivity, burning, and pain (Kaelin, 2004). There may also be discharge from the nipple. Approximately half of women diagnosed with Paget's also have a lump in the breast (Kaelin, 2004).

- Bone or joint pains can sometimes be manifestations of metastatic breast cancer, as can jaundice or neurological symptoms. These symptoms are "non-specific", meaning they can also be manifestations of many other illnesses (8). Most symptoms of breast disorder do not turn out to represent underlying breast cancer. Benign breast diseases such as mastitis and fibroadenoma of the breast are more common causes of breast disorder symptoms (Shaaban et al., 2002). The appearance of a new symptom should be taken seriously by both patients and their doctors, because of the possibility of an underlying breast cancer at almost any age (6).

2.1.4 STAGES OF BREAST CANCER

Breast cancer staging is based on the TNM system, defined by the American Joint Committee on Cancer, which takes into account tumor (T) size, the extent of regional lymph node (N) involvement, and the presence or absence of metastasis (M) beyond the regional lymph nodes. Using this system, whose criteria and details are outlined in Table 2.1, breast cancer is staged from 0 to IV. Stage 0 implies in situ cancer, while stages I to IV indicate invasive cancer, with IV implying metastatic spread to distant organs.

Primary Tumor (T) †	Regional Lymph Node Status (N)	Distant Metastasis (M)	Stage Groupings
Carcinoma in situ	No evidence of cancer in nearby nodes	No	**Stage 0**
Tumor ≤ 2 cm	No evidence of cancer in nearby nodes	No	**Stage I**
No evidence of primary tumor	Metastasis to 1–3 nodes	No	**Stage IIA**
Tumor ≤ 2 cm*	Metastasis to 1–3 nodes		
Tumor > 2 cm but ≤ 5 cm	No evidence of cancer in nearby nodes		
Tumor > 2 cm but ≤ 5 cm	Metastasis to 1–3 nodes	No	**Stage IIB**
Tumor > 5 cm	No evidence of cancer in nearby nodes		
No evidence of primary tumor	Metastasis to 4–10 nodes	No	**Stage IIIA**
Tumor ≤ 2 cm*	Metastasis to 4–10 nodes		
Tumor > 2 cm but ≤ 5 cm	Metastasis to 4–10 nodes		
Tumor > 5 cm	Metastasis to 1–3 nodes		
Tumor > 5 cm	Metastasis to 4–10 nodes		
Tumor of any size with direct extension to chest wall or skin	No evidence of cancer in nearby nodes	No	**Stage IIIB**
Tumor of any size with direct extension to chest wall or skin	Metastasis to 1–3 nodes		
Tumor of any size with direct extension to chest wall or skin	Metastasis to 4–10 nodes		
Any tumor designation	Metastasis to > 10 nodes	No	**Stage IIIC**
Any tumor designation	Any lymph node designation	Yes	**Stage IV**

"†" Size measurements are for the tumor's greatest dimension.

"*"Includes microinvasion of 0.1 cm or less in greatest dimension.

Table 2.1: Criteria for staging breast tumors according to the American Joint Committee on Cancer's TNM classification (Greene et al., 2002).

2.1.3 RISK FACTORS

Following risk factors for breast cancer have been identified and unanimously agreed upon.

2.1.5.1 REPRODUCTIVE FACTORS

2.1.5.1.1 Menstrual history: Women who have had more menstrual cycles because they started menstruating at an early age (before age 12) and/or went through menopause at a later age (after age 55) have a slightly higher risk of breast cancer (Helmrich et al., 1983; Winer et al., 2000). This may be related to a higher lifetime exposure to the hormones like estrogen and progesterone (Henderson et al., 1988).

2.1.5.1.2 Parity, Reproductive history and Breast feeding: Parity and an early first full-term pregnancy (FFTP) both have been shown to decrease the long-term breast cancer risk (Lambe et al., 1996; Sinha et al., 1988). Nulliparous women are also at increased risk of acquiring breast cancer (Huo et al. 2008). Before pregnancy, mammary gland cells are in a vulnerable undifferentiated state but differentiate to functioning milk-producing structures during pregnancy which are refractory to carcinogenesis (Russo et al., 1994, 1997, 2001). It is thought that pregnancies and FFTP in particular, consecutively decrease the pool of vulnerable breast cells (Russo et al., 1994, 1997, 2001). The undifferentiated cells found in the breast of nulliparous women never undergoes through the process of differentiation, retaining a high concentration of epithelial cells that are targets for carcinogens and are therefore susceptible to undergo neoplastic transformation (Boulanger et al., 2005; Henry et al., 2004; Wagner et al., 2002). Breast feeding also reduces BC risk and is thought to exert its effects through hormonal mechanisms (Huo et al., 2008; Lord et al., 2008; Zeng et al., 2010).

2.1.5.1.3 Post-menopausal hormone therapy (PHT): This therapy also known as hormone replacement therapy (HRT) and menopausal hormone therapy (MHT), is used by many older women to relieve the symptoms of menopause and to help prevent osteoporosis (thinning of the bones). The long-term (more than five years) use of postmenopausal estrogen therapy (ERT) or combined estrogen/progestin hormone replacement therapy (HRT) may be associated with an increase in breast cancer risk (Porch et al., 2002; Beral, 2004).

2.1.5.1.4 Abortion/miscarriage history: Some studies have reported an increased risk of breast cancer among women who have had induced abortions (Newcomb et al., 1996; Zeng et al., 2010). In incomplete pregnancy, the breast is exposed only to the high estrogen levels of early pregnancy and thus may be responsible for the increased risk seen in these women. However, some other studies have found no association between abortions and increased risk of breast cancer (Erlandsson et al., 2003)**.**

2.1.6.1 ENVIRONMENTAL AND LIFE STYLE FACTORS

2.1.6.1.1 Environmental pollutants: Certain compounds in the environment have been found to be associated with increased breast cancer risk (Cohn et al., 2007; Engel et al., 2005; Romieu et al., 2000; Teitelbaum et al., 2007). For example, substances found in some plastics, certain cosmetics and personal care products, pesticides, and PCBs (polychlorinated biphenyls) seem to have such properties.

2.1.6.1.2 Life-style: Studies have consistently shown that the risk of breast cancer is lower among physically active premenopausal women than among sedentary women (Bernstein et al., 1994; Friedenreich et al., 2001). Physical activity during adolescence may be especially protective, and the effect of physical activity may be strongest among women who have at least one full-term pregnancy.

2.1.6.1.3 Exercise: A woman's exposure to estrogen is lowered by exercise, which affects the menstrual cycle and can inhibit ovulation. Research suggests that the less a woman ovulates (that is, the fewer ovulation cycles she has), the lower her risk of breast cancer. So exercise - with its apparent ability to affect estrogen and, likewise, ovulation - may indirectly lower the risk of breast cancer (Bray et al., 2004).

2.1.6.1.4 Alcohol Use and smoking: The risk of breast cancer is increased among women who consume alcohol or smoke (Atkinson, 2003; Chen et al., 2002; Hamajima et al., 2002; Palmer & Rosenberg, 1993). Studies have shown that consumption of more than two drinks a day leads to an increased level of estrogen in the blood leading to increased risk of breast cancer.

2.1.6.1.5 Dietary fat: There are conflicting results concerning the relationship between dietary fat and breast cancer. Many U.S. studies have found no association between the two; however, international findings suggest that breast cancer rates are minimal in countries where the standard diet is low in fat (Schulz et al., 2008).

2.1.6.1.6 Radiation Exposure: A significantly increased risk of breast cancer has been found in women who have received radiation therapy in the chest area during childhood or young adulthood. This association has been observed both among atomic bomb survivors and among women who received high-dose radiation for medical purposes (Dershaw et al., 1992; Mattsson et al., 2002).

2.1.6.1.7 Body fat: Obesity has been consistently associated with an increased risk of breast cancer among postmenopausal women (Brown & Allen, 2002; Calle et al., 2003; Hirose et al., 2001). This relationship may be mediated again by estrogen production. Fat cells produce some estrogen and obese postmenopausal women, therefore, tend to have higher blood estrogen levels than lean women.

2.1.6.1.8 Race and ethnicity: Breast cancer is diagnosed more often in white women than Latina, Asian, or African American women (Frost et al., 1996; Joslyn et al., 2000).

2.1.6.2 GENETIC FACTORS

2.1.6.2.1 Gender: Simply being a woman is the main risk factor for developing breast cancer (Kelsey & Gammon, 1990). The reason women develop breast cancer more is not merely because of more breast cells than men, but lies in the fact that their cells are constantly exposed to the growth-promoting effects of the female hormones estrogen and progesterone. Although men do develop breast cancer, the disease is about 100 times more common among women than men (Wu et al., 2002).

2.1.6.2.2 Age: The chance of getting breast cancer goes up as a woman gets older (Edwards et al., 2002; Ries et al., 2000; Wu et al., 2002). About 1 out of 8 invasive breast cancers are found in women younger than 45, while about 2 out of 3 invasive breast cancers are found in women at an age 55 or older.

2.1.6.2.3 Breast cancer: A woman who has breast cancer in one breast has a 3- to 4-fold increased risk of developing a new cancer in the other breast or in another part of the same breast. (Hartmann et al., 2005).

2.1.6.2.4 Family history: A woman's risk of developing breast cancer is higher if her mother, sister, or daughter had breast cancer. The risk is also higher if one of her family member got the disease before age 40 (Claus et al., 2003). Having other relatives with breast cancer (in either her mother's or father's family) may also increase a woman's risk to the disease.

2.1.6.2.5 Genetic Alterations: Genetic susceptibility to cancer is triggered in several ways; the best understood causal mechanism being due to inactivating germline mutations in tumor suppressors and/or oncogenes (Levine, 1998; Cooper, 1990). The genetic events affecting oncogenes often result in increased stimulatory function, whereas those affecting tumor suppressor genes may cause loss of inhibitory function.

Mutations targeting Oncogenes result in the hyper-activation of these genes in cancer cells, giving those cells new properties, such as hyperactive growth and division, recalcitrance against programmed cell death, loss of respect for normal tissue boundaries and the ability to become established in diverse tissue environments (Bishop, 1989; Varmus, 1989). This can be achieved by a number of simple molecular mechanisms, including point mutations that constitutively activate an enzyme, deletions that remove negative regulatory regions from proteins (Bishop, 1991), or increased expression resulting from promoter deregulation or from multiplication of the number of copies of the gene by a mechanism called amplification (Savelyeva & Schwab, 2001). Activation of an oncogene is a dominant mechanism, since alteration of a single allele is sufficient to confer a gain of function for onset of cancer progression.

Mutations targeting Tumor suppressor genes (TSGs) results in the loss of normal functions essential for the maintenance of normal proliferation, accurate DNA replication, control over the orientation and adhesion within tissues, and interaction with protective cells of the immune system. Loss of function of a tumor suppressor gene, is typically recessive (Fearon, 1998) and can happen due to gene inactivation mechanisms like mutations, methylation or loss of alleles (most often through the loss of entire chromosomal sections encompassing several dozen genes), small deletions or insertions that scramble the reading frame of the gene, transcriptional silencing by alteration of the promoter region, or point mutations that change the nature of the residues that are crucial for the activity of the corresponding protein. TSGs are classified into two major groups; first group genes are nicknamed “gatekeepers” which are negative regulators of the cell-cycle,

acting as brakes to control cell division. The genes of the second group are called "care-takers" as their primary aim is not to control the speed or timing of cell-division but rather its accuracy. Care-taker genes are usually involved in the DNA repair and in controlling genomic instability. Their inactivation does not enhance cell proliferation per se but primes the cell for rapid acquisition of further genetic changes (Kinzler & Vogelstein, 1997).

The combined activation of oncogenes and inactivation of tumor suppressor genes drives the progression of cancer. The most evident biological consequences of these alterations are autonomous cell proliferation, increased ability to acquire genetic alterations, due to dysregulated DNA repair, ability to grow in adverse conditions due to decreased apoptosis, capacity to invade tissues locally and to form distant metastasis, and ability to activate the formation of new blood vessels (Bishop, 1989; Varmus, 1989). None alone is sufficient in itself, but cancer arises when these five biological phenomena interact together into a chain of coordinated events that profoundly modifies the normal cellular pattern of growth and development.

Genetic susceptibility as a result of highly penetrant germ line inactivation in cancer predisposing genes have a risk of approximately 5-10% for breast cancers, 10% for ovarian cancer and 25% for the early onset of breast cancer (Palma et al., 2006).The dissection of the genetics of BC gained momentum more than 10 years ago with the identification of the two main BC associated tumor suppressor genes BRCA1 and BRCA2. (Claus et al., 1996; Hall et al., 1990; Miki et al., 1994; Wooster et al., 1995). In addition, many other genes have been found to associate with BC, including p53, ATM, CHEK2, MDM2, PTEN, the GADD repair group, the HER2/neu oncogene, etc (Campeau et al., 2008; Lavin, 1998; Nechushtan et al., 2009; Walsh et al., 2006). However, mutations in these genes account for only a minor fraction of breast cancers. Some of these genes directly influence BC risk, whereas others are involved in the general processes of cancer growth and metastasis.

2.1.6.2.5 BRCA2

BRCA2 is a well established BC susceptibility gene, mutated form of which when inherited strongly predispose to breast cancers (Welcsh & King, 2001). Germ-line mutations of BRCA2 are predicted to account for approximately 35% of families with multiple cases, early onset female breast cancer, and they are also associated with an increased risk of male breast cancer and ovarian cancer (Gayther et al., 1997; Thorlacius et al., 1996). The candidate tumor suppressor gene BRCA2 was discovered in 1994 by Professor Michael Stratton and Dr Richard Wooster (Institute of Cancer Research, UK) and was localized on long (q) arm of chromosome 13 at position 12.3 by linkage analysis and cloned in 1995 (Wooster et al., 1994, 1995). More precisely, the BRCA2 gene is located from base pair 31,787,616 to base pair 31,871,804 on chromosome 13. The BRCA2 gene spans approximately 70kb in the genome and is composed of 27 exons. The BRCA2 cDNA consists of 11,385 bp and encodes a protein of 3,418 amino acids (385kDa molecular weight). Unlike most human genes, the coding sequence is AT rich (>60%).

The protein encoded by this gene is involved in the repair of chromosomal damage with an important role in the error-free repair of DNA double strand breaks as well as transcriptional regulation (Scully & Livingston, 2000; Zheng, 2000). In normal cells, BRCA2 help ensure the stability of the cell's genetic material (DNA) and help prevent uncontrolled cell growth. The BRCA2 gene acts as a classical tumor suppressors and can be rendered nonfunctional in cancer cells as a result of germline mutations in any one of the second allele (the Knudson two hit hypothesis).

The BRCA2 is essential for homologous DNA recombination (HDR), a process for the error-free repair of DNA double-stranded breaks (DSBs), in the cells of higher eukaryotes (Kojic et al., 2002; Moynahan et al., 2001). BRCA2 orthologues exist in most eukarya, with the notable exception of yeast (Lo et al.,

2003). These breaks can be caused by natural and medical radiation or other environmental exposures, and also occur when chromosomes exchange genetic material in preparation for cell division. Loss of these controls following BRCA2 inactivation may be a key event leading to genomic instability and tumorigenesis.

The BRCA2 protein is known to interact with major proteins involved in DNA repair by homologous recombination and, in particular the RAD51 protein (recombinase) (Chen et al., 1998). RAD51 family members are homologous to the bacterial RecA and yeast Rad51 (Yang et al., 2005). The protein is highly conserved in most eukaryotes, from yeast to humans (Davies et al., 2001; Pellegrini et al., 2002; Yuan et al., 1999). BRCA2 is shown to regulate both the intracellular localization and DNA-binding ability of this protein (Sharan et al., 1997; Yuan et al., 1999). RAD51 is indispensable in catalyzing the primary reaction required for HR, and its association with BRCA2 is required to facilitate this effect (Davies et al., 2001). The primary reaction begins when broken DNA is end-resected to generate ssDNA, upon which RAD51 oligomerizes to form an active nucleoprotein filament and this multimerization is controlled by its interaction with BRCA2 in vertebrates. The RAD51 nucleoprotein filament catalyzes strand exchange by invading and pairing with a homologous DNA duplex, used as a template for repair. Several functions have been ascribed to the BRCA2-RAD51 interaction, both in vivo and in vitro, in regulating these events. They include the sequestration of RAD51 in undamaged cells and its mobilization after damage (Yu et al., 2003), targeting of RAD51 to DSBs (Yuan et al., 1999), control of RAD51 oligomerization (Davies et al., 2001; Pellegrini et al., 2002) and modulation of sequential DNA substrate selection by RAD51, a process that orders the strand exchange reaction itself (Carreira et al., 2009; Shivji et al., 2009).

BRCA2 utilizes two motifs with apparently distinct properties to bind RAD51. A conserved region in the exon11 encodes short sequence of eight 35-peptide motifs called BRC repeats which are conserved in both their sequence and spacing. Each of the eight BRC repeats in human BRCA2, despite only

subtle sequence variation, exhibits a varying affinity for RAD51 (Chen etal., 1998; 1999; Wong et al., 1997). BRC3 and BRC4 have the strongest interaction with RAD51 (Davies & Pellegrini, 2007; Esashi et al., 2007; Saeki et al., 2006). BRC repeats, in isolation, or in the context of BRCA2 , bind RAD51 complexes on DNA (Galkin et al., 2005; Shivji et al., 2006) and differentially regulate RAD51 assembly on ssDNA versus dsDNA, promoting loading onto ssDNA, whilst concurrently impeding loading onto dsDNA, thereby stimulating strand exchange (Carreira et al., 2009; Shivji et al., 2009). A second region at the extreme carboxyl (C) terminus binds RAD51 (Mizuta et al., 1997; Sharan et al., 1997) in a manner regulated by the phosphorylation of a key serine residue (Ser3291 in human BRCA2) by cyclin-dependent kinase (CDKs) (Esashi et al., 2005) and is shown to stabilize RAD51 oligmeric assemblies in vitro both on and off DNA. Recent cellular studies suggest that the C-terminal RAD51-binding region is dispensable for the execution of HDR in vivo, and instead, coordinates the termination of HDR with entry into mitosis (Ayoub et al., 2009). Thus, the binding of BRC repeats of BRCA2 to RAD51 may be the primary mode of interaction between the two proteins relevant to HDR.

Cells that are defective for BRCA2 are hypersensitive to agents that crosslink DNA strands or that produce breaks in double-stranded DNA, such as cisplatin and mitomycin C as RAD51 foci do not form after DNA damage (Moynahan et al., 2001; Yuan et al., 1999). In these cells, double-strand breaks are repaired by an error-prone mechanism such as non-homologous end joining leading to chromosomal rearrangements (Patel et al., 1998; Yu et al., 2000). The resulting chromosomal instability is a crucial feature of carcinogenesis. The levels of expression of BRCA1, BRCA2 and RAD51 increase in cells when they enter S phase, indicating that they function during or after DNA replication. So, BRCA1 and BRCA2 function in a common pathway that is responsible for the integrity of the genome and the maintenance of chromosomal stability (Venkitaraman, 2002). Studies have shown that exogenous BRCA2 expression in cancer cells inhibits p53's transcriptional activity, and RAD51 co-expression

enhances inhibitory effects of BRCA2 (Marmorstein et al., 1998). Studies have also indicated that RAD51 physically associates with the p53 tumor suppressor protein (Buchhop et al., 1997).

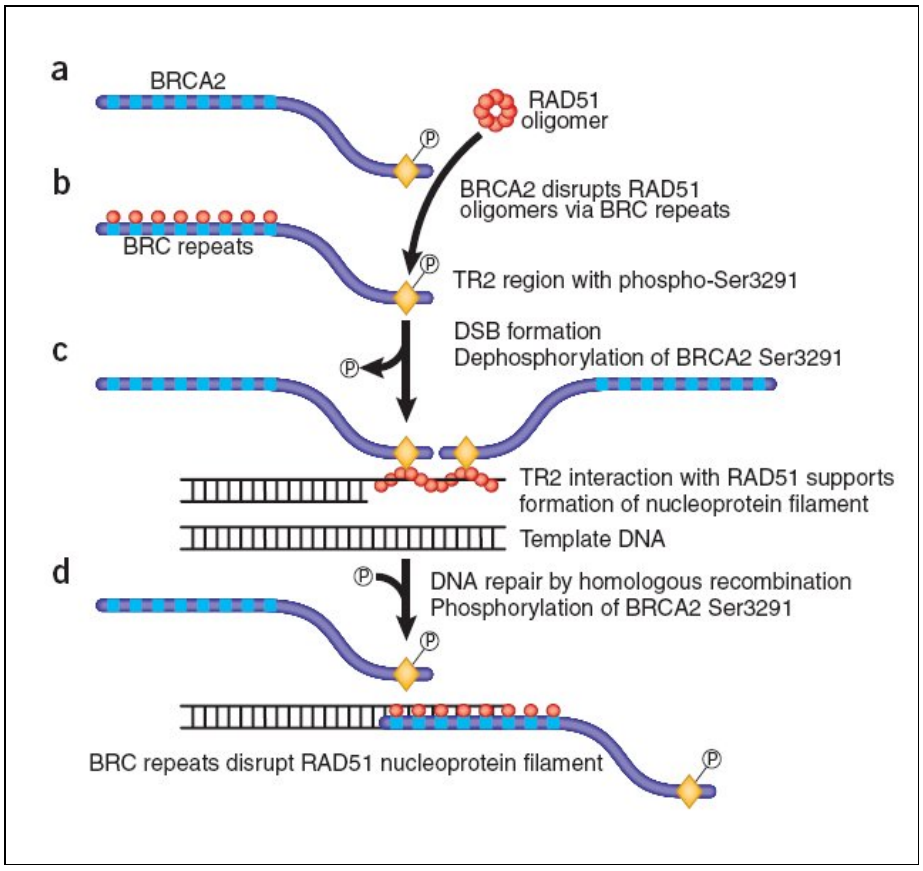


Fig. 2.1: The BRCA2-RAD51 interaction in DSB repair (Lord & Ashworth, 2007).

These findings demonstrate that BRCA2 physically and functionally interacts with two key components of cell cycle control and DNA repair pathways and therefore participates with p53 and RAD51 in maintaining genome integrity (Marmorstein et al., 1998).

Attempts to discover other proteins capable of interacting with BRCA2 led to the identification of DSS1 (deleted in split-hand/split-foot syndrome 1), a highly acidic 70 residue polypeptide. The DNA repair activity of BRCA2 is regulated by DSS1 that seems to function as a necessary cofactor (Kojic et al., 2003). DSS1 binds to the carboxy- terminal region of BRCA2 (distal to the BRC region) which also includes the NLS (Marston et al., 1999). BRCA2 localization to nuclear foci requires its association with the partner and localizer of BRCA2 (PALB2), mutations in which are associated with cancer predisposition (Xia et al., 2006). PALB2 supports BRCA2 stability and determines its localization in the nucleus after DNA damage (Xia et al., 2006). Relocation of PALB2 and BRCA2 to damaged chromatin is regulated by BRCA1 protein (Zhang et al., 2009). These three proteins form a complex in which PALB2 acts as a bridge between BRCA1 and BRCA2 (Zhang et al., 2009). In cells depleted of PALB2, DNA repair pathway dependent on the BRCA1/2 is disrupted (Xia et al., 2006; Zhang et al., 2009). Deletion mapping has localized the PALB2 – interacting region of BRCA to residues 10-40 at the N-terminus of the protein (Xia et al., 2006). The discovery of PALB2 as a crucial factor in localizing BRCA2 to sites of DNA damage provides the main role of amino terminal region of BRCA2 is to provide a binding site for PALB2 (Oliver et al., 2009).

BRCA2 possesses a putative transcriptional activation domain in exon 3, suggesting a role of BRCA2 in the regulation of gene expression and chromatin remodelling (Milner et al., 1997). Its involvement in transcriptional activation (Fuks et al., 1998; Milner et al., 1997), is reinforced by the discovery of EMSY, a protein able to interact with chromatin-regulatory proteins, as a binding partner for the BRCA2 (exon3) N-terminus (Hughes-Davies et al., 2003). When EMSY protein is overexpressed, it inhibits transcriptional activation of BRCA2. The PALB2–binding site on BRCA2 directly overlaps that of EMSY (Hughes-Davies et al., 2003). Tumors with EMSY amplifications show chromosomal instability and mitomycin-C sensitivity characteristic of cells defective in BRCA2 or PALB2 (Raouf et al., 2005; Turner et al., 2004; Xia et al., 2006). BRCA2 also

regulates the activity of other genes and plays a critical role in embryo development. BRCA2 has been shown to interact with BRE (Dong et al., 2003), Filamin (Yuan and Shen, 2001), Replication protein A1 (Wong et al., 2003), BRCC3 (Dong et al., 2003), BARD1 (Dong et al., 2003; Ryser et al., 2009), FANC G (Hussain et al., 2003), and BCCIP (Liu et al., 2001).

