CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of breast cancer

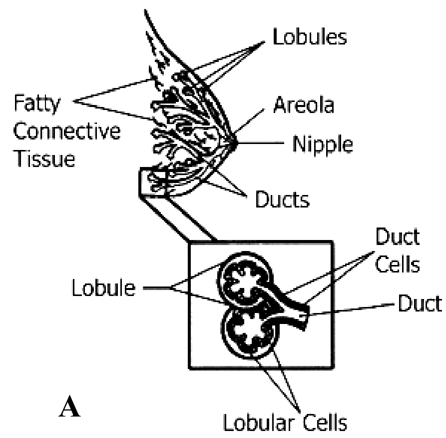
2.1.1 Breast anatomy and physiology