Germline mutations in the BRCA2 gene are associated with an increased susceptibility to breast cancer (in both males and females) and they also confer an increased risk of early onset ovarian, prostate, and pancreatic cancer. Mutations are distributed throughout the entire coding region of BRCA2 and to date numerous deleterious mutations have been reported. BRCA2 mutations are usually insertions or deletions of a small number of DNA base pairs in the gene or are nonsense mutations that lead to the introduction of a stop codon (Ford et al., 1998). However 38% of BRCA2 mutations are missense mutations that alter one amino acid, but do not truncate the protein and are of uncertain significance. These mutations invariably generate a shortened (truncated) and non-functional BRCA2 protein. As a result of these mutations, the protein product of the BRCA2 gene is abnormal and does not function properly.

Wooster et al., were first to identify 6 different germ line mutations in the BRCA2 gene in families with BC, each causing serious disruption to the open reading frame of the transcriptional unit (Xia et al., 2001). Since then, over 300 distinct mutations in BRCA2 have been described. The spectrum of BRCA2 mutations has been characterized in different populations worldwide, with significant variation of the relative contribution of these genes to hereditary cancer between populations. Various population-based studies have shown population specific BRCA2 founder mutations and also variable number of novel mutations in different populations, and thus have defined high and low risk subsets for developing breast cancer based on ethnic origin (Oddoux et al., 1996; Thorlacius et al., 1997). Uniquely, amongst Jewish Ashkenazi individuals, one founder mutation 6174delT in BRCA2 account for the majority of cases in high-risk families (Oddoux et al., 1996). Founder mutations have also been identified

in Icelandic and Polish populations. The spectrum of mutations also varies between populations, with some showing a high frequency of unique mutations, for example in Italy (Santarosa et al., 1998, 1999). Population specific mutations have also been described in the Netherlands (Peelen et al., 1997), Sweden (Hakansson et al., 1997), France (Stoppa et al., 1997), Spain (Diez et al., 2003) and other countries (Szabo and King, 1997).

Population or subgroup	BRCA2 mutation(s)
Ashkenazi Jewish	6174delT (Oddoux et al., 1996)
Dutch	5579insA (Verhoog et al., 2001)
Finns	T8555G, 999del5 (Huusko et al., 1998)
Finns	8765delAG (Tonin et al., 1999)
Germans	C61G (Backe et al., 1999)
Hungarians	9326insA (Van Der Looij et al., 2000)
Icelandics	999del5 (Thorlacius et al., 1996; Thorlacius et al., 1997)
Italians	8765delAG (Pisano et al., 2000)
Pakistanis	3337C>T (Liede et al., 2002)

Table 2.2: Different examples of founder mutations in the BRCA2 gene.

In addition, limited data indicate that the frequencies of specific BRCA2 mutations may vary among individual racial and ethnic groups in the United States, including African Americans, Hispanics, Asian Americans, and non-Hispanic whites (John et al., 2007; Malone et al., 2006; Vogel et al., 2007).

The Fanconi – anemia: FA is a rare recessive disease of childhood that features skeletal abnormalities, abnormal skin pigmentation, short stature and micropthalmia (Grompe & Andrea, 2001; Joenje & Patel, 2001). People who have two mutated copies of the BRCA2 gene have FA (Howlett et al., 2002). This condition is caused by extremely reduced levels of the BRCA2 protein in cells, which allows the accumulation of damaged DNA. Patients with FA are prone to several types of leukemia; solid tumors, particularly of the head, neck, skin, and reproductive organs; and bone marrow suppression (reduced blood cell production that leads to anemia).

Breast cancer incidence rate varies atleast tenfold worldwide (Parkin et al., 2001) largely because of range of socio-economic, reproductive, hormonal, nutritional and genetic factors (Mcpherson et al., 2000). The high-incidence areas include North America and European countries (Parkin et al., 2001). South-east Asia contributes over 1/5th of the worldwide prevalence of breast cancer with Pakistan having highest incidence among Asian population after Israel (Bhurgri et al., 2000). In India, although overall incidence of BC in Indian population is low compared to Western populations (ASR of 23.5 vs. 90.7), the incidence of early onset disease (<40yrs) does not show significant geographic variation (ASR range worldwide of 12-33), suggesting that in the Indian population a greater proportion of BC is due to early onset disease compared to Western populations (Parkin et al., 1997). According to the National Cancer Registry Project (NCRP) based on cancer registries at six hospitals, the average age of patients was found to range from 44.2 yrs (Dibrugarh) to 49.6 yrs (Bangalore and Chennai) (Jussawalla and Jain, 1997). Kashmir, which lies at an altitude of 1800-2400 km from the sea-level and borders the low-incidence country, India and the high-incidence area of Pakistan, is distinct from other areas in term of its unique geographical locale, intra-community marriages, tradition, culture, food habits and ethnicity. Over the past few years, the valley has witnessed an increase in incidence and occurrence of breast cancer in its unexplored ethnic population. In Kashmiri women, breast cancer is the second most common cancer after uterine

cervix. The age adjusted rate as per our hospital records is approximately 12.6 per 105 individuals per year with male to female ratio 0.02:1 (personal communication). The average age at onset of disease is lower (than western and other parts of India) and familial and breast cancer cases are also prevalent in the valley.

Despite the large number of BRCA2 gene mutations reported worldwide, only few reports on the prevalence of BRCA2 mutation in India are available. Valarmathi et al., (2003) has reported three deleterious BRCA2 mutations including three frame shift mutations (c.5227dupT, c.5242dupT, c.6180dupA) and four novel missense variants in BRCA2. On analyzing a group of patients with a family history of breast and/or ovarian cancer and early onset BC, Raj Kumar et al., (2003) has reported one non-sense mutation in BRCA2 gene. In 2006, Saxena et al., (2002) have reported three pathogenic protein-truncating mutations unique to Indian women (6376insAA in exon 11, 8576insC in exon 19, and 9999delA in exon 27) along with one missense mutation (A2951T), and one silent substitution (1593A>G) in BRCA2 in a cohort of 204 Indian BC patients. Mutation analysis of BRCA2 gene in 102 breast/ovarian cancer patients from 96 different breast ovarian cancer families has identified 16 distinct germ line alterations in 27 individuals (26.47%) of which 11 were (69%) in exon 11. It was Saxena et al., (2006), who first attempted to analyze BRCA2 gene in North India and reported sequence variant in exon 18 (8345A>G) of BRCA2 resulting in a conservative substitution of asparagine to serine substitution at codon 2706 (N2706S) and these missense variants likely represent population-specific polymorphisms. There is a further need of screening large number of samples from Indian population to investigate the role of BRCA2 gene mutations in the high-risk group of familial as well as early onset cases, which forms the largest group of breast cancer patients in the Indian population. Conversely, the impact of BRCA2 mutations on prognosis and survival of breast cancer patients is still debated in Indian population as compared to other populations where its use in screening of people which are at high of breast cancer has already started.

2.1.6.2.6 CONNEXIN 43

The formation of multi-cellular organisms necessitated the development of different forms of cell-cell communication to coordinate the activity of different groups of cells. Specialized but distinct structures have evolved independently to provide direct cell-to-cell communication in plants (plasmodesmata - Meiners et al., (1988)), fungi (septal pores – Belozerskaya, 1998) and animals (gap junctions - Finbow et al., 1983). In animals, the maintenance of this homeostatic balance is controlled by the global interplay between three major communicative networks, namely extra-, intra- and intercellular mechanisms. Direct intercellular communication is mainly mediated by gap-junctions. Gap- junctions are intercellular channels that typically gather in groups of 10-10,000 so called plaques, at the membrane surface. They arise from the interaction of two opposing hemi-channels or connexons, one contributed by each communicating cells and the connexon is in turn composed of six connexin proteins (Bruzzone et al., 1996; White & Paul., 1999). In humans, more than twenty connexin species have yet been characterized. Connexin proteins either assemble into a hemichannel with either identical connexin subunits (homomeric) or different connexins (heteromeric) although only certain combinations are allowed (Falk et al., 1997; Segretain & Falk, 2004).

Historically, these structures have been described as a particular group of cell junctions being specialized in the control of direct exchange of essential cellular metabolites like secondary messengers, ions and other small molecules of <1,000 Da between adjacent cells (Flagg-Newton et al. 1997; Loewenstein, 1981; Loewenstein & Kanno, 1966). However studies have shown now that gap junctions play a crucial role in various physiologic functions such as regulation of cell proliferation, cell differentiation, cell apoptosis and tissue development (El Sabban et al., 2003; Gramsch et al., 2001; Ma & Dahl, 2006; Vinken et al., 2006; Wiszniewski et al., 2000). Down regulation of intercellular communication via gap junctions, either by down regulation or mistargeting of connexins (due to

post-translational processing, germline or somatic mutations), appears to play a role in many types of pathologies, including cancer (Laird et al., 2006; Mesnil et al., 2005; Severs et al., 2004).

Cx43 is the most widely expressed connexin being present in at least 34 tissues and 46 cell types (Laird, 2006), and it is the predominant connexin expressed in most cell lines. It is known as the "heart gap junction protein" since it was first identified in heart muscle tissue (Beyer et al., 1987). The human Cx43 gene is located at position 6q22.3 while there is also Cx43 processed pseudogene located on chromosome 5, which has got all the features of an expressed gene (Fishman et al., 1991). Cx43 has a common gene structure, consisting of two exons separated by one intron sequence. The intron, which is of variable length, is situated within the 5'-untranslated region, while the second exon contains the connexin coding sequence, and the 3'-untranslated region (Willecke et al., 2002). The Cx43 gene sequence remains relatively conserved among species, indicating its presence is very important for survival, as shown by Cx43 knockout mice dying early in development because of heart malformations (Reaume et al., 1995). The C-terminal region of the Cx43 contains most of the regulatory and protein-protein interaction domains. Mice that are engineered to lack the C-terminus of Cx43 die shortly after birth (Maass et al., 2004). The C terminus of Cx43 protein which is approximately 150 amino-acids long, contains multiple serine/ tyrosine residues that are phosphorylation targets for various kinases including protein kinase A (PKA) (Yogo et al., 2006), protein kinase C (PKC) (Solan & Lampe, 2008) p34cdc2/cyclin B kinase (p34cdc2) (Kanemitsu et al., 1998), casein kinase 1 (CK1) (Cooper & Lampe, 2002), mitogen-activated protein kinase (MAPK) (Cameron et al., 2003; Norris et al., 2008), and p60src kinase (src) (Loo et al., 1995). Phosphorylation of these sites is responsible for the regulation of GJ channel gating as well as GJ trafficking and degradation. Cx43 does not contain serine residues in its cytoplasmic loop, and neither there are any reports of phosphorylation of the N-terminal domain of connexin43. The C-terminal of Cx43 serves as an interactive platform for a variety of cellular

proteins which are participants in the field of growth control; their interaction (and regulation of the interaction) with Cx43, or other connexins anchored to the plasma membrane may affect their subcellular localization and thus their sites of action.

Initially all effects of connexin were attributed to direct cell to cell diffusion. However some of the connexin functions seem to occur unrelated to channel function. Cx43 has been shown to inhibit cell proliferation when GJC was blocked by pharmacological inhibitors, or channel formation is prevented (Moorby & Patel, 2001). In addition, Cx43 point-mutants that did block GJC still reduced cell growth, and expression of the C-terminal tail alone gave similar results. Studies have shown that Cx43 over-expression can inhibit cell proliferation, accompanied by the decreased stability of Skp2, a protein involved in cell cycle regulation. (Zhang et al., 2003). Studies have also shown that the non-channel forming carboxy-terminal domain of Cx43 decreases cell proliferation as efficiently as the full length Cx43. The binding sites through which different proteins interact with the gap junction lie in the connexin molecule's cytoplasmic carboxy terminal (CT) domain (Giepmans, 2004; Hunter et al., 2005). This domain is well known to house a series of functionally important sites, notably those involved in channel gating and those for phosphorylation and thus consists a main and important part of Cx43.

Loss of Cx43 has been shown to correlate with tumorigenesis (Yamasaki et al., 1995), and its upregulation has been shown to restore normal phenotypes in vitro and reduce tumor growth in vivo (Huang et al., 1998; Rose et al., 1993). Cx43 expression is known to be reduced in most cancer cell types, like epithelial, endothelial, mesothelial, neuroblastoma, breast and in many other cell types (Holder et al., 1993; Goodenough et al., 1996; Krutovskikh et al., 1994; Trosko et al., 2003; Yamasaki et al., 1995). In most cases, the loss of Cx43 mediated GJIC is due to a reduction in both protein and RNA levels. However, Cx43 trafficking to the cell membrane can also be ineffective, leading to the accumulation of cytoplasmic Cx43 that never forms GJs (Krutovskikh et al., 2000). Moreover,

other studies have suggested that the rate of Cx43 degradation can also determine the overall intracellular Cx43 level (Musil et al., 2000). The frequent impairment of Cx43 in early transformation (Yamasaki et al., 1995) suggests its aberrant expression is involved in the process of carcinogenesis and its expression is disadvantageous to cancer cell survival. It has long been shown that Cx43 over-expression leads to the reversal of the differentiation phenotype to that usually seen in normal cells and inhibition of neoplastic potential (Chen et al., 1995; King et al., 2000; Koffler et al., 2000).

Connexin 43 is the predominant gap-junction protein expressed in normal breast tissue and plays important role in normal mammogenesis, lactogenesis and involution (Monaghan et al., 1996). Cx43 is not expressed in normal breast stem cells but is expressed in the normal breast epithelial cells derived from these breast stem cells (Chang et al., 2001; Nomata et al., 1996; Trosko et al., 2000). Studies have shown that Cx43 is down regulated at the mRNA and protein level in human breast tumors and several human mammary tumor cell lines (Lee et al., 1992). Decreased expression of connexin gap junctions is seen in breast cancer at various stages of progression and restoration of gap-junction intercellular communication by up-regulation of connexins has been shown to restore normal phenotypes in vitro and reduce tumor growth in vivo (Hirschi et al., 1996; Laird et al., 1999). Various studies have shown that down-regulation of Cx43 plays role in primary tumor formation as well as its metastasis in breast tissue.

Primary breast cancer is generally comprised of tumor cells and surrounding connective tissue. This arrangement creates multiple cell-cell interactions among tumor cells and between tumor cells and normal neighboring stromal cells. Among various patterns of cell-cell interactions, GJIC involving Cx43 is considered among the earliest alterations associated with malignant cell transformation. Studies have shown down-regulation of connexin 43 gap-junction in human breast cancer tissues compared with the non-neoplastic breast tissue surrounding primary tissue and re-expression of Cx43 reverses the malignancy of human mammary carcinoma cells (Laird et al., 1999; Naus et al., 1992;

Zhongyong et al., 2008). Furthermore, data obtained with rat mammary carcinoma induced by DMBA also demonstrates that the loss of connexin 43-gap junction is a common feature of mammary neoplastic transformed. Reexpression of Cx43 suppressed the cancer phenotype, a regained ability to differentiate into three-dimensional (3D) structures in the presence of a basement membrane in human mammary carcinoma cells, MDA-MB-435 and reduction of tumor-growth in animal models (Qin et al., 2002; Zhongyong, 2008). Studies have also shown that down-regulation of endogenous Connexin 43 expression by small interfering RNA promoted a more aggressive phenotype in human breast cancer cell lines (Shao et al., 2005). In this study, it was seen Cx43 protein reduced fibroblastic growth factor receptor (FGFR) expression and possibly affected the expression of other proteins involved in tumor progression. Importantly, not only has the over-expression of Cx43 or Cx26 been found to restore growth control in human breast tumor cells, but connexin-expressing tumor cells partially revert to a less malignant phenotype (Hirschi et al., 1996).

Recent studies have also shown Cx43 upregulation by the drugs genistein and quercetin leads to GJIC-independent inhibition of cell proliferation (Conklin et al., 2007). Cx43 plays role in tumorigenesis probably by inhibiting angiogenesis independently of cell communication as when Cx43 expression was inhibited by RNAi in breast cancer Hs578t cells, resulting in faster growth and increased aggressiveness of the cells, TSP-1 expression was reduced while expression of proangiogenic vascular endothelial growth factor was increased (Shao et al., 2005). A similar shift in the balance toward antiangiogenic factors was observed in MDA-MB-231 cells overexpressing Cx43, and conditioned media from these cells inhibited in vitro endothelial cell tubulogenesis and migration (McLachlan et al., 2006). Additionally, xenografts of Cx43-overexpressing MDA-MB-231 cells showed reduced tumor angiogenesis. Altogether these results suggest that Cx43 acts as a tumor suppressor by mechanisms related to cell proliferation, migration and angiogenesis, supporting

a causal relationship between physiologic changes in Cx43 levels and aggressive malignant breast tumor cell phenotypes.

Tumor metastasis is a multi-step process that involves the dissociation of tumor cells from the primary tumor followed by their entry into the circulation, extravasation from the vascular system and proliferation into tumor masses at secondary tissue sites. Both cell-cell and cell-matrix interactions are important regulators at different stages of this metastatic cascade (Cairns et al., 2003). Down-regulation of connexins has shown to promote metastasis as exogenous expression of the breast metastasis suppressor 1 gene in MDA-MB-435 cells led to upregulation of Cx43 and restoration of GJIC, providing evidence that connexins act as tumor suppressors in metastasis (Saunders et al., 2001). Functional in vitro studies have observed that Cx26 and Cx43 expression leads to a decrease in the cell's migratory potential and ability to invade through a basement membrane matrix (Momiyama et al., 2003) accompanied by a slight reduction in matrix metalloproteinase activity (Kalra et al., 2006). Additionally, studies have also shown that the number of metastases to lungs was reduced in mice injected with Cx43 expressing breast cancer cells relative to mice injected with vector controls (Zhongyang et al., 2008).

The movement of cancer cells across the endothelial cell barrier as they move in and out of the blood vessels has been shown to be a key step in metastasis and studies have shown that connexins play an important role in tumor cell vascular intravasation and extravasation (Zhou et al., 2008). In one study, GJIC was reduced in endothelial cells when they were co-cultured with breast cancer cells, presumably weakening cell-cell contacts and making it easier for the cells to cross the endothelial barrier during both intravasation and extravasation (Cai et al., 1998). Another study showed that overexpressing Cx43 in GJIC-deficient HBL100 breast cancer cells allowed them to form heterocellular junctions capable of dye transfer with cells from a human microvascular endothelial cell line that expresses Cx43 and this increased tumor cell diapedesis. This increase in transendothelial migration was blocked when the endothelial

culture was pretreated with GJIC inhibitors or when the breast cancer cells expressed a membrane localizing, nonfunctional chimeric mutant of Cx43 (Pollmann et al., 2005). This study implies that both homocellular GJIC in the endothelium and heterocellular GJIC between breast cancer cells and the endothelium facilitate transendothelial migration. A series of studies performed on the effects of metastatic breast cancer cells on osteoblast differentiation performed with MDA-MB-231 and MC3T3-E1 cells showed inhibition of osteoblast differentiation by conditioned medium from breast cancer cells (Mercer et al., 2004; Mercer & Mastro, 2005). Studies have demonstrated that Cx43 expression in MDA-MB-231 results in decreased expression of OB-cadherin (Li et al., 2008) and found a similar trend in Cx43 expressing MDA-MB-435 cells. A significant decrease in N-cadherin, a protein which is involved in increased motility, invasion and metastases of breast cancer cells (Hazan et al., 2000; Nieman et al., 1999) seen in Cx43 over expressing MDA-MB-231 cells (Zhongyong et al., 2008) clearly show that it contributes to decreased metastasis in vivo. The anti metastatic effect of Cx43 may be related to enhanced sensitivity to apoptosis as Cx43 enhances apoptosis in response to chemotherapeutic agents or low serum in human glioblastoma cells (Huang et al., 2001). Studies have also demonstrated that Cx43 expression is directly correlated with the expression of BAK (Bcl-2 homologous antagonist/killer), a pro-apoptotic gene of the Bcl-2 family, in human breast cancer tissue (Kanczuga-Koda et al., 2005). Cx43 suppressed the cancer phenotype in human mammary carcinoma cell, MDA-MB-435 and cell growth in culture and in animal models.

There remains little doubt that down regulation of Cx43 plays a very important role in the primary tumor formation and its metastasis in mammary glands. However the mechanism by which Cx43 down regulation occurs in breast cancer is far from clear. Several mechanisms like Cx43 promoter hyper-methylation or a cancer-specific reduction of Cx43 expression /trafficking by the modulation of various components of the Cx43 life cycle appear to be involved in

the down regulation of connexins in mammary glands, but irreversible mutational alterations have not yet been proved to be among them.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS*

Chemical Name

Absolute ethanol

Acetic Acid

Agarose

Ammonium acetate

Betaine

Bromophenol Blue

Bovine Serum Albumin (BSA)

Chloroform

Dimethyl sulfoxide (DMSO)

Dithiothritol (DTT)

Ethidium Bromide

Ethylene diamine tetra acetate (EDTA)

Isoamyl alcohol

Isopropanol

Nitric acid

Phenol

Pottassium bicarbonate

Sodium acetate

Sodium bisulphite ($Na_2S_2O_3$)

Sodium chloride

Sodium dodecyl sulphate

Sodium hydroxide

Silicon dioxide

Sodium Iodide

Tris Base

3.1.2 ENZYMES

Taq Polymerase

Proteinase K

3.1.3 MISCELLANEOUS MATERIALS

1kbp DNA Ladder

dNTP mix

**All the chemicals are of Standard Molecular Biology Grade.*

3.1.4 REAGENTS

3.1.4.1 Reagents for DNA Extraction

Nuclear Lysis Buffer (NLB)

Ammonium acetate (4M)

EDTA (0.5M)

Proteinase K (20mg/ml)

2M Tris – HCl

70% Ethanol

Isopropanol

NaCl (5M)

SDS (10%)

3.1.4.2 Reagents for Agarose Gel Electrophoresis

1.5% Agarose

1X Tris Acetate EDTA (TAE) buffer (pH 8)

Ethidium bromide

3.1.4.3 Reagents for DNA Amplification

dNTP mix (dATP, dTTP, dGTP, dCTP in equal molar concentrations)

Primers

Taq polymerase

Taq Buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 1.5mM $MgCl_2$) (pH 8.7)

3.1.4.4 Reagents for PCR product Purification

Low melting Agarose

Tris Acetate EDTA (TAE)

Sodium iodide solution (Sodium Iodide, sodium bisulphate)

Glass milk (Nitric acid treated Glass beads)

Wash buffer (Tris HCL pH 7.4 and Absolute Ethanol in 1:1 ratio)

3.1.4.5 Instruments

Centrifuge 5810R, Eppendorf, Germany

Table top centrifuge, Eppendorf, Germany

Deed freezer, LabTech Co. Ltd. Korea

Ice maker, LabTech Co. Ltd. Korea

Thermocycler, Applied Biosystems

Electrophoresis set, Wealter Corp, Taiwan.

Vortex-2 GENIE, Science Industries, Inc., USA

Ultraviolet Transilluminator, Bio Doc ITTM System, USA

Water bath, LabTech Co. Ltd. Korea.

Autoclave, Diahan Lab Tech Co. Ltd. Korea.

Measuring balance, Denver Instrument Company.

3.1.4.6 Computer software used

ChromasMFC Version 2.22, Technelysium Pvt. Ltd., USA and ClustalX version 2.

3.2 METHODOLOGY

3.2.1 PATIENTS AND SAMPLES

Patients presenting for treatment of Breast cancer for the first time at the Sheri-I-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir between Jan 2008 to June 2010 were recruited for the study with prior informed consent. Patients underwent fine needle aspiration cytology and histopathological examination to establish the clinical profile. Out of the total 50 breast cancer cases under study, blood samples and surgically resected breast tissue (which included tumor tissue, normal tissue, and lymph node/s wherever involved) was collected from 50 sporadic breast cancer cases. All samples were snap-frozen at –80°C until analysis. A questionnaire was used to collect the information on Clinico-epidemiological characteristics such as age, family story of disease, body mass, menopause status, site of tumor, marital status, provisional diagnosis, lymph node/s involved and clinical tumor stage of patients.

Clinico-Epidemiological Features	**Patient Details**												
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13
Age	35	48	42	51	38	54	42	65	70	52	37	38	65
Sex[1]	F	F	F	F	F	F	F	F	F	F	F	F	F
Rural/Urban[2]	R	R	U	U	R	R	R	R	R	R	R	R	R
Breast Involved[3]	R	L	R	L	L	R	L	L	R	R	L	L	L
Menopausal Status[4]	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Post	Post	Pre	Pre	Post
Provisional Diagnosis[5]	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC
Lymph Node Status[6]	Y	Y	Y	Y	Y	N	Y	Y	Y	N	Y	Y	N
Clinical Tumor Stage[7]	IV	IIa	IIb	IIb	IIa	IV	IIIa	IIIa	IIIb	IIIb	IIa	IIIb	IIIa

Clinico-Epidemiological Features	**Patient Details**												
	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26
Age	35	60	60	50	55	50	60	60	55	35	29	50	35
Sex[1]	F	F	F	F	F	F	F	F	F	F	F	F	
Rural/Urban[2]	R	U	U	R	U	R	R	R	R	R	R	R	U
Breast Involved[3]	R	L	R	L	L	R	L	L	L	L	L	L	R
Menopausal Status[4]	Pre	Post	Post	Post	Post	Post	Post	Post	Post	Pre	Pre	Post	Pre
Provisional Diagnosis[5]	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC
Lymph Node Status[6]	Y	N	N	Y	Y	Y	Y	N	Y	N	Y	Y	N
Clinical Tumor Stage[7]	IV	IIb	IIb	IIb	IIIb	IV	IIIa	IV	IIb	IIb	IIIb	IV	IIa

Clinico-Epidemiological Features	Patient Details												
	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	C37	C38	C39
Age	60	50	60	60	55	50	50	55	35	50	29	40	40
Sex[1]	F	F	F	F	F	F	F	F	F	F	F	F	F
Rural/Urban[2]	R	R	R	R	U	R	U	R	U	R	R	R	R
Breast Involved[3]	L	R	L	R	L	R	L	L	R	L	L	L	L
Menopausal Status[4]	Post	Post	Post	Post	Post	Post	Post	Post	Pre	Post	Pre	Pre	Pre
Provisional Diagnosis[5]	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC
Lymph Node Status[6]	Y	Y	N	N	Y	N	Y	Y	Y	Y	Y	Y	Y
Clinical Tumor Stage[7]	IIa	IIIa	IIIb	IIb	IIa	IV	IIIa	IIb	IIIb	IIb	IIIb	IV	IV

Clinico-Epidemiological Features	Patient Details										
	C40	C41	C42	C43	C44	C45	C46	C47	C48	C49	C50
Age	38	37	52	70	65	42	54	38	51	40	48
Sex[1]	F	F	F	F	F	F	F	F	F	F	F
Rural/Urban[2]	R	R	R	U	R	R	R	R	R	R	R
Breast Involved[3]	L	L	L	L	R	R	L	L	R	L	L
Menopausal Status[4]	Pre	Pre	Post	Post	Post	Pre	Post	Pre	Post	Pre	Post
Provisional Diagnosis[5]	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC	IDC
Lymph Node Status[6]	Y	Y	N	N	Y	Y	Y	Y	N	Y	Y
Clinical Tumor Stage[7]	IIa	IIa	IIb	IIIa	IIIa	IIb	IV	IIIa	IIIb	IV	IV

[1]Sex: M= male, F= female; [2]Rural/Urban: Rural= R, Urban= U; [3]Breast Involved: Right= R, Left= U; [4]Menopausal Status: Pre= Premenopausal, Post= Postmenopausal; [5]Provisional Diagnosis: IDC= Infiltrating Ductal Carcinoma, IBC= Inflammatory Breast Carcinoma, Pagt= Pagets disease; [6]Lymph Node Status: Y= Yes, N= No; [7]Clinical Tumor Stage: II(a,b)= When tumor size ranges from >2cm and >5cm and spreads to ipisilateral axillary nodes but no metastasis, III(a,b)= Tumor of nodes but no metastasis and IV: Tumor extends to chest wall, any number of nodes involved and metastasis.

Table 3.1: Clinico-epidemiological details of the study subjects.

3.2.2 DNA EXTRACTION FROM TISSUE

1. Breast tissue (0.1g) was taken in a sterile petriplate and was finely chopped using sterile surgical blade.
2. Nuclear Lysis Buffer solution (3ml) was added to it and the mixture was transferred to sterile propylene tube, 200µl of SDS (10%) and 40µl of proteinase K (20 mg/ml) was added.
3. Mixture was incubated overnight at 45ºC in water bath.
4. 4ml of ammonium acetate (4M) and 3ml of chloroform was added and mixed gently followed by centrifugation at 3000rpm for 30 minutes at 4ºC.
5. The supernatant obtained was transferred to a fresh sterile 50ml falcon tube and kept at -20ºC for 30 minutes. Isopropanol (5-10ml) was added along the sides of tube and mixed gently. White thread like precipitate of genomic DNA was seen.
6. Precipitated DNA was retrieved carefully and washed 2-3 times with 70% ethanol. The obtained DNA pellet was allowed to air dry at room temperature.
7. The air dried pellet was dissolved in autoclaved MQ water or Tris – HCl (pH=8) and incubated for 5 minutes at 65^{o}C, followed by storage at -20 ^{o}C.

3.2.3 QUANTIFICATION

The absorbance of the DNA samples was recorded at 260nm on a double beam spectrophotometer and the concentration was determined by the following equation.

DNA (µg/ml) = A260×50×dilution factor.

The purity of DNA was established by using A260/A280 ratio.

3.2.4 ANALYSIS OF DNA

The integrity of DNA isolated was assessed by agarose gel electrophoresis. Agarose gel was prepared by boiling agarose powder in 1X TAE buffer followed by cooling to 50-60°C. Ethidium bromide was added to a final concentration of 0.5µg/ml. The gel solution was mixed by gentle swirling and was poured into an electrophoresis plastic tray (with sealed edges) with comb inserted. The gel was kept at room temperature for approximately 20 min to set into semisolid form. 3µl of DNA sample was mixed with 1µl of loading dye and loaded in to gel slots along with molecular size marker in the last lane. Electrophoresis was carried out at 100 volts for 40 minutes. DNA band pattern was visualized and photographed. The samples showing bright, intact bands with no fragmentation or shearing and without any apparent contamination or streaking were chosen for further analysis.

3.2.5 AMPLIFICATION OF DIFFERENT EXONS OF BRCA2

PCR amplification using 10 set of primers (Table 3.2) was done to amplify exon 2, 9, 11.1, 11.2, 11.3, 11.4, 11.5, 18, 20 and 25 of BRCA2. PCR was carried out under conditions indicated for individual set of primers (Table 3.2). PCR assays were carried out in a reaction volume of 50µl containing 100–200 ng of genomic DNA,0.2mM dNTPs (Sigma), 1X PCR buffer (Sigma), 0.5U of Taq DNA Polymerase (Sigma) and 10 pmol of each primer (Sigma). The PCR protocol and programme are given in Table 3.4 and 3.5 respectively.

3µl of PCR product was analyzed on 1.5% agarose gel by electrophoresis. The concentration of amplified PCR products was approximated by comparison with the intensity of the corresponding bands in the 1Kb DNA ladder.

Amplicon	Primer Sequence (5' to 3')	Annealing Temperature	Product size (bp)
Exon 2	F CTC AGT CAC ATA ATA AGG AAT R ACA CTG TGA CGT ACT GGG TTT T	58	256
Exon 9	F CTA GTG ATT TTA AAC TAT AAT TTT G R GTT CAA CTA AAC AGA GGA CT	53	164
Exon 11.1	F ATT TAG TGA ATG TGA TTG ATG G R TGA TTC TTT GCC TCT AGA AA	55	521
Exon 11.2	F CAA AAG TGG AAT ACA GTG ATA C R TCT GTT TCA TGA AGT TCC TT	58	507
Exon 11.3	F TTC AAA AAT AAC TGT CAA TCC R TCT TTG AAG AAC ATT TTG CT	54	488
Exon 11.4	F ACA AAT GGG CAG GAC TCT TAG G R TCT GCA TTC CTC AGA AGT GG	61	508
Exon 11.5	F GAA TCA GGA AGT CAG TTT GA R TAT CAG TTG GCA TTT ATT ATT TTT	57	521
Exon 18	F GTG ACT TGT TTA AAC AGT GGA AR ATT GAG CAT CCT TAG TAA GCA	56	500
Exon 20	F AAG TGA ATA TTT TTA AGG CAG TT R TAT ATG GTA AGT TTC AAG AAT	53	296
Exon 25	F TTA GAG TTT CCT TTC TTG CAT C R AAG CTA TTT CCT TGA TAC TGG A	61	470

Table 3.2: Primers used for screening different exons of BRCA2.

3.2.6 AMPLIFICATION OF CONNEXIN 43

PCR primers were designed with mismatches to the pseudogene sequence to specifically amplify GJA1 and exclude any contribution from processed pseudogene (GJAIP) that has been identified in humans. The desired amplicons were obtained using Nested PCR method. In the first round, 1331bp product which contains the entire coding region plus a small portion of an intron at the 5' UTR was amplified using primers "A". In the second round, the generated amplicons were used as template to specifically amplify the N-terminal, mid-region and C-terminal region of Connexin 43 using respective primers (Table 3.3). PCR assays were carried out in a reaction volume of 50µl containing 100–200 ng of genomic DNA, 0.2mM dNTPs (Sigma), 1X PCR buffer (Sigma), 0.5 U of Taq DNA Polymerase (Sigma) and 10 pmol of each primer (Sigma). The Primer details, PCR protocol and programme are given in tables 3.3, 3.4 and 3.5.

3µl of PCR product was analyzed on 1.5% agarose gel by electrophoresis. The concentration of amplified PCR products was approximated by comparison with the intensity of the corresponding bands in the 1Kb DNA ladder.

Primer Name	Primer sequence (5' to 3')	Annealing Temperature	Product size (bp)
A	F CGTGAAACCGTTGGTAGTA R GCACTTTCTACAGCACCTT	54°C	1331
B	F CGTGAAACCGTTGGTAGTA R CCCCTCGCATTTTCACCTTACC	62°C	552
C	F TTTGAGGTGGCCTTCTTGCTGA A TAGGCGAGAGGGGAGCGGT	63°C	350
D	F TGGGTACAAGCTGGTTACTG R GCACTTTCTACAGCACCTT	58°C	365

Table 3.3: Primers used for screening Connexin 43.

REAGENT	VOLUME REQUIRED	FINAL CONCENTRATION
Taq Buffer (10X)	5.0 µl	1 X
dNTP mix (2mM)	5.0 µl	0.2mM
Forward Primer (10pmoles/µl)	1.0 µl	0.2pmole/µl
Reverse Primer (10pmoles/µl)	1.0 µl	0.2pmole/µl
Taq DNA Polymerase (5U/µl)	0.1 µl	0.01 units/ µl
Genomic DNA	2.0 µl	
MilliQ water	35.9 µl	
Total volume	50 µl	

Table 3.4: PCR protocol

STEP	TEMPERATURE °C	TIME	
Initial Denaturation	95	6 min	
Denaturation	95	30 sec	35 Cycles
Annealing	X	45 sec	
Extension	72	1 min	
Final Extension	72	10 min	

Table3.5: PCR cycling parameters

3.2.7 PURIFICATION OF THE PCR PRODUCTS

Prior to sequencing every PCR product was purified (Vogelstein and Gillespie 1976) as follows:

1. PCR product was loaded on a 1.5% agarose gel and the electrophoresis was carried out at 100 volts until the dye had migrated for a sufficient distance through the gel.
2. The band of interest was cut using a sharp clean surgical blade/scalpel.
3. Each excised gel piece was dispensed in approx. 3 times sodium iodide and the tubes were incubated in a water bath at 45°C-55°C for 5 minutes or till the gel melts completely. The tubes were gently shaken during this process.
4. 10-15 µl of glass milk was added to each tube and incubated on ice for 15 min, vortexing slightly 2-3 times during incubation.
5. The tubes were centrifuged in a microfuge at maximum speed for 90 sec and the supernatant discarded.
6. Wash buffer (500µl) of wash buffer was added to the glass milk pellet to resuspend it into it completely by vortexing, followed by centrifugation at 14,000 rpm.
7. Washing was repeated twice.
8. Pellet was allowed to air dry and 10-15µl of MilliQ water was added, as per the volume of the glass-milk added at step 4.
9. Tubes were incubated in a water bath at 45-55°C for 5 minutes and then centrifuged at maximum speed for 45 minutes.
10. The supernatant (purified DNA fragment) was aspirated out carefully without disturbing the pellet and stored in the fresh eppendorf tubes.
11. The quality and the concentration of the purified DNA fragment was checked on 1.5% agarose gel.

3.2.8 DNA SEQUENCING

Sequencing was done commercially using the services of The Centre for Genomic Applications (TCGA), Delhi. DNA sequences of the amplicons were obtained in fasta and pdf formats. For analyzing the sequencing data in fasta format, software programs ClustalX version 2 (for sequence alignment) (Larkin et al., 2007; Thompson et al., 1997) and Chromas Pro Version 1.49 beta 2 (for detailed inspection of the individual chromatograms), were used. The pdf file of each DNA sequence was used for visual inspection of the sequencing chromatogram using Acrobat Reader 8.0.

3.2.9 STATISTICAL ANALYSIS

All statistical analysis was performed using SPSS program. Odds ratio was performed to determine the association of presence of mutations with various Clinico-epidemiological characteristic such as age, menopause status, site of tumor, provisional diagnosis, lymph node/s involved and clinical stage of tumor. Statistical significance was considered when $p \leq 0.05$. The prevalence of BRCA2 mutations obtained in Kashmiri patients was compared with compiled data reported for breast cancer in BIC database (www.ngri.nih.gov/intramuraresearch/BIC) and other areas including India.

CHAPTER 4

RESULTS

CHAPTER FOUR

RESULTS

4.1 GENOMIC DNA INTEGRITY

Genomic DNA isolated from tissue samples was assessed for its integrity, concentration and purification by agarose gel electrophoresis and spectrophotometry. Concentration of the samples ranged between 200ng/µl to 800ng/µl, adequate for amplification. The purity determined by OD260/OD280 ratio was in the range of 1.60-1.92 in all DNA preparations. Gel analysis indicated high molecular weight DNA in nearly all samples, with some showing slight degradation. DNA having molecular weight more than 23kb is considered to be fairly intact and appropriate for PCR.

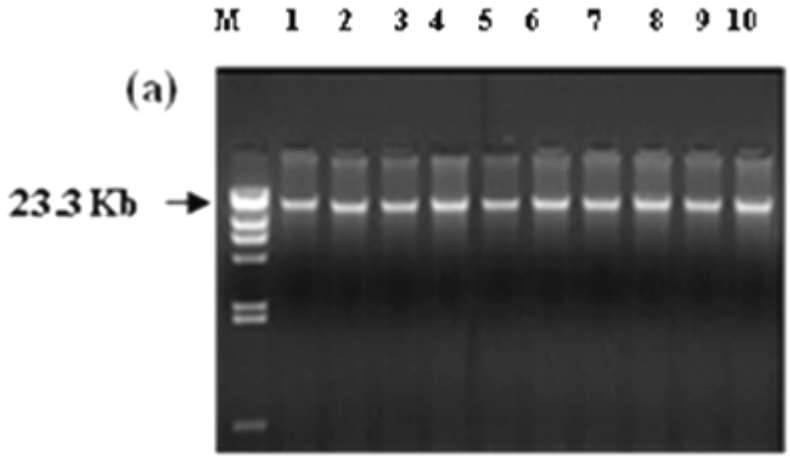


Fig. 4.1: Genomic DNA extracted from blood samples run on 0.8% agarose gel. (a) Lane represented by M shows marker (Hind III digested λ DNA) and lanes 1-10 show analysis of genomic DNA (b) Lane 1 shows separation pattern of Hind III digested λ DNA and sample DNA was run in the remaining lanes.

4.2 BRCA2 ANALYSIS

4.2.1 PCR AMPLIFICATION OF BRCA2

Ten set of primers (Table 3.2) were used to amplify exon 2, exon 9, exon 11 (11.1, 11.2, 11.3, 11.4, 11.5), exon 18, exon 20 and exon 25 of BRCA2 respectively strictly in accordance with conditions described in methods. PCR product (5µl) was analyzed on the 1% agarose gel. After visualizing under UV light, it was seen that all the exons were successfully amplified from DNA samples, generating specific PCR products strictly as per the expected sizes and were sufficient to be purified and sequenced. Prior to sequencing, the final concentration of amplicons was adjusted to 50ng/µl.

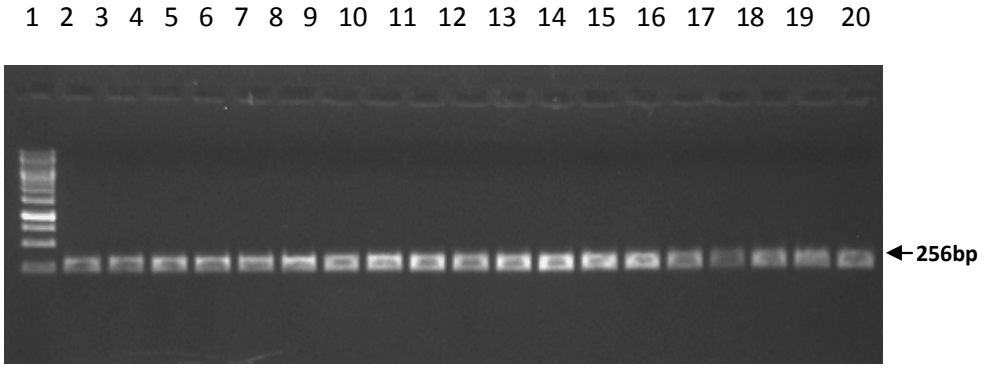


Fig. 4.2a: Exon 2 amplification product (256bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

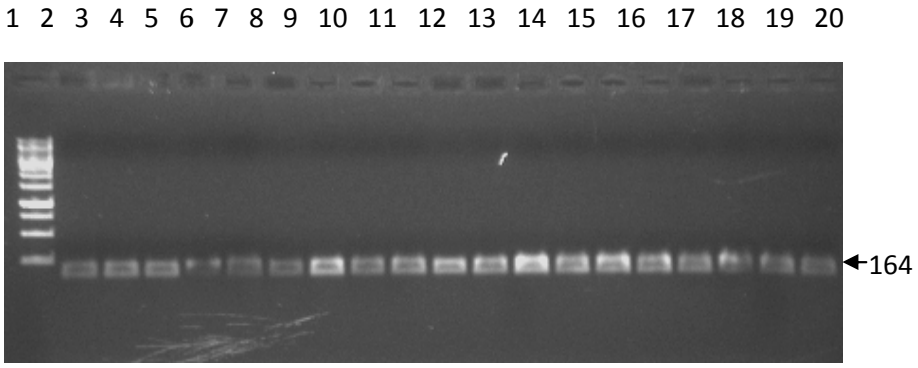


Fig. 4.2b: Exon 9 amplification product (164bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

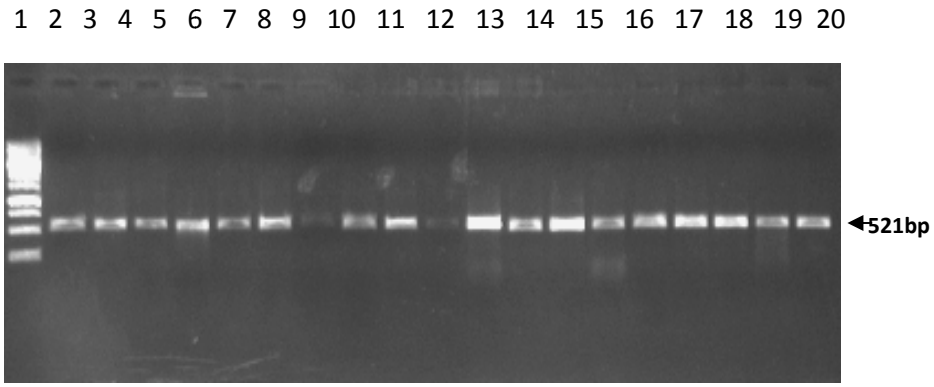


Fig. 4.2c: Exon 11.1 fragment amplification product (521bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

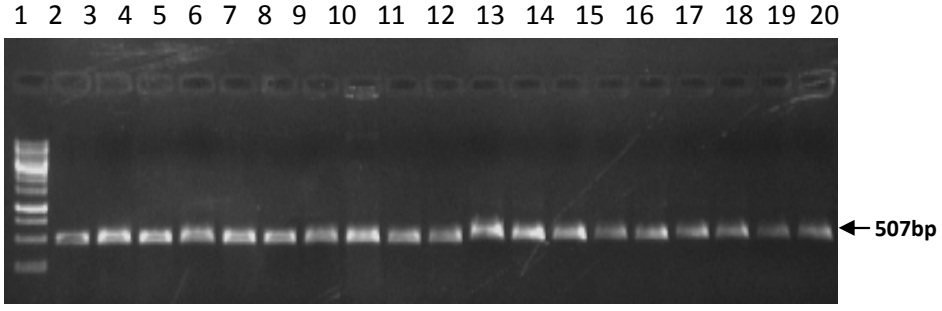


Fig. 4.2d: Exon 11.2 fragment amplification product (507bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

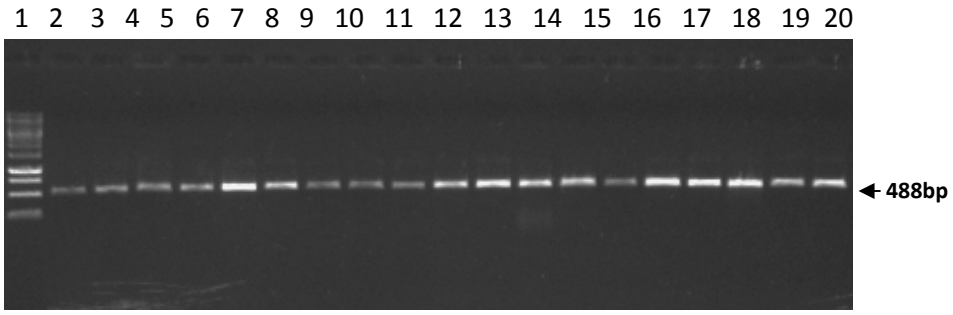


Fig. 4.2e: Exon 11.3 fragment amplification product (488bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

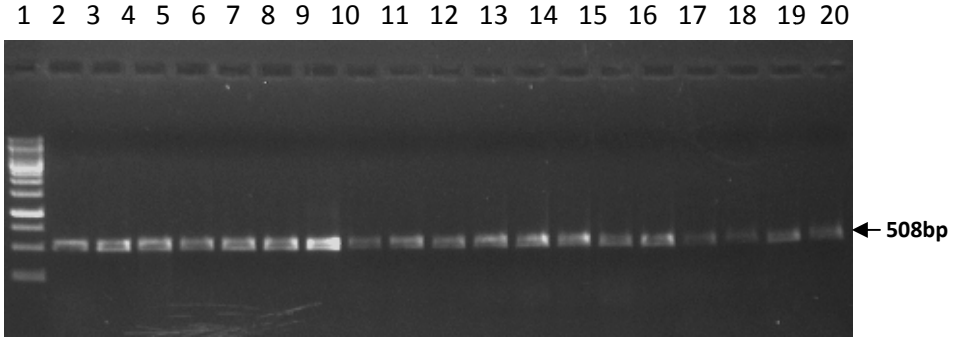


Fig. 4.2f: Exon 11.4 fragment amplification product (508bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

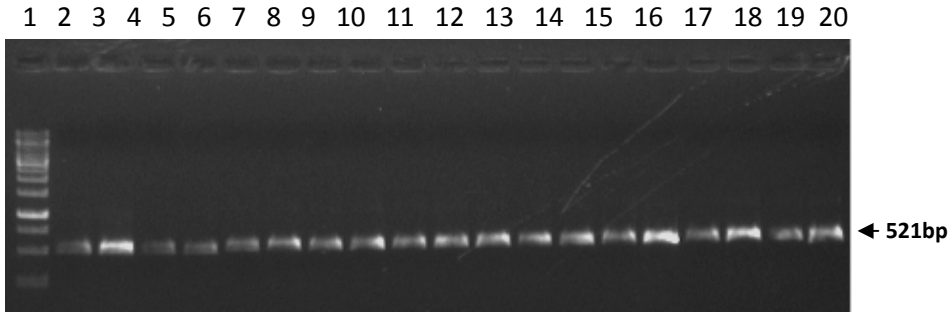


Fig. 4.2g: Exon 11.5 fragment amplification product (521bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

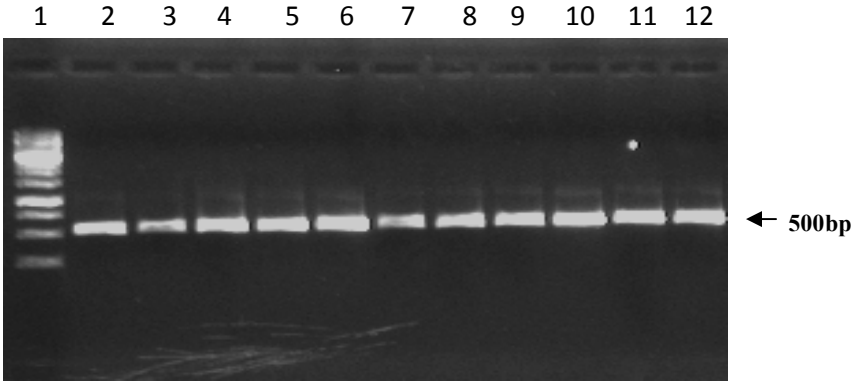


Fig. 4.2h: Exon 18 amplification product (500bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-12 show analysis of 5µl aliquot of PCR product.

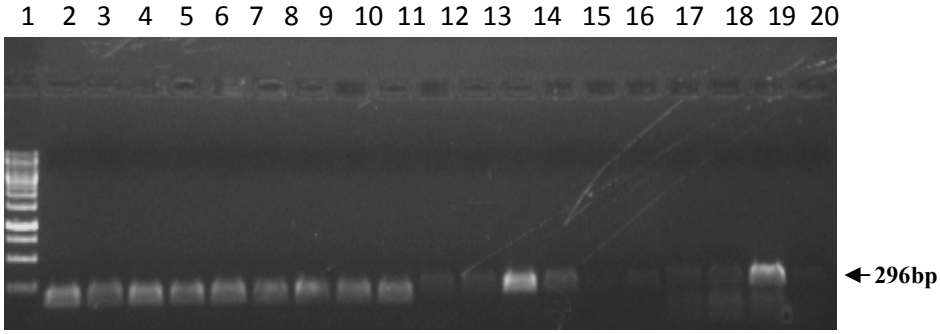


Fig. 4.2i: Exon 20 amplification product (296 bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product

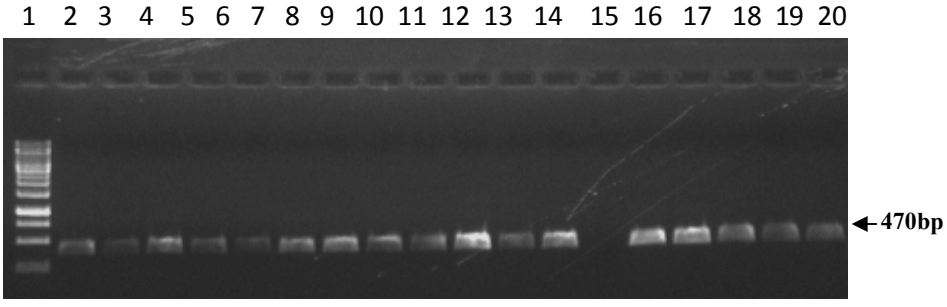


Fig. 4.2j: Exon 25 amplification product (470bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-20 show analysis of 5µl aliquot of PCR product.

4.2.2 SEQUENCE ANALYSIS

The sequencing data was obtained commercially from the Center for Genomics Application, New Delhi. DNA sequences of the amplicons wee obtained in fasta and pdf formats. The fasta files were analyzed using *ClustalX* software for sequence alignment and by *Chromas Pro* software for the detailed inspection of the chromatograms individually. The pdf file of each DNA sequence was used for visual inspection of the entire sequencing chromatograms.

Mutational screening of commonly mutated exons (2, 9, 11, 18, 20, 25) of BRCA2 in 50 sporadic tumor samples of breast cancer patients from Kashmir revealed a total of five variations, out of which four were somatic and one was germline in nature. The germline nature of the variation was established as the variation observed in tumor was also identified in its adjacent normal tissue, blood and lymph node of the same patient. All somatic variations were located in exon 11 of BRCA2, and the germline variation was observed in UTR region of exon 2 screened. Mutation pattern of BRCA2 revealed only missense mutations and no insertion or any kind of deletion was seen in any of the samples. Four mutations observed in exon eleven are G:C > A:T transition at position 846 and 868 and A:T > G:C transitions at codon positions 991 and 1131 (Table 4.1). Codons coding for amino-acid positions at 846 and 868 were seen to be present in heterozygous state in normal breast tissue samples whereas this heterozygous nature of both the codons was seen to be lost in associated tumor samples in 44 out of 50 patients (88%). It was also seen that the loss of heterozygosity at theses two codon positions was always associated and none of the two was individually lost in any of the samples. C > A transition at amino-acid position 846 is a silent mutation whereas the C > A transition at 868 amino-acid position is a mis-sense mutation leading to the replacement of proline by threonine. Mutation at amino-acid position 991 leading to replacement of aspargine by aspartic acid was present in 24% (12/50) of all cancer cases whereas the A to C transition at

amino-acid position 1131 was a silent mutation and was found in 20% (10/50) of all cancer patients. The germline variation A:T > G:C transition observed in UTR region of exon 2 at contig position 13870572 was observed in 32% (16/50) of all patients. C to A transition at amino-acid position 868 is novel whereas others variations have already been reported. However no mutation of any kind either germ-line or somatic was observed in other four exons screened i.e., exon 9, 18, 20 and 25.

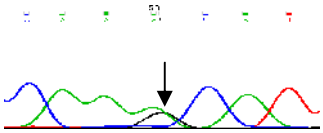
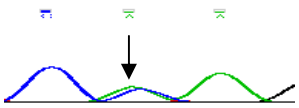
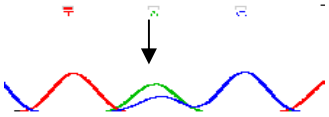
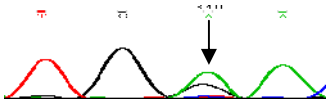
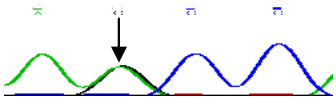
Exon	Codon Position	rs no	Observed base pair change	Amino acid change	Chromatogram
2	5' UTR	1799943	A to G	NIL	
11	846	11571654	C to A	NIL	
11	868	Novel	C to A	Thr to Pro	
11	991	1799944	A to G	NIL	
11	1131	1801406	A to G	NIL	

Table 4.1: Mutations found in different exons of Brca2 in ethnic Kashmiri population.

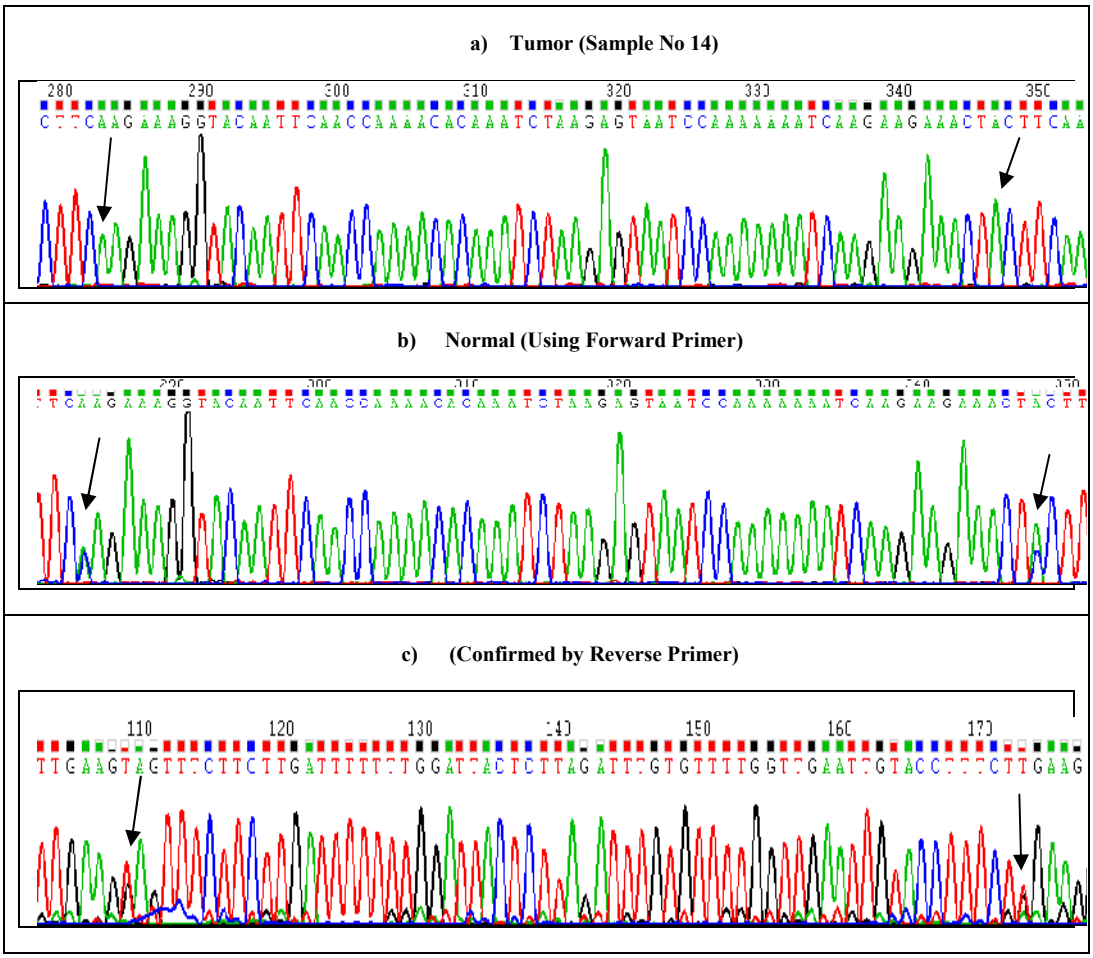


Figure 4.3a: Partial chromatograms representing the normal and mutant (Shown by arrows) profile in Exon 11 of two linked mutations (C>A) at codon position 846 and 868 a) Tumor b) Normal (forward primer) c) SNPs confirmed by reverse primer

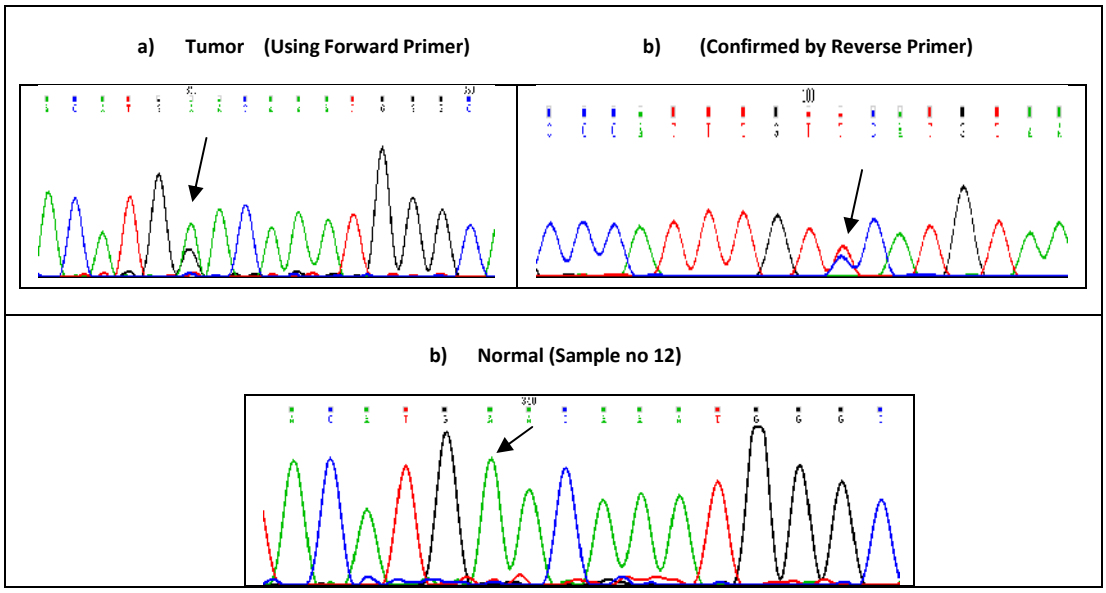


Figure 4.3b: Partial chromatograms representing the normal and mutant (Shown by arrows) profile in Exon 11 at codon position 991 (A>G) a) Tumor (Forward primer) b) SNP confirmed by reverse primer c) Normal.

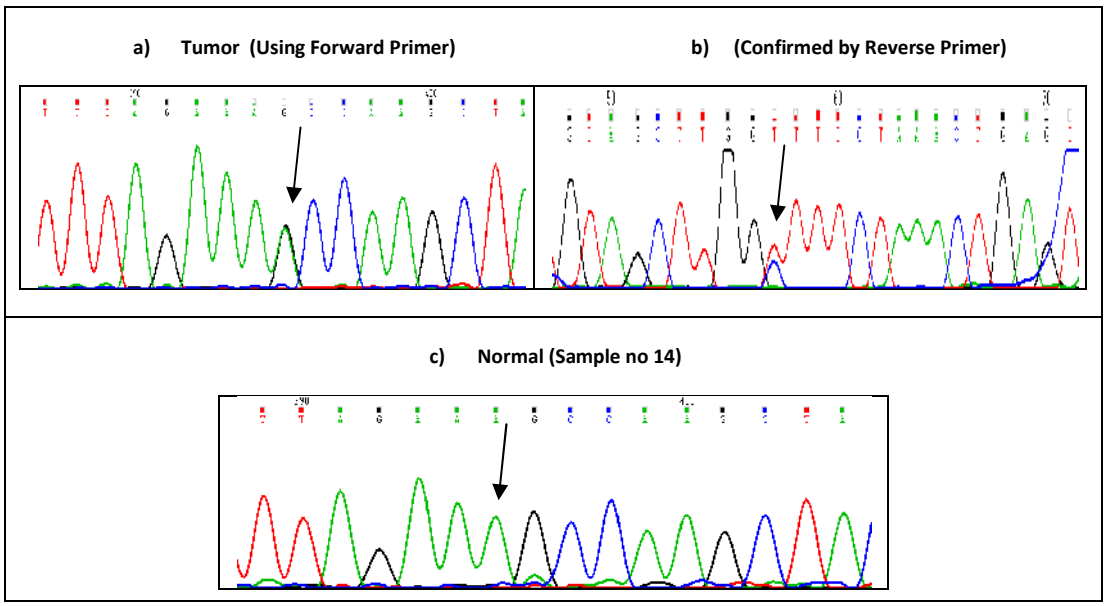


Figure 4.3c: Partial chromatograms representing the normal and mutant (Shown by arrows): profile in (1) Exon 11 at codon position 1131 a) Tumor (forward primer) b) SNP confirmed by reverse primer c) Normal.

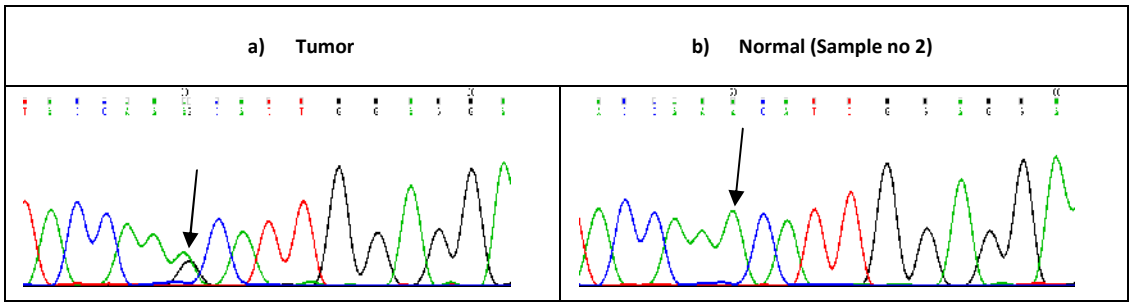


Figure 4.3d: Partial chromatograms representing the normal and mutant (Shown by arrows): profile in (1) Exon 2 in the UTR region at Contig position 13870572 a) Tumor b) Normal.

RVASPSRKVQ FNQNTNLRVI QKNQEETPSI SKITVNPDSE ELFSDNENNF VFQVANERNN
LALGNTKELH ETDLTCVNEP IFKNSTMVLY GDTGDKQATQ VSIKKDLVYV LAEENKNSVK
QHIKMTLGQD LKSDISLNID KIPEKNNDYM NKWAGLLGPI SNHSFGGSFR TASNKEIKLS
EHNIKKSKMF FKDIEEQYPTSL

Normal translation of BRCA2 gene

RVASPSRKVQ FNQNTNLRVI QKNQEETTSI SKITVNPDSE ELFSDNENNF VFQVANERNN
LALGNTKELH ETDLTCVNEP IFKNSTMVLY GDTGDKQATQ VSIKKDLVYV LAEENKNSVK
QHIKMTLGQD LKSDISLNID KIPEKNNDYM DKWAGLLGPI SNHSFGGSFR TASNKEIKLS
EHNIKKSKMF FKDIEEQYPTSL

Translation of BRCA2 gene (Affected): Showing codon 846 (Silent mutation), codon 868 pro to thr (red in normal, green in affected), codon 991 asn to asp (red in normal, orange in affected), codon 1131 (silent mutation).

Table 4.2: Translation of normal and affected BRCA2 gene exon 11.

Patient ID	Age	Rural/ Urban	Menopausal Stage	Provisional Diagnosis	Lymph node/s	Clinical Tumor Stage	Codon No	Base change	Mutation (cDNA sequence)	Amino-acid change
C1	35	R	Pre	IDC	Y	IV	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C2	48	R	Post	IDC	Y	IIa	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C4	51	U	Post	IDC	Y	IIb	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C5	38	U	Pre	IDC	Y	IIa	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C6	54	R	Post	IDC	N	IV	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C7	42	R	Pre	IDC	Y	IIIa	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C8	65	R	Post	IDC	Y	IIIa	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C9	70	R	Post	IDC	Y	IIIb	846 868 1131	TC**C**>TC**A** **C**CT>**A**CT AA**A**>AA**G**	2538C>A 2602C>A 3393A>G	 Pro>Thr
C10	52	R	Post	IDC	N	IIIb	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C11	37	R	Pre	IDC	Y	IIa	846 868 1131	TC**C**>TC**A** **C**CT>**A**CT AA**A**>AA**G**	2538C>A 2602C>A 3393A>G	 Pro>Thr
C12	38	R	Pre	IDC	Y	IIIb	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr
C13	65	R	Post	IDC	N	IIIa	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C14	35	R	Pre	IDC	Y	IVa	846 868 1131	TC**C**>TC**A** **C**CT>**A**CT AAA>AAG	2538C>A 2602C>A 3393A>G	 Pro>Thr
C15	60	U	Post	IDC	N	IIb	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C17	60	R	Post	IDC	Y	IIb	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C18	50	U	Post	IDC	Y	IIIb	846 868 1131	TC**C**>TC**A** **C**CT>**A**CT AA**A**>AA**G**	2538C>A 2602C>A 3393A>G	 Pro>Thr
C19	50	R	Post	IDC	Y	IV	846 868 1131	TC**C**>TC**A** **C**CT>**A**CT AA**A**>AA**G**	2538C>A 2602C>A 3393A>G	 Pro>Thr
C20	60	R	Post	IDC	Y	IIIa	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C21	60	R	Post	IDC	N	IV	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C23	35	R	Pre	IDC	N	IIb	846 868	TC**C**>TC**A** **C**CT>**A**CT	2538C>A 2602C>A	 Pro>Thr
C24	29	R	Pre	IDC	Y	IIIb	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C25	50	R	Post	IDC	Y	IV	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C26	35	U	Pre	IDC	N	IIa	846 868 991	TC**C**>TC**A** **C**CT>**A**CT **A**AC>**G**AC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C27	60	R	Post	IDC	Y	IIa	846 868 1131	TC**C**>TC**A** **C**CT>**A**CT AA**A**>AA**G**	2538C>A 2602C>A 3393A>G	 Pro>Thr
C28	50	R	Post	IDC	Y	IIIa	846	TC**C**>TC**A**	2538C>A	

							868	CCT>ACT	2602C>A	Pro>Thr
C29	60	R	Post	IDC	N	IIIb	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C31	55	R	Post	IDC	Y	IIa	846 868 1131	TCC>TCA CCT>ACT AAA>AAG	2538C>A 2602C>A 3393A>G	 Pro>Thr
C32	50	R	Post	IDC	N	IV	846 868 991	TCC>TCA CCT>ACT AAC>GAC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C33	50	U	Post	IDC	Y	IIIa	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C34	55	R	Post	IDC	Y	IIb	846 868 991	TCC>TCA CCT>ACT AAC>GAC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C35	35	U	Pre	IDC	Y	IIIb	846 868 991	TCC>TCA CCT>ACT AAC>GAC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C36	50	R	Post	IDC	Y	IIb	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C37	29	R	Pre	IDC	Y	IIIb	846 868 991	TCC>TCA CCT>ACT AAC>GAC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C38	40	R	Pre	IDC	Y	IV	846 868 1131	TCC>TCA CCT>ACT AAA>AAG	2538C>A 2602C>A 3393A>G	 Pro>Thr
C39	40	R	Pre	IDC	Y	IV	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C41	37	R	Pre	IDC	Y	IIa	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C42	52	R	Post	IDC	N	IIb	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C43	70	U	Post	IDC	N	IIIa	846 868 1131	TCC>TCA CCT>ACT AAA>AAG	2538C>A 2602C>A 3393A>G	 Pro>Thr
C44	65	R	Post	IDC	Y	IIIa	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C46	54	R	Post	IDC	Y	IV	846 868 991	TCC>TCA CCT>ACT AAC>GAC	2538C>A 2602C>A 2971A>G	 Pro>Thr Asn>Asp
C47	38	R	Pre	IDC	Y	IIIa	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C48	51	R	Post	IDC	N	IIIb	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr
C49	40	R	Pre	IDC	Y	IV	846 868 1131	TCC>TCA CCT>ACT AAA>AAG	2538C>A 2602C>A 3393A>G	 Pro>Thr
C50	48	R	Post	IDC	Y	IV	846 868	TCC>TCA CCT>ACT	2538C>A 2602C>A	 Pro>Thr

Table 4.3: Clinico-epidemiological details and nature of BRCA2 mutations in sporadic breast cancer patients from Kashmir valley.

4.2.3 BRCA2 MUTATIONS AND CLINICO-EPIDEMIOLGICAL FEATURES

The presence of BRCA2 mutations when compared with various clinico-epidemiological attributes of sporadic breast cancer patients showed some association though statistically not significant, with early onset and late onset breast cancer cases, menopausal stage and advanced clinical stage (III and IV) of the disease (Table 4.4a & Table 4.4b). However there was no association seen with positive lymph node status and breast involved.

Features	Codon position 846 and 868	P-value	Odds Ratio
Age			
≤ 45 years	16/20	0.65	1.38
≥ 45 years	28/30		(0.1-17.67)
Rural	36/40		0.44
Urban	8/10	0.5	(0.032-6.18)
Menopause Status			
Pre-menopausal	16/20	0.65	1.38
Post-menopausal	28/30		(0.1-17.67)
Breast Involved			
Right	14/16	0.69	1.4
Left	30/34		
Provisional Diagnosis			
IDC	44/50		
IBC	0/0	N/A	
Paget's Disease	0/0		
Lymph Nodes Involved			
Yes	32/36	0.64	0.75
No	12/14		(0.05-9.8)
Tumor Details			
Clinical Tumor Stage			
II (a,b)	14/20	0.54	0.5 (0.03-6.6)
III (a,b)	18/18	0.3	4 (0.27-58.56)
IV	12/12	0.48	2 (0.2-19.9)

Table 4.4a: Association of two associated mutations at codon position 846 (rs11571654) and 868 (Novel) on exon 11 of BRCA2 with Clinico-epidemiological features of sporadic breast cancer patients of Kashmir (n=50).

Features	Codon position 991	P-value	Odds Ratio
Age			
≤ 45 years	6/20	0.46	1.7
≥ 45 years	6/30		(0.1-4.7)
Rural	10/40		0.75
Urban	2/10	0.65	(0.06-8.38)
Menopause Status			
Pre-menopausal	6/20	0.46	1.7
Post-menopausal	6/30		(0.26-10.92)
Breast Involved			
Right	4/16	0.65	1.08
Left	8/34		(0.15-7.64)
Provisional Diagnosis			
IDC	10/50	N/A	N/A
IBC	0/0		
Paget's Disease	0/0		
Lymph Nodes Involved			
Yes	10/36	0.44	0.43
No	2/14		(0.04-4.5)
Tumor Details			
Clinical Tumor Stage			
II (a,b)	4/20	0.66	1.14(0.12-10.38)
III (a,b)	4/18	0.53	1.75(0.17-17.68)
IV	4/12	0.48	2 (0.2-19.9)

Table 4.4b: Association of mutation at codon position 991 (rs1799944) on exon 11 of BRCA2 with Clinico-epidemiological features of sporadic breast cancer patients of Kashmir (n=50).

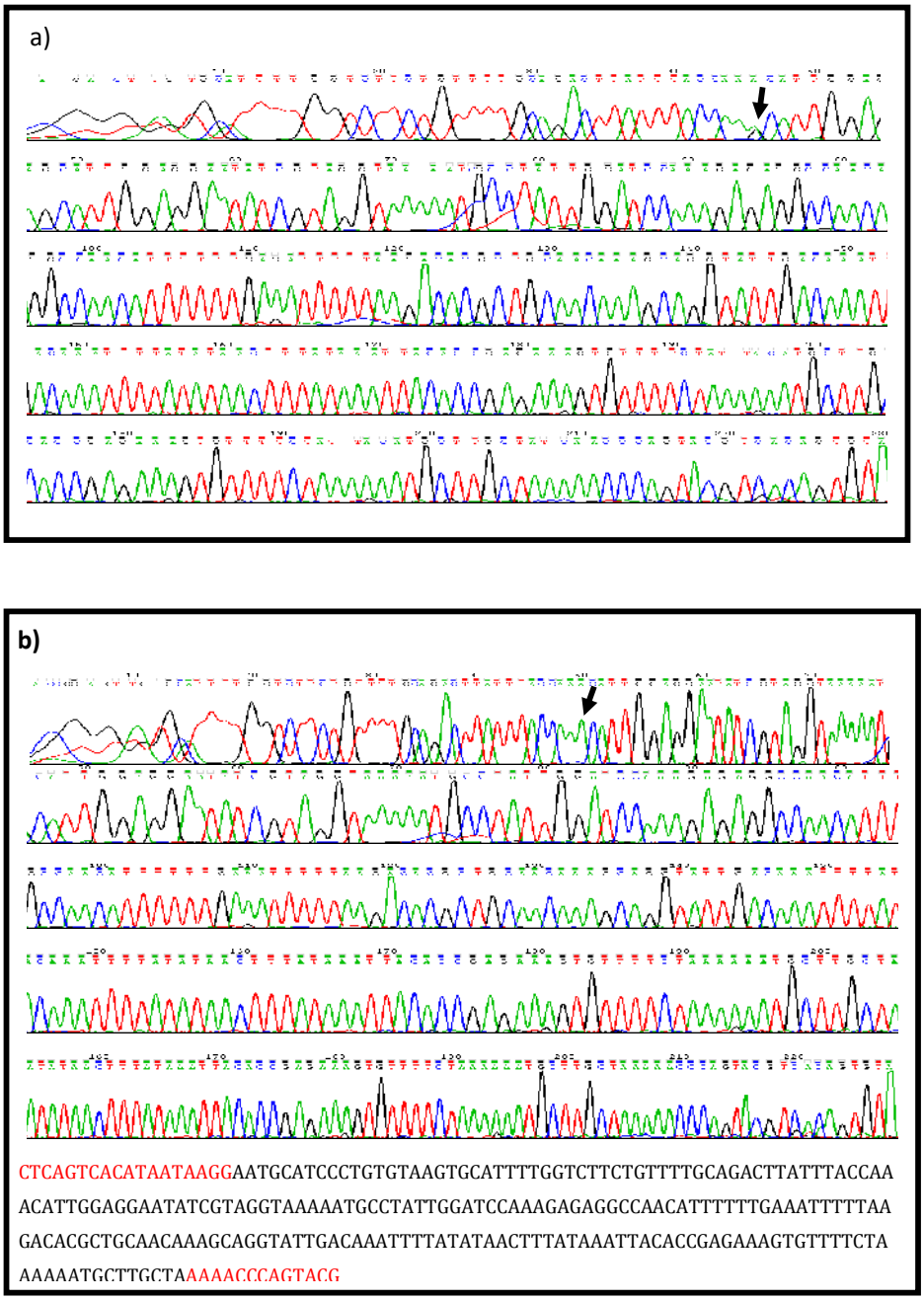


Figure 4.4a: Representative sequencing chromatogram showing the mutant and normal profile (shown by black arrows) in Exon 2 of BRCA2 gene of a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

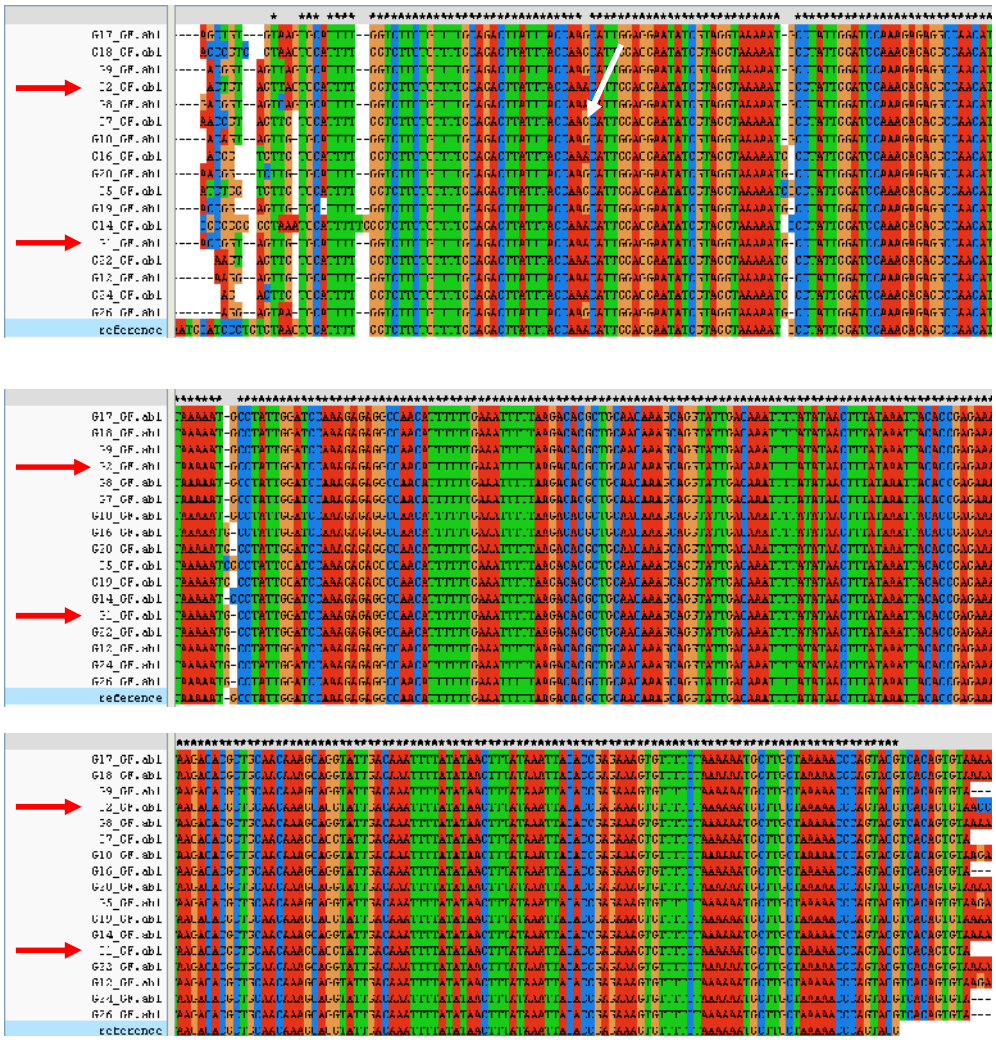
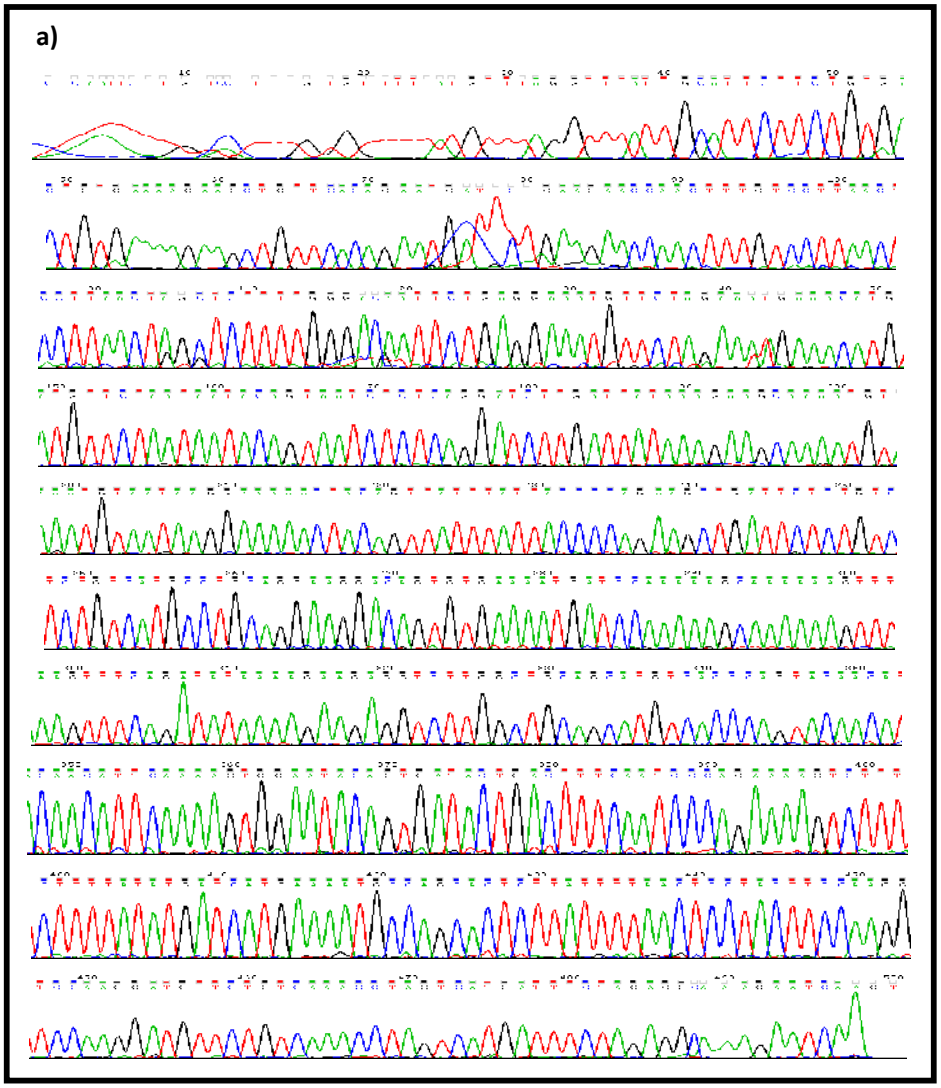


Figure 4.4b: **Multiple sequence alignment of samples generated from Exon 2 amplification of BRCA2. The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by red arrows), using Clustal X software. White arrow shows the nucleotide position where single base pair change was observed.**


a)

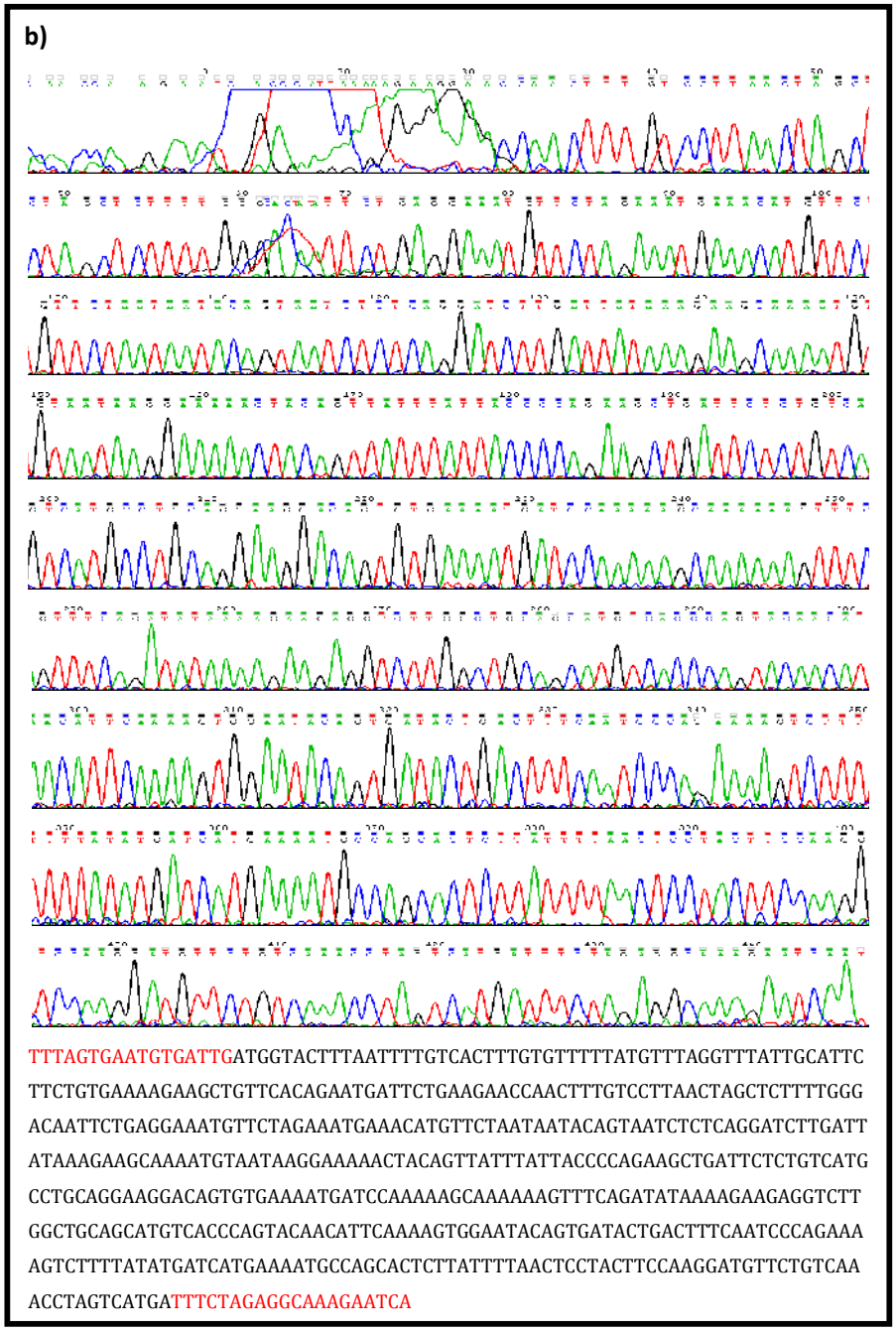
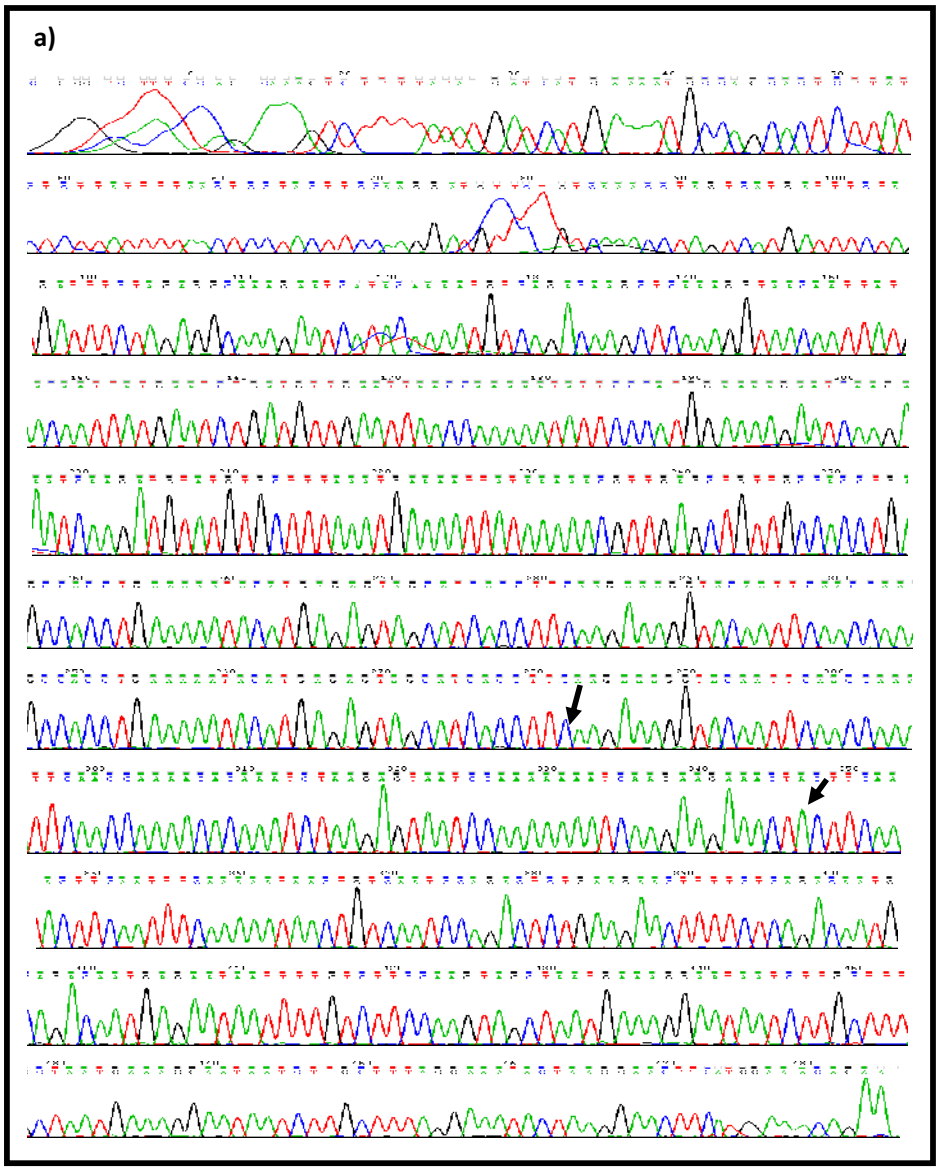


Figure 4.4c: Representative sequencing chromatogram of 11.1 fragment of exon 11 (BRCA2) of a) Tumor sample b) Normal sample along with its NCBI fasta sequence.


a)

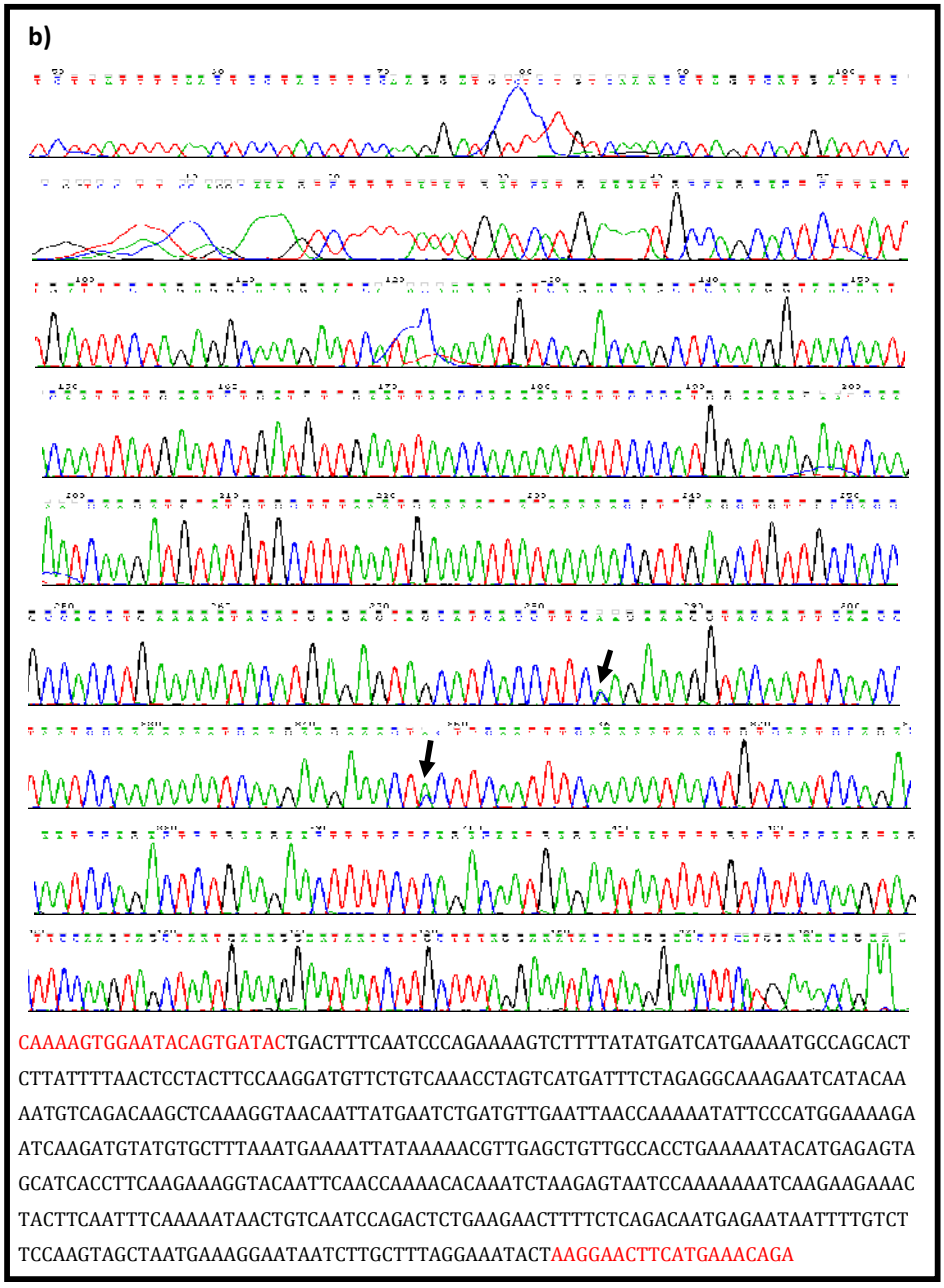


Figure 4.4d: Representative sequencing chromatogram showing the normal and mutant profile (shown by black arrows) in 11.2 fragment of exon 11 (BRCA2) of a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

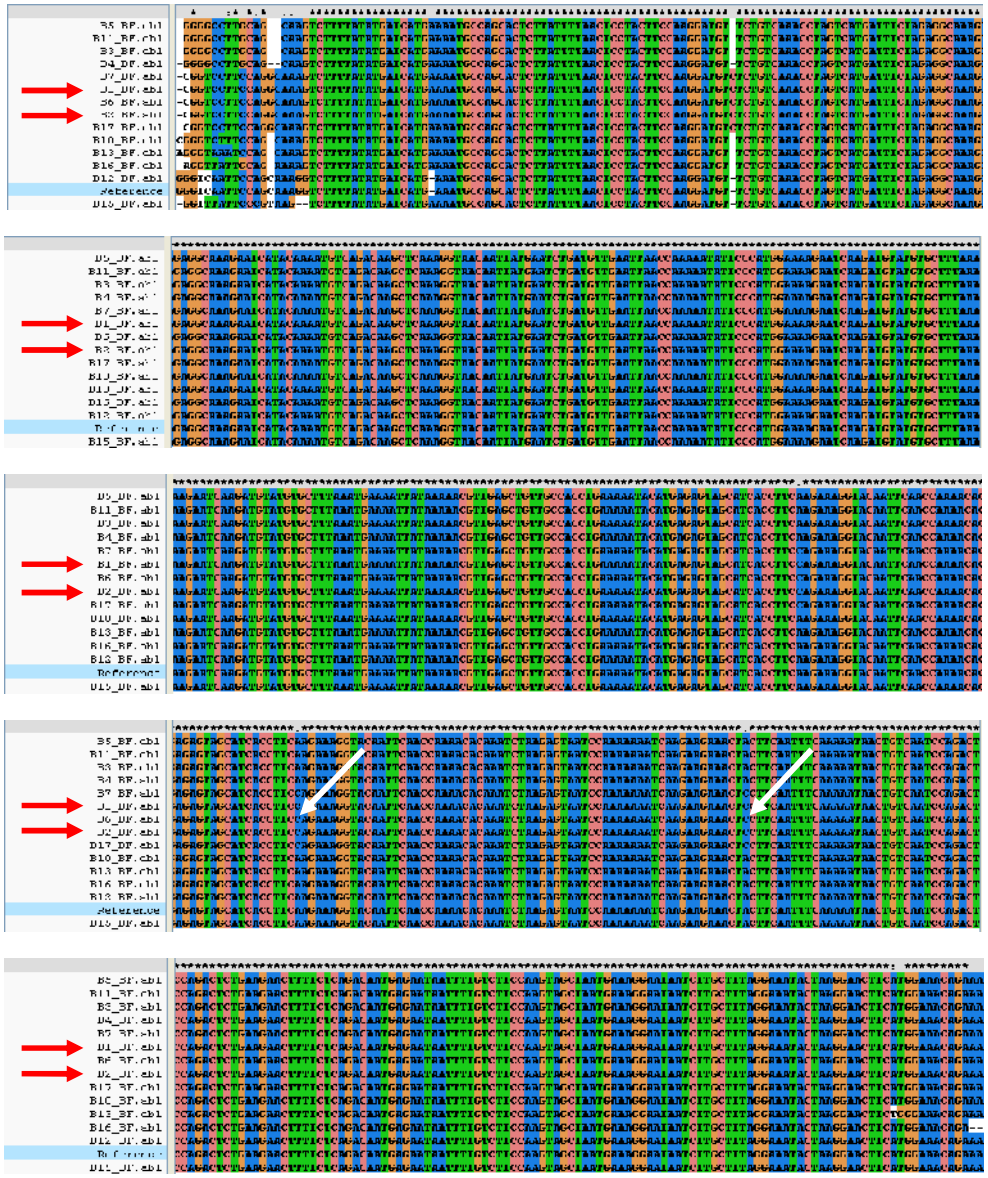
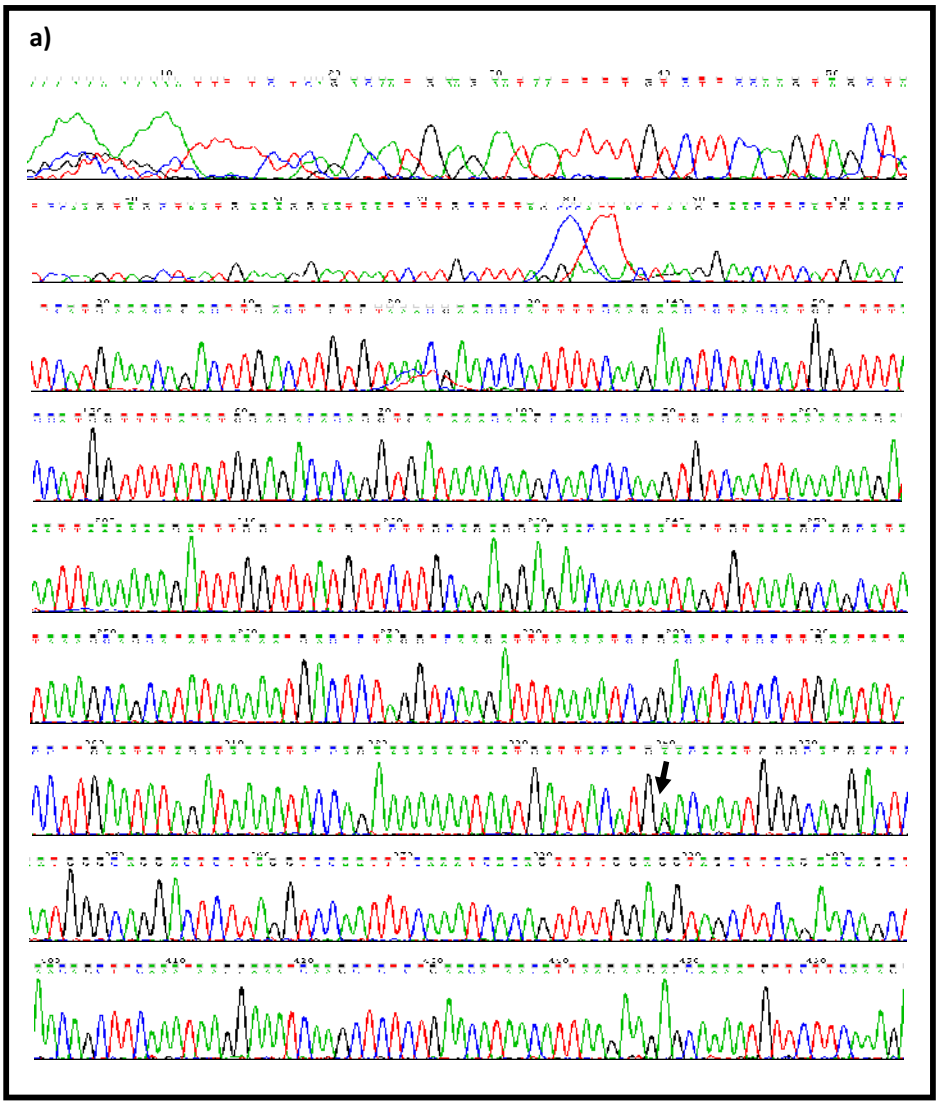


Figure 4.4e: Multiple sequence alignment of samples generated from 11.2 fragment amplification of exon 11 (BRCA2). The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by red arrows), using Clustal X software. White arrow shows the nucleotide position where single base pair change was observed.


a)

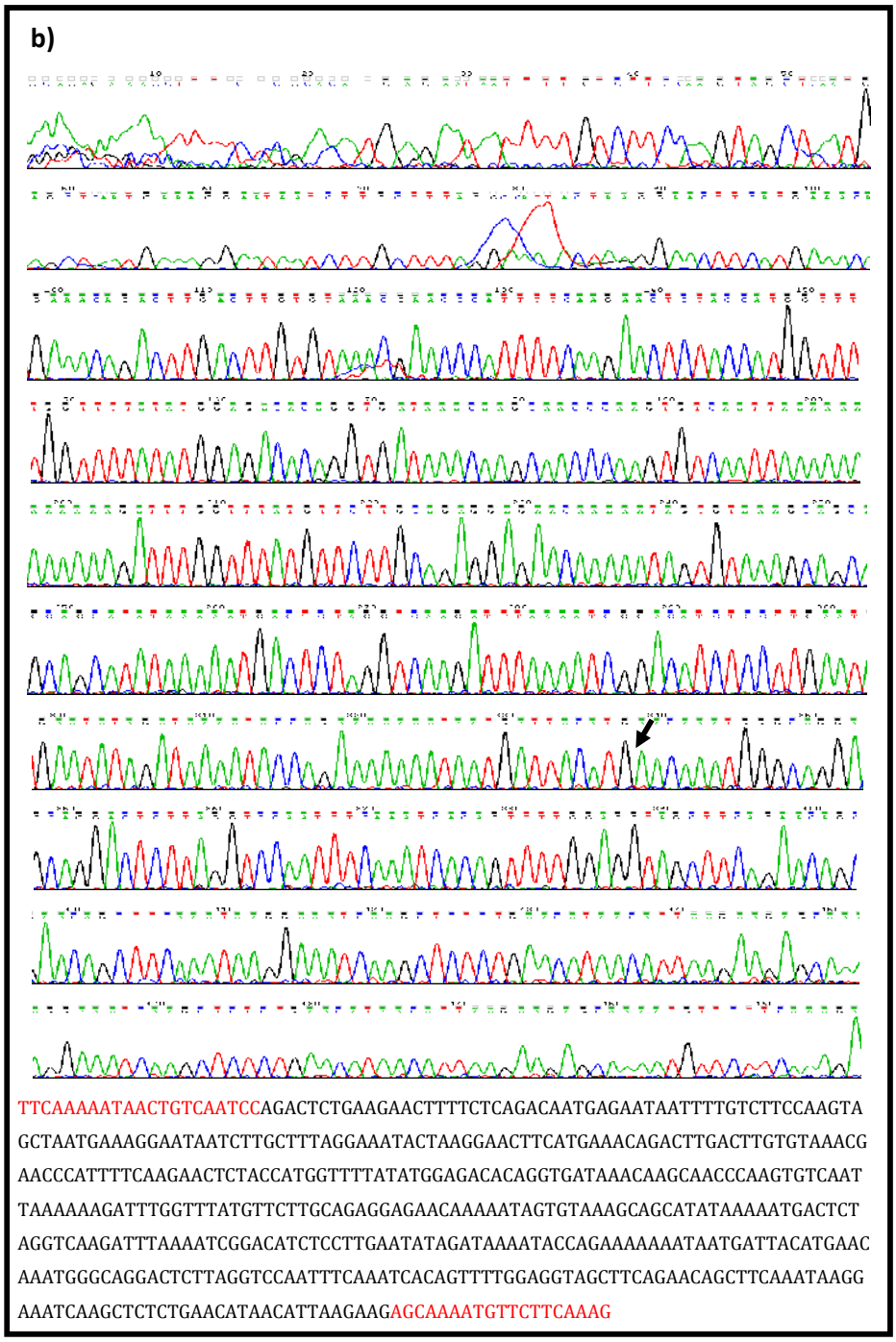


Figure 4.4f: Representative sequencing chromatogram showing the normal and mutant profile (shown by black arrows) in 11.3 fragment of exon 11 (BRCA2) of a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

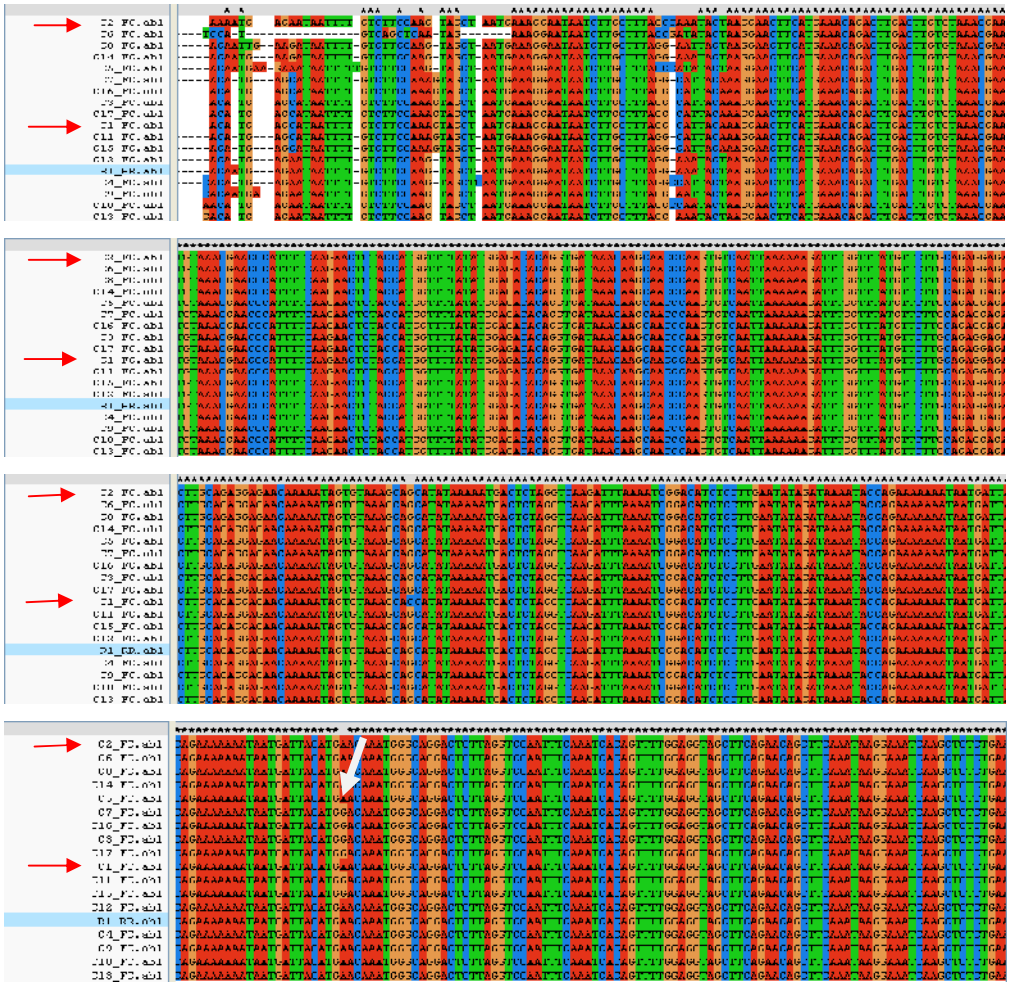
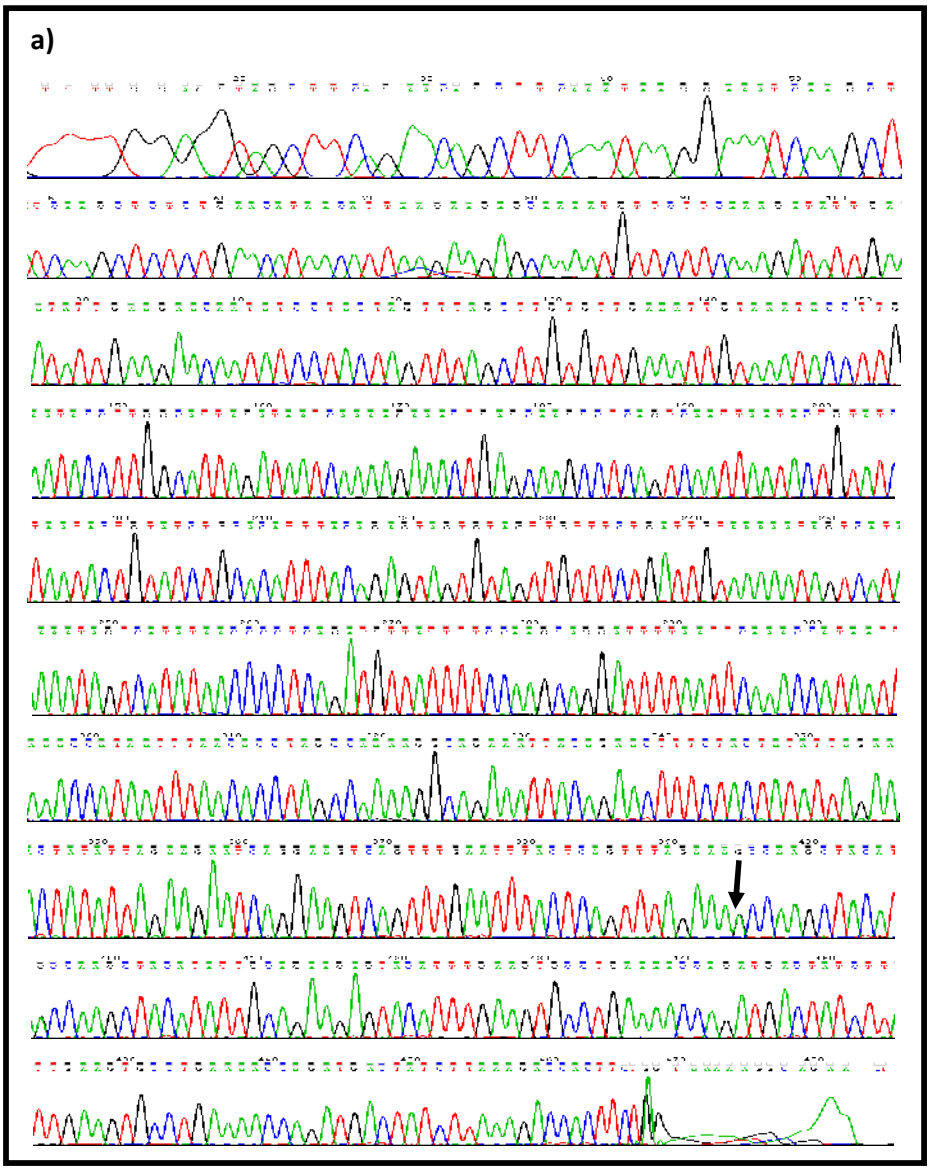


Figure 4.4g: **Multiple sequence alignment of samples generated from 11.3 fragment amplification of Exon 11 (BRCA2). The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by red arrows), using Clustal X software. White arrow shows the nucleotide position where single base pair change was observed.**


a)

ACAAATGGGCAGGACTCTTAGGTCCAATTTCAAATCACAGTTTTGGAGGTAGCTTCAGAACAGCTTCAAATAA
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CAATTAATACTGTATCTGCACATTTACAGAGTAGTGTAGTTGTTTCTGATTGTAAAAATAGTCATATAACCCC
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GAACTTTCTACTATATTAGAAGAATCAGGAAGTCAGTTTGAATTTACTCAGTTTAGAAAACCAAGCTACATAT
TGCAGAAGAGTACATTTGAAGTGCCTGAAAACCAGATGACTATCTTAAAGACCACTTCTGAGGAATGCAGA

Figure 4.4h: Representative sequencing chromatogram showing the normal and mutant profile (shown by black arrows) in 11.4 fragment of exon 11 (BRCA2) a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

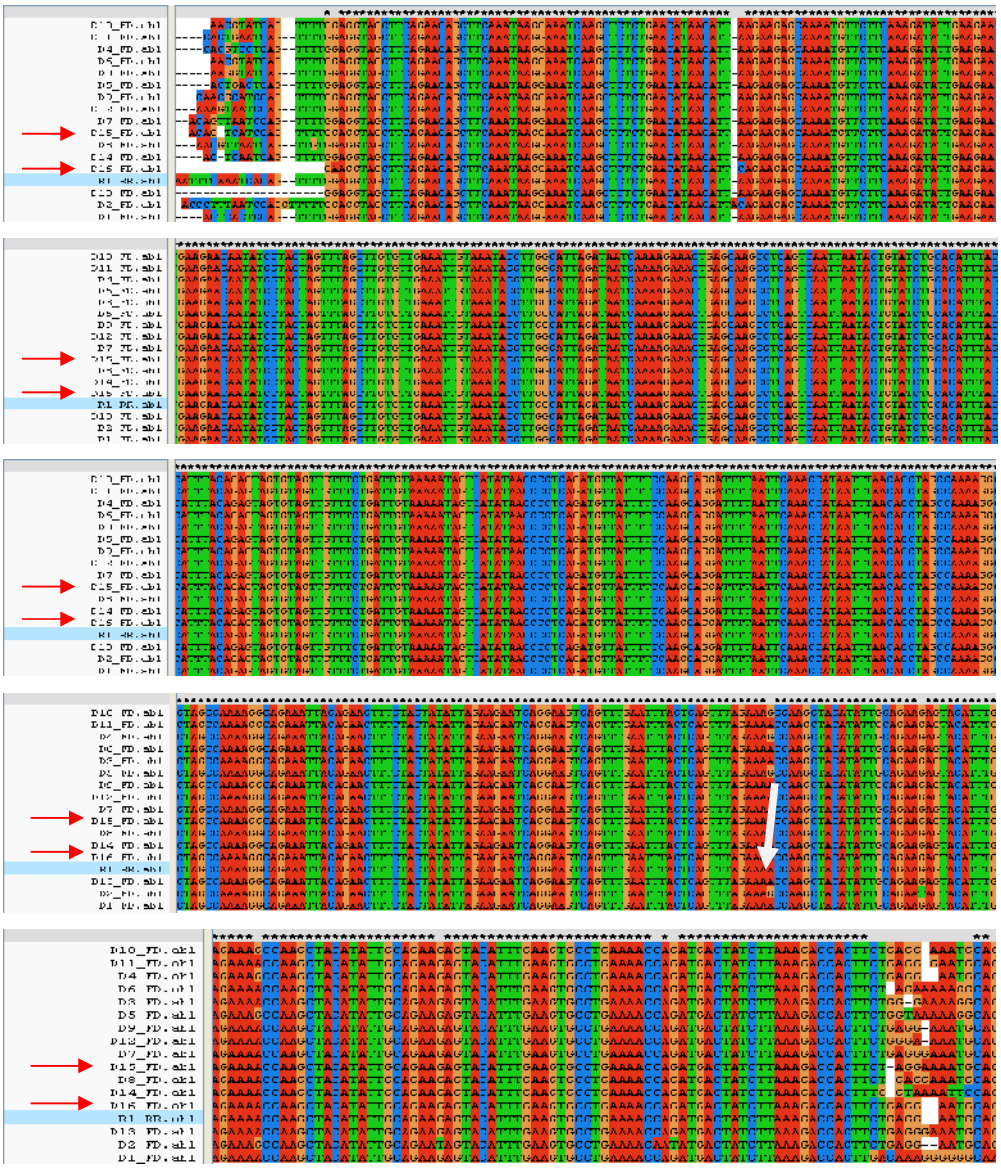
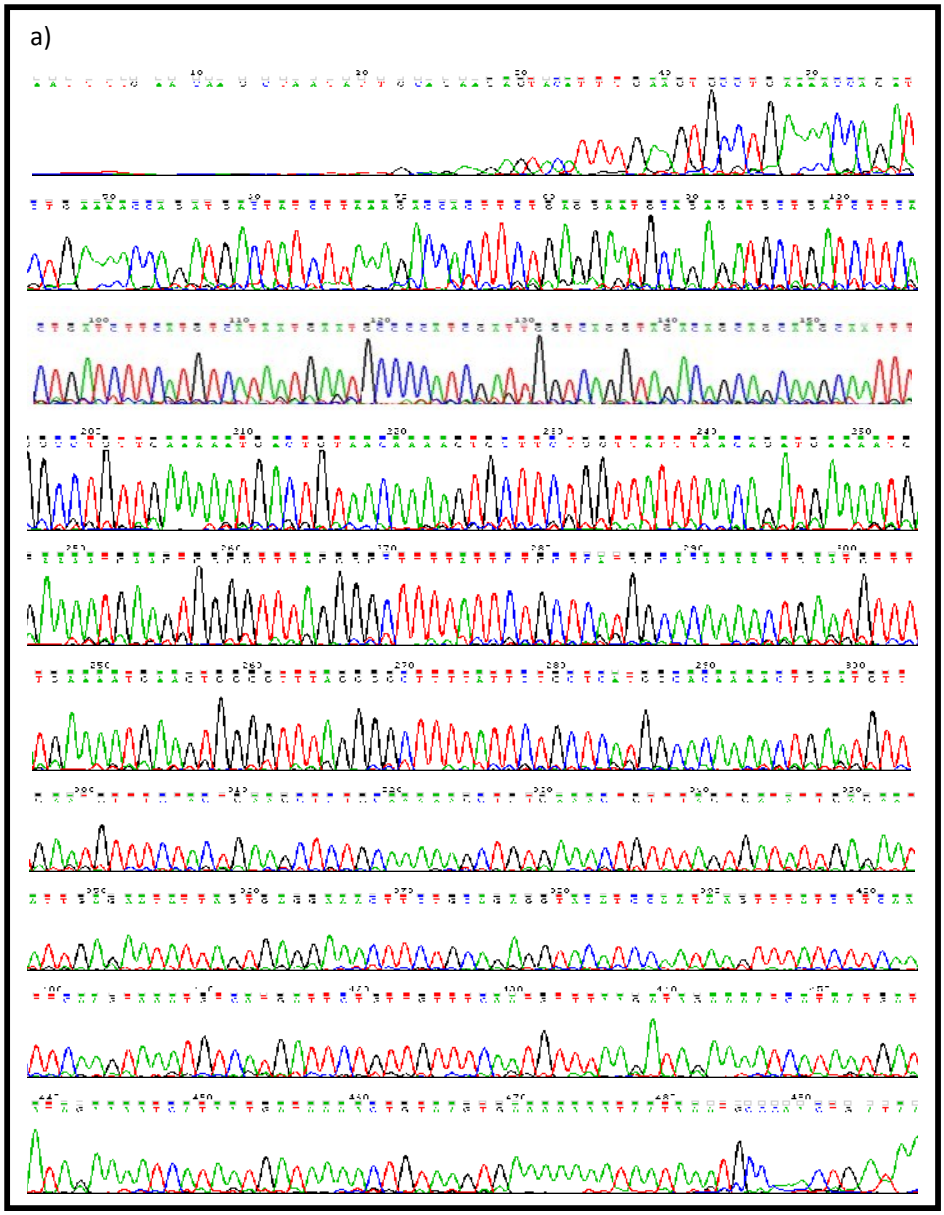


Figure 4.4i: Multiple sequence alignment of amplified samples generated from 11.4 fragment of Exon 11 (BRCA2). The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by red arrows), using Clustal X software. White arrow shows the nucleotide position where single base pair change was observed.


a)

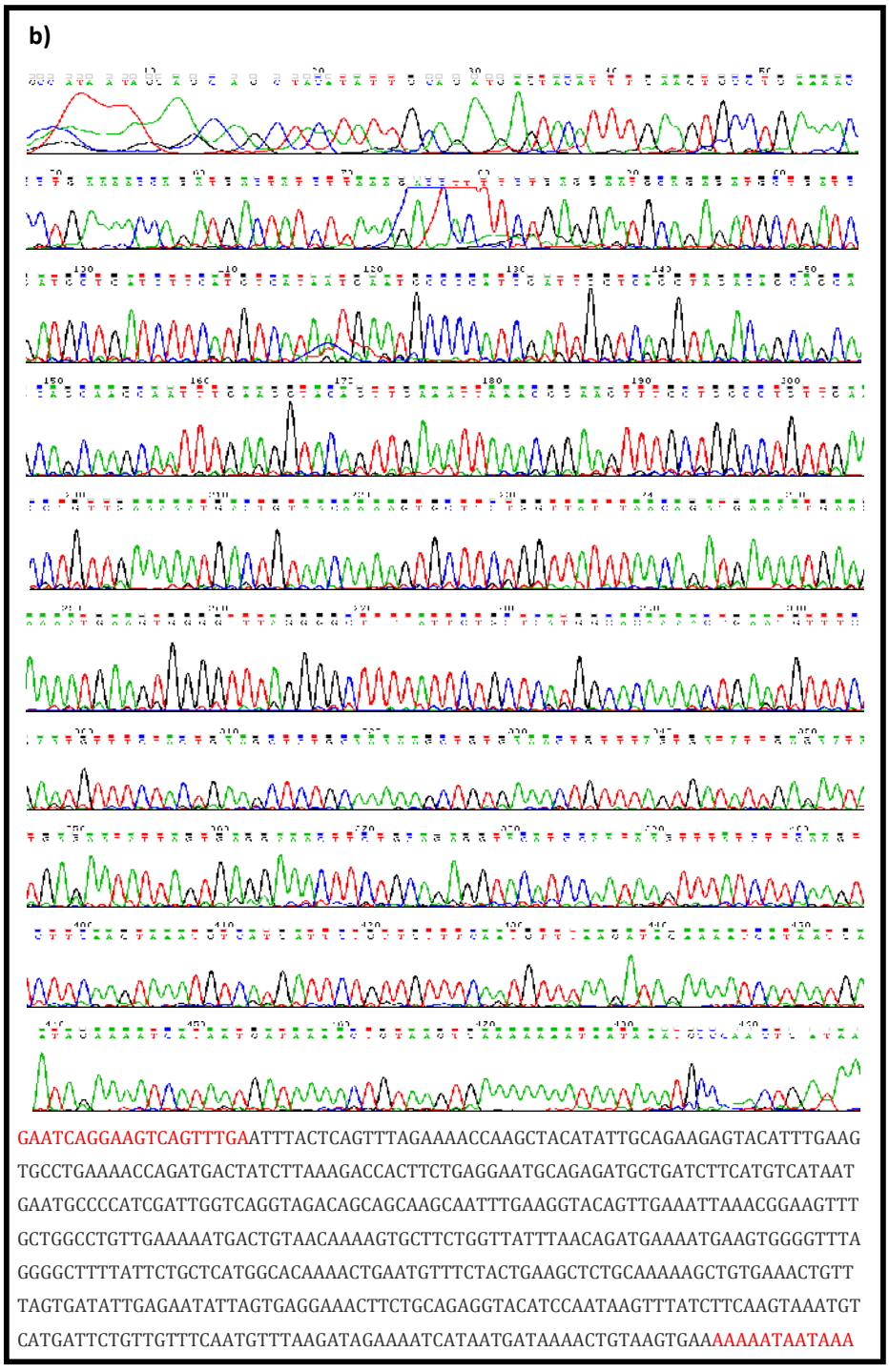


Figure 4.4j: Representative sequencing chromatogram of 11.5 fragment of exon 11 (BRCA2) a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

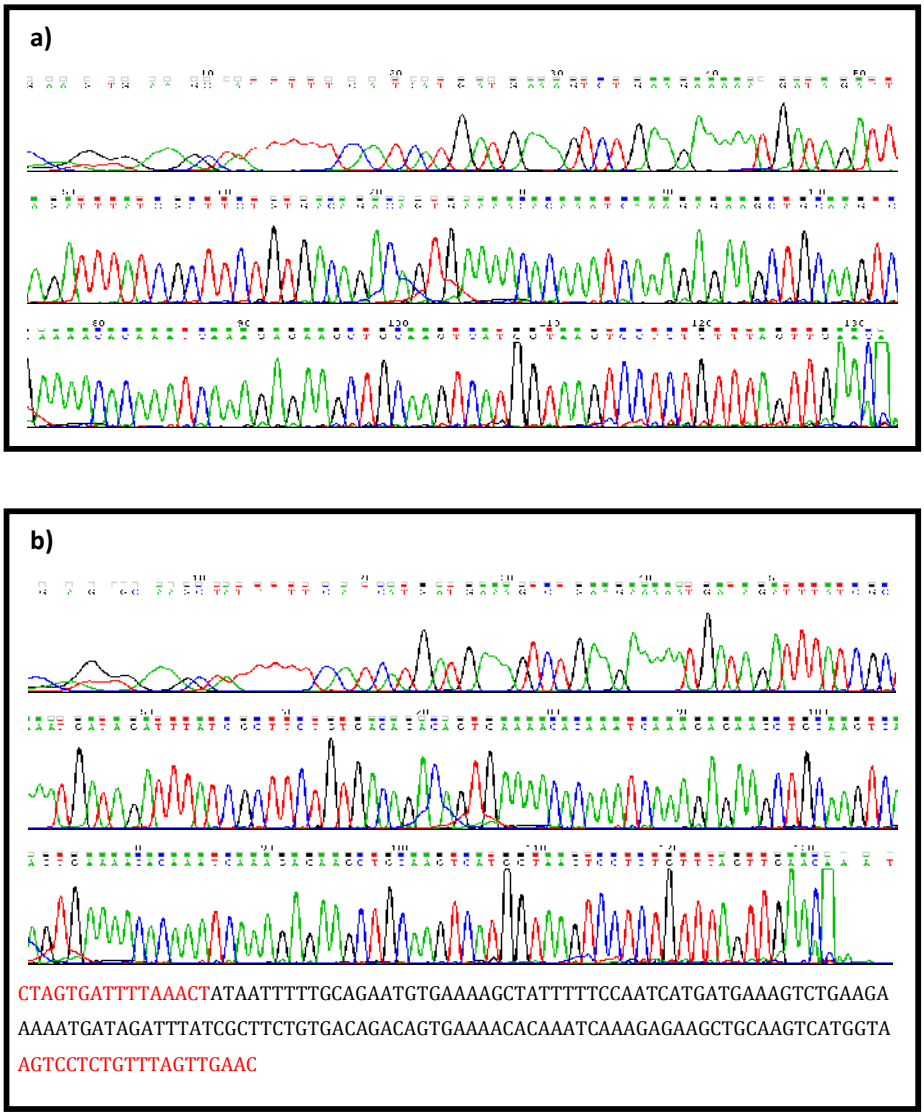
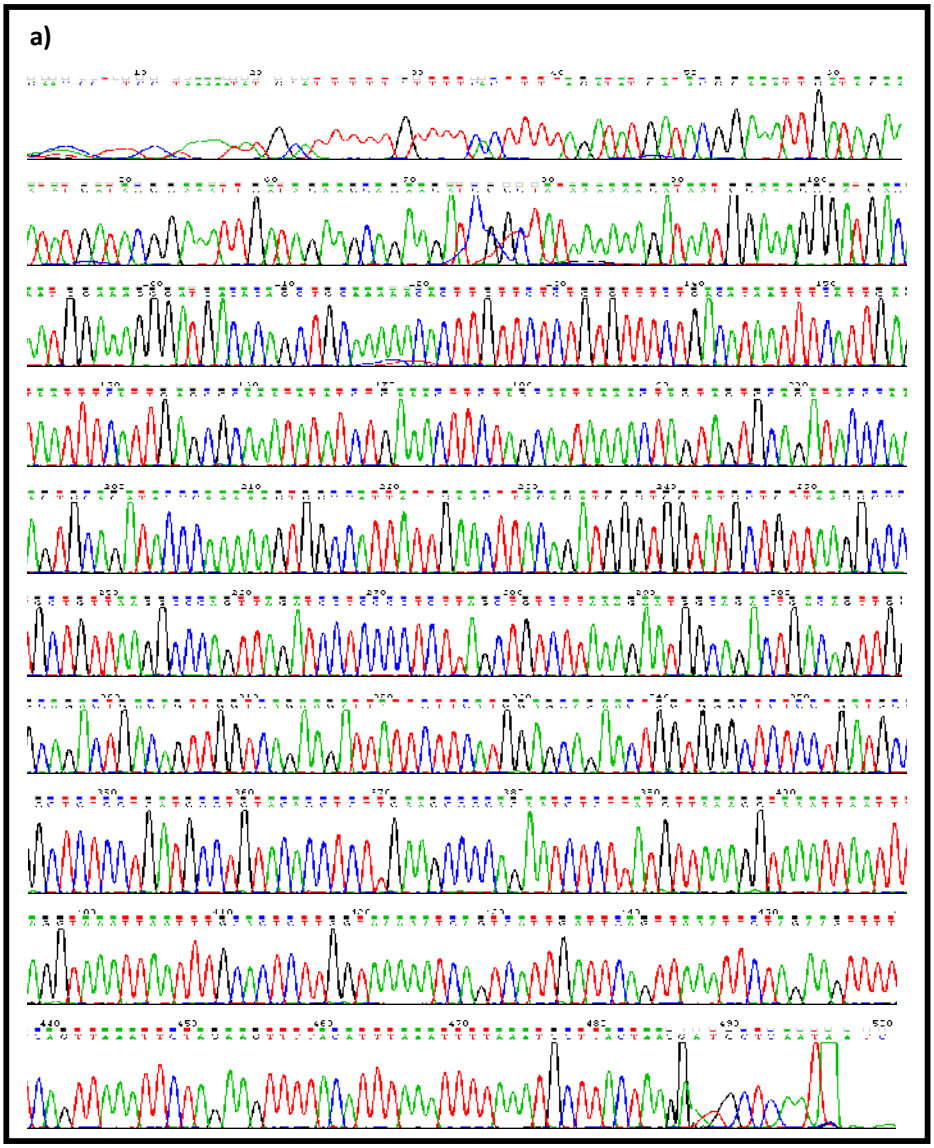


Figure 4.4k: Representative sequencing chromatogram of Exon 9 of BRCA2 gene a) Tumor sample b) Normal sample along with its NCBI fasta sequence.


a)

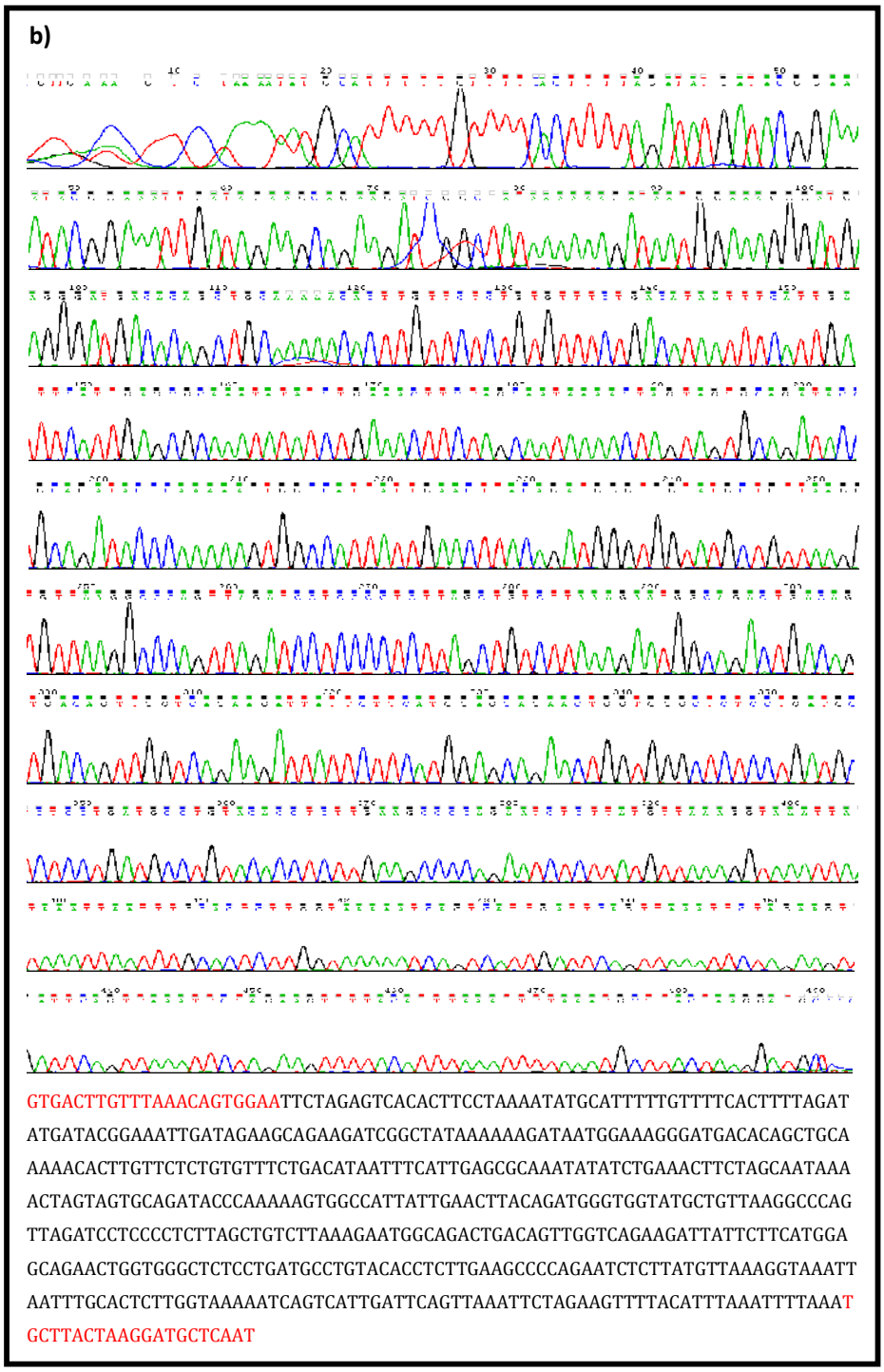
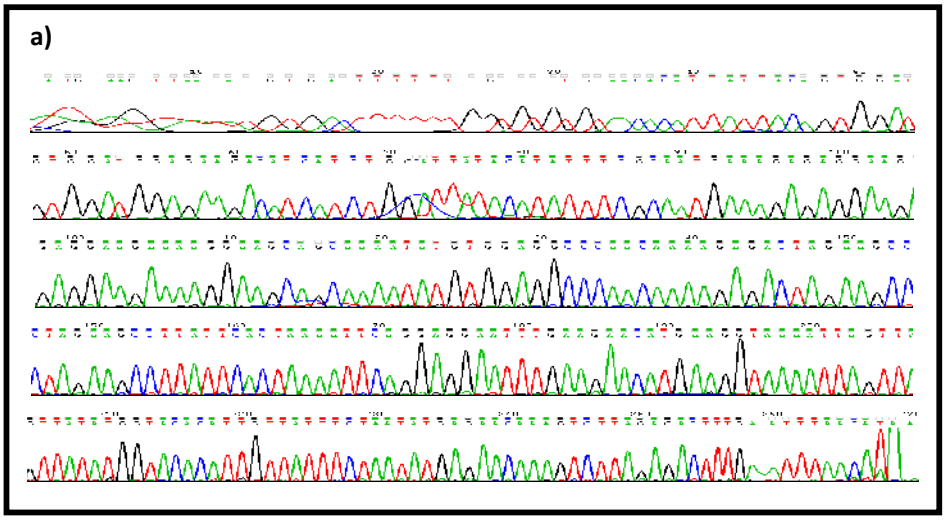


Figure 4.4l: Representative sequencing chromatogram of Exon 18 of BRCA2 gene a) Tumor sample b) Normal sample along with its NCBI fasta sequence.



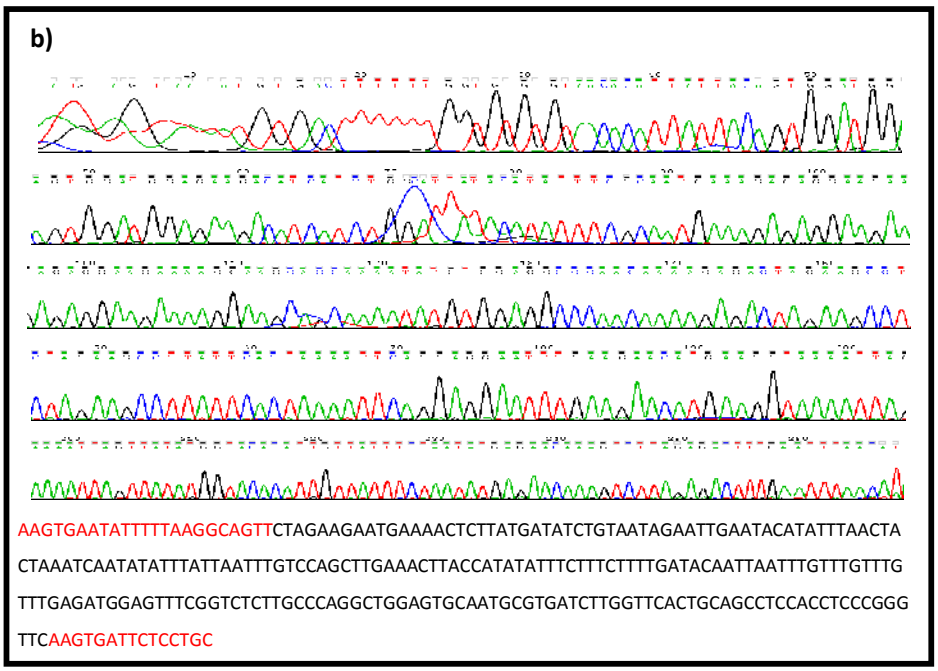


Figure 4.4m: Representative sequencing chromatogram of Exon 20 of BRCA2 gene a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

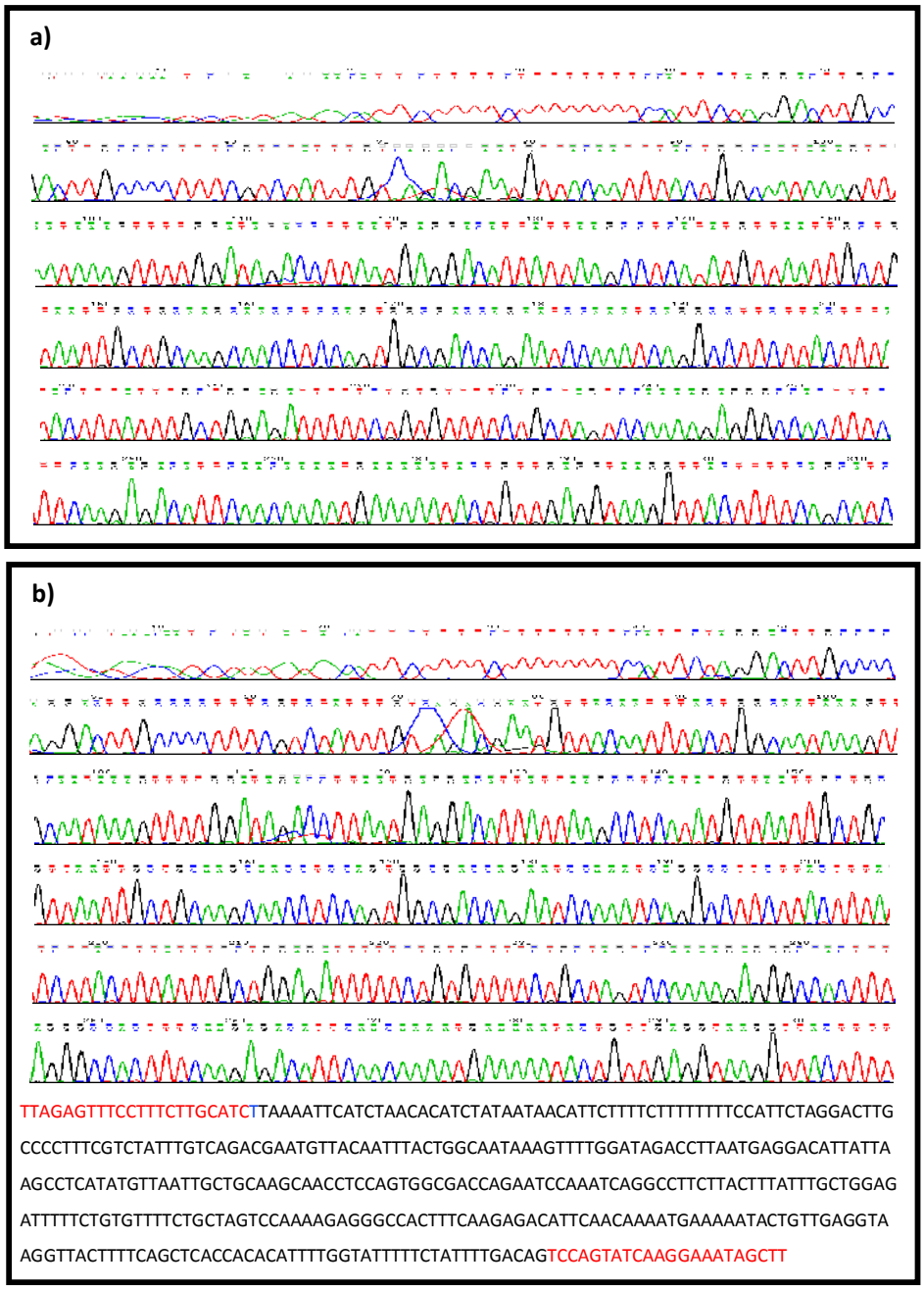


Figure 4.4n: Representative sequencing chromatogram of Exon 25 of BRCA2 gene a) Tumor sample b) Normal sample along with its NCBI fasta sequence.

4.3 CONNEXIN 43 ANALYSIS

4.3.1 AMPLIFICATION OF CONNEXIN 43

Four sets of primers were used to amplify coding region of Connexin 43 using Nested PCR method. In the first round, 1331bp product which contains the entire coding region plus a small portion of an intron at the 5' UTR was amplified using set 'A'. In the second round, the generated amplicons were used as template to specifically amplify the N-terminal, mid-region and C-terminal region of Connexin 43 using primer sets 'B', 'C' and 'D' strictly in accordance with the conditions described in methods (Table 3.3). PCR product (5µl) was analyzed on the 1% agarose gel. After visualizing under UV light, it was seen that the amplicons were successfully amplified from DNA samples, generating specific PCR products strictly as per the expected size and were sufficient to be purified and sequenced. Prior to sequencing, the final concentration of amplicons was adjusted to 50ng/µl.

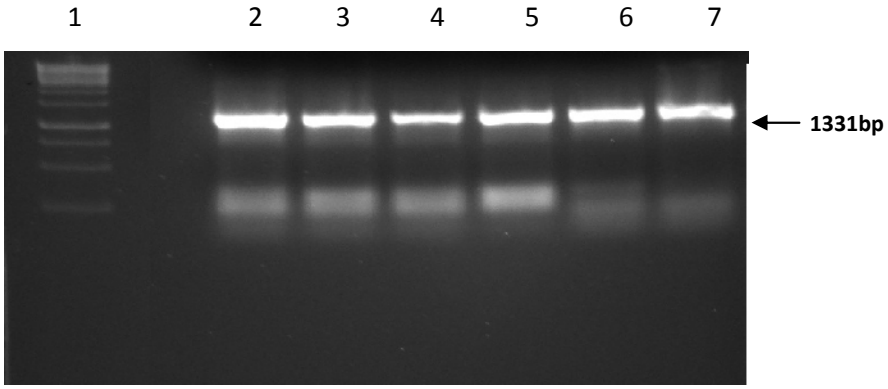


Fig 4.5a: Amplification product of entire coding region of Cx 43 along with small portion of UTR (1331bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-7 show analysis of 5µl aliquot of PCR product.

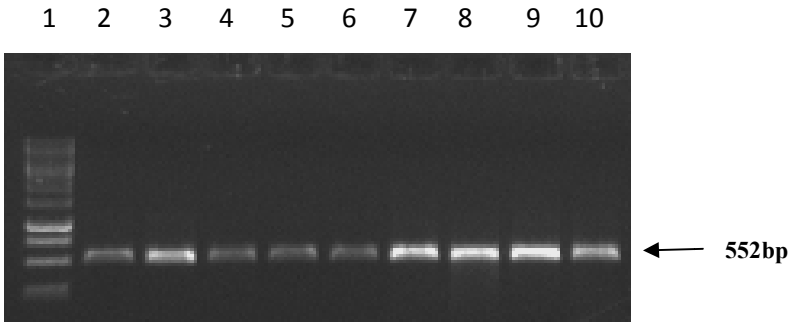


Fig 4.5b: Amplification product of N-terminal region of Cx43 (552bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. Lane 1 shows the separation pattern of 1 kb ladder, 2-10 show analysis of 5µl aliquot of PCR product.

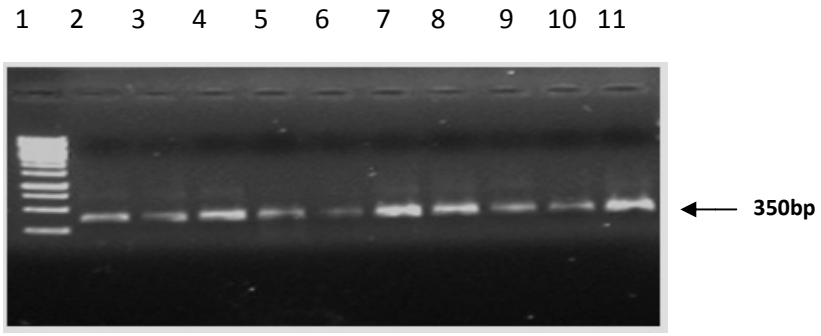


Fig 4.5c: Amplification product of mid-region of Cx43 (350bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. a) Lane 1 shows the separation pattern of 1 kb ladder, 2-11 show analysis of 5µl aliquot of PCR product.

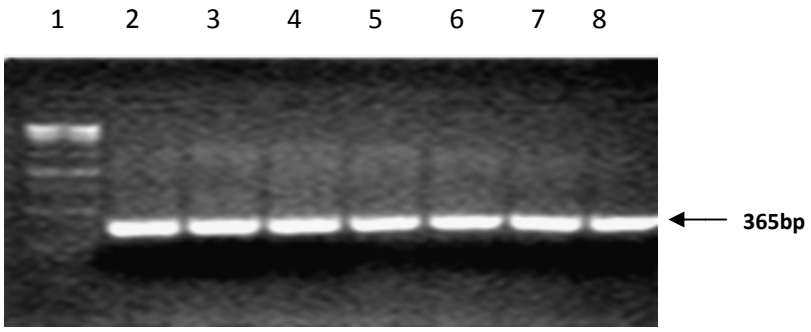


Fig 4.5d: Amplification product of C-terminal region of Cx43 (365bp). PCR products generated from various samples run on 1.5% agarose gel with 1 kb DNA ladder marker. a) Lane 1 shows the separation pattern of 1 kb ladder, 2-8 show analysis of 5µl aliquot of PCR product.

4.3 SEQUENCE ANALYSIS

The sequencing data was obtained commercially from the Center for Genomics Application, New Delhi. DNA sequences of the amplicons were obtained in fasta and pdf formats. The fasta files were analyzed using *ClustalX* software for sequence alignment and by *Chromas Pro* software for the detailed inspection of the chromatograms individually. The pdf file of each DNA sequence was used for visual inspection of the entire sequencing chromatograms.

Sequence analysis (which included thorough analysis of the chromatograms and multiple sequence alignments) of all the three fragments of Connexin 43 gene in 50 sporadic tumor samples of breast cancer patients from Kashmir did not reveal any kind of variation with respect to standard Connexin 43 sequence available in NCBI database in any of the samples screened.

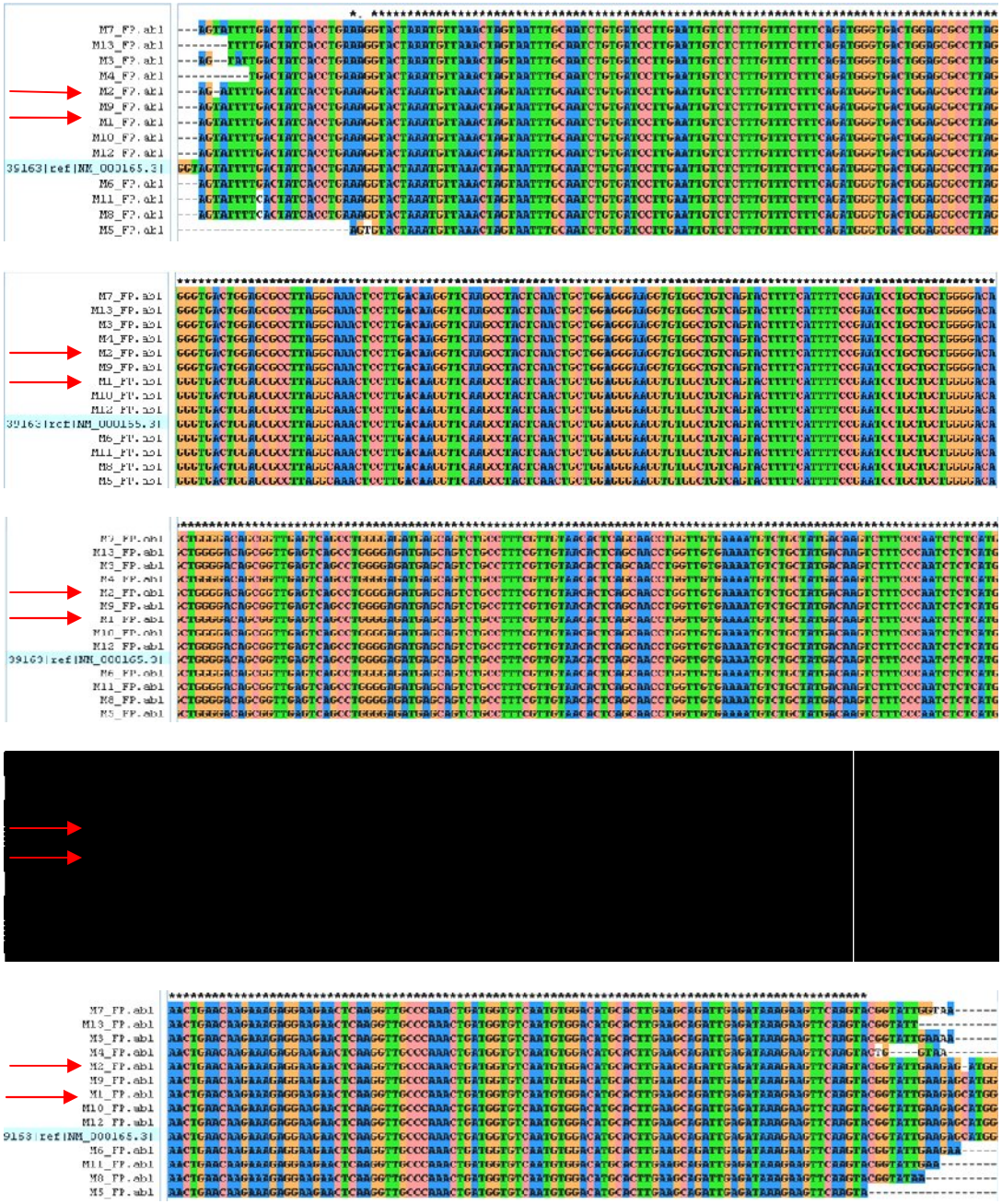


Figure 4.6a: Multiple sequence alignment of samples generated from N-terminal region amplification of Cx43. The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by arrow head), using Clustal X software.

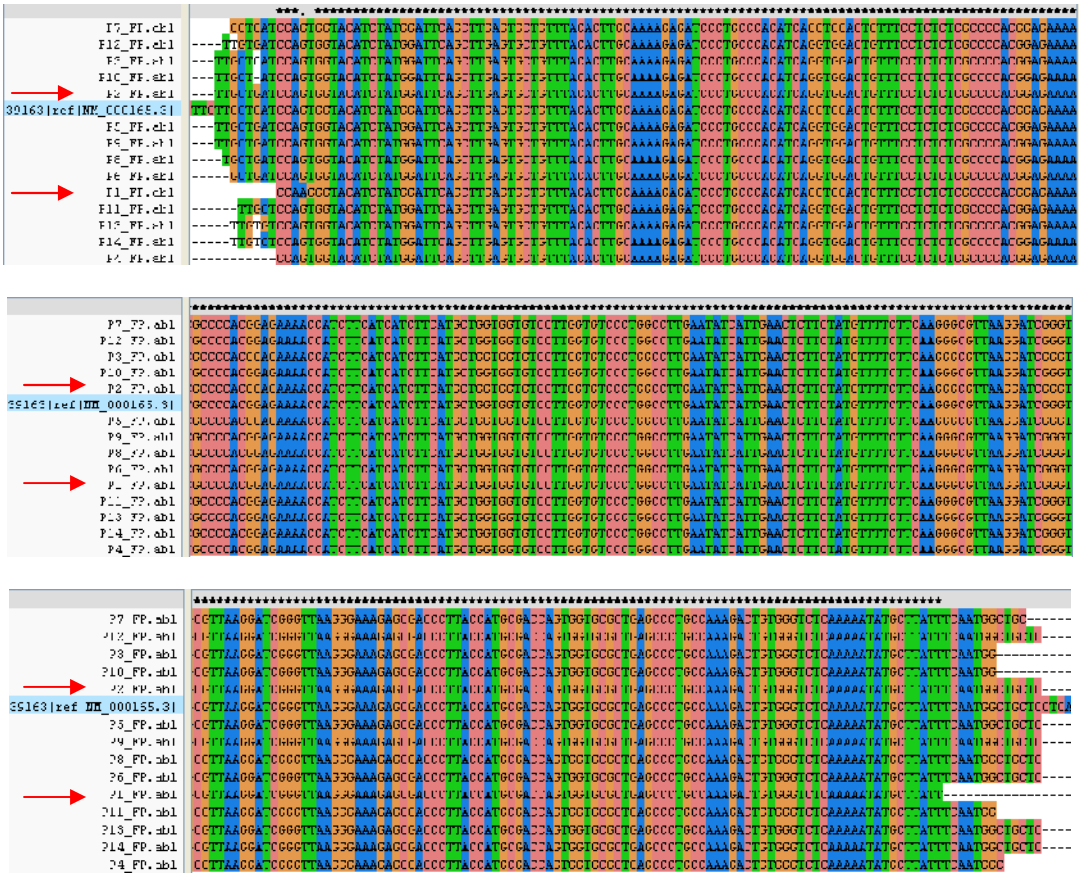


Figure 4.6b: Multiple sequence alignment of samples generated from mid-region amplification of Cx43. The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by arrow head), using Clustal X software.

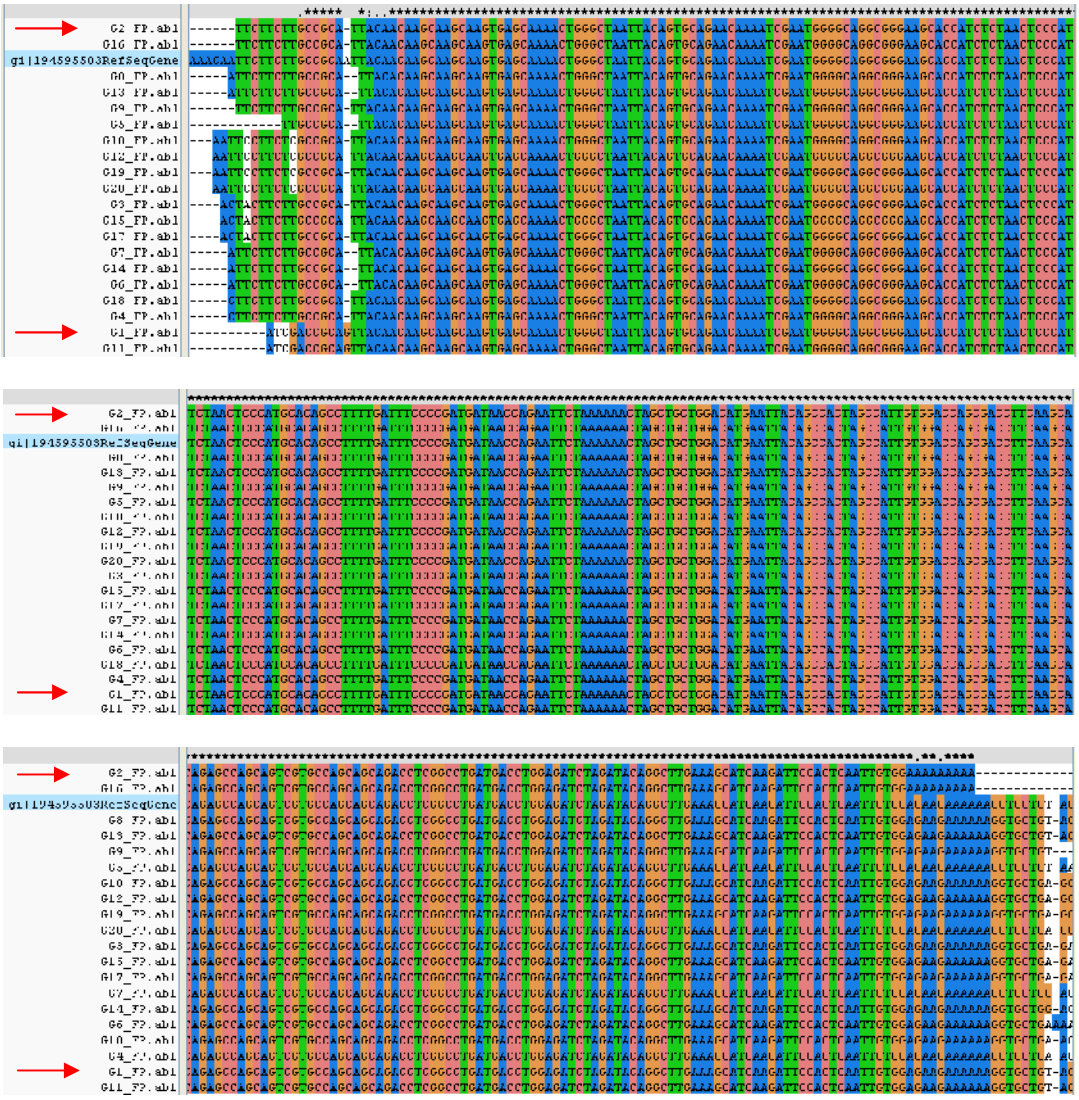


Figure 4.6c: Multiple sequence alignment of samples generated from C-terminal region amplification of Cx43. The fasta sequences of samples were aligned with the reference sequence available from NCBI website (blue higlight), in addition to the sequence of a normal DNA samples (pointed out by arrow head), using Clustal X software.

CHAPTER 5

DISCUSSION

CHAPTER FIVE

DISCUSSION

The contribution of mutations in different genes like BRCA1 and BRCA2 to the incidence and prevalence of breast cancer has been established worldwide and many more genes which could play role in the tumorigenesis of this disease at the primary or secondary level are unveiled continuously. Some of them directly influence BC risk, whereas others are involved in the general process of cancer growth and metastasis. However, the role of these genes in pre-disposing Kashmiris to breast cancer is least explored represented by only a few studies. Kashmir, which lies at an altitude of 1800-2400km from the sea-level, is distinct from other areas in term of its location, culture, food habits and ethnicity. In the study population, consanguinity is the choice and the risk factors associated with the breast cancer in other regions like the nulliparity (Gail et al., 1989; Winer et al., 2000), consumption of alcohol (Longnecker et al., 1994) are lacking.

BRCA2 is one the well established breast cancer susceptibility gene mutated form of which when inherited strongly predisposes to breast cancer. In an attempt to screen the BRCA2 gene in Kashmiri population to establish its role in predisposing Kashmiri population to breast cancer, we have located four somatic mutations in BRCA2 gene at codon positions 846 (rs11571654), 868 (Novel), 991 (rs1799944), 1131 (rs1801406) and one germ-line variation in the UTR region of exon 2 at contig position 13870572 (rs1799943).

The amino-acid change at 991 position seen in 24% (12/50) of the patients is a mis-sense change and this change substitutes asparagine by aspartic acid. This amino-acid lies in the BRC1 repeat of exon 11, which constitutes one of those 4 repeats of BRCA2 protein that have been seen to be highly conserved in mammals (Bork et al. 1996; Bignell et al. 1997). These BRC repeats in BRCA2 protein helps in directly binding BRCA2 to RAD51, a critical protein for

DNA recombination and doube-stranded DNA repair (Benson et al., 1994; Donovan et al., 1994; Hays et al., 1995). The association of this mis-sense mutation to breast cancer susceptibility has been recently reported in Cyprus population (Maria et al., 2009). The authors have found significant association of this change with the increased risk of breast cancer. (p=0.01 and P=0.0076). A moderately strong association of this BRCA2 polymorphism with malignant melanoma risk has also been reported (p=0.02 after Bonferroni correction) (Debniak et al., 2007). In-silico prediction methods also suggest that this is a non-tolerated amino-acid substitution within the limits of confidence in the alignments (Fackenthal et al., 2004). The role of this SNP in breast cancer has also been investigated in the Multi-ethnic cohort study and no association has been found (Freedman et al., 2004). Apart from this mis-sense mutation, a novel missense mutation is reported at position 846 replacing amino-acid proline by threonine. This novel missense mutation was seen in 88% of the Breast cancer patients and was always linked with another silent mutation at amino-acid position 826. This change was observed in the N-terminal region of exon 11 and lies very close to the highly conserved BRC1 repeat. This amino-acid change can be very significant as proline belonging to non-polar aliphatic group has been replaced by threonine belonging to a group of polar and uncharged amino-acids. The proline reduces the structural flexibility of protein at that position as the secondary amino (imino) group of proline is held in rigid conformation, thus this substitution can play important role in altering the functional properties of the protein. However, the strong association of this mis-sense mutation with another silent mutation at position 846 delivers something very strong and needs to further elucidated. It may be possible that the duo change is actually making carriers more susceptible to the breast cancer in this cohort of population. The germline variation G > A observed in the UTR region of the exon 2 was observed in 32% (16/50) of total patients. This variation observed is just 25 bases ahead of initiation codon in exon 2, thus there is a high probability that it may play an important role in RNA processing. Though this variant has been classified as a

variant of no clinical significance in the Breast Cancer Information Core Database (BIC), yet large and well controlled population based studies needs to be done to establish the full range of risks associated with this variant, as the association may be missed if only a small number of cancers are studied.

Apart from these mis-sense mutations, no deletion or insertion was seen. No mutation of any kind either germ-line or somatic was observed in exon 9, 18, 20 and 25 screened. Nonetheless, the number of disease associated BRCA2 mutations in Kashmiri sporadic breast cancer cases is very low and this study is comparable to the data available from other places. A mutational analysis of BRCA2 gene in North-Indian population has revealed no major sequence variation in BRCA2 except few mis-sense mutations (Saxena et al., 2002). They have not also reported any kind of deleterious or truncation mutation in any patient.

The comparison of various clinico-epidemiological attributes of sporadic breast cancer patients to these somatic mutations had shown some kind of association although not significant with early onset and late onset breast cancer cases, menopausal status, advanced clinical stage (III and IV) of the disease. However there was no significant association seen with positive lymph node status and breast involved. Clinical tumor stage (III and IV) is a finding which assumes significance in view of the fact that it reflects poor prognosis.

Decreased expression of Connexin 43 gap junctions is seen in breast cancer at various stages of progression and restoration of gap-junction intercellular communication by up-regulation of Connexin43 has been shown to restore normal phenotypes (Laird et al., 1999; Mehta et al., 1991). Studies have shown that the tumor-suppressive capacity of individual connexins is very connexin-specific and tissue-specific, implying that mutational deactivation of certain connexins with particularly strong tumor-suppressive potency, but not others, may be etiologically implicated in tumorigenesis in tissues where they are expressed. Since Connexin 43 is predominantly expressed in gap junction protein in normal breast tissue and is a disease susceptibility gene for breast cancer, we

attempted to study whether Connexin 43 expression is impaired in neoplastic breast tissue due to any mutations in Kashmiri cohort of population.

Compared to BRCA2, our results did not show any kind of variation whether somatic or germline in the coding region of Connexin 43 in any of the patients. As connexin genes have been suggested as tumor suppressor genes, it is expected that mutational deactivation of these genes during carcinogenesis can constitute strong evidence in favor of their tumor-suppressive capacity. In contrast, our results suggest that Cx43 gene mutations are rare in breast cancer. The lack of Cx43 mutations in human breast cancer samples makes it apparent that mutation of Cx43 is a rather rare event in tumor formation. Thus our study indicates that Connexin 43 is not down regulated in breast tumors due to any mutations. Instead some other regulatory mechanism like hypermethylation or mutation of the promoter region of the gene may play an important role in the down regulation of this gene.

Although some studies have reported mutations in Cx43 in advanced stage of the cancer (Dubina et al., 2002); on the contrary, we also did not report any kind of variation in the patients who were in the advanced stage of the disease. Thus our studies strongly implicate that mutational deactivation does not play any role in primary tumor formation or its metastasis in the breast. Our studies also imply (like Lee et al., 1991) that connexins can be described as class II tumor suppressors as the genes encoding connexins are not mutated while the protein expression levels are frequently altered, presumably due to the mutation of an upstream regulator or other factors. However it is debatable how far downstream connexins are from the primary assault and whether their regulation is a key player in carcinogenesis or simply a distant secondary effect.

In summary, the lack of Cx43 mutations and a high prevalence of somatic mis-sense mutations in BRCA2 suggest no role of Cx43 mutations in breast cancer predisposition in Kashmiri population but a definite involvement of BRCA2 mutations in the disease. Although no insertion or deletion was reported, but these mis-sense variations cannot be nullified as the prevailing polygenic

model of breast cancer risk suggests that a moderate number of genes, each conferring a small amount of risk alone (relative risk 1.3-1.5), together would combine multiplicatively, resulting in modest susceptibility to breast cancer (Antoniou et al., 2002). According to this model, more than 100 genes may contribute to breast cancer susceptibility. Each gene could have either common or rare variants and the women carrying more variant alleles would be at greater risk than those carrying fewer. Thus our results implicate that these somatic mis-sense variants may probably be contributing to the breast cancer susceptibility along with variations in other low penetrating genes in sporadic type of breast cancer in this cohort of population.

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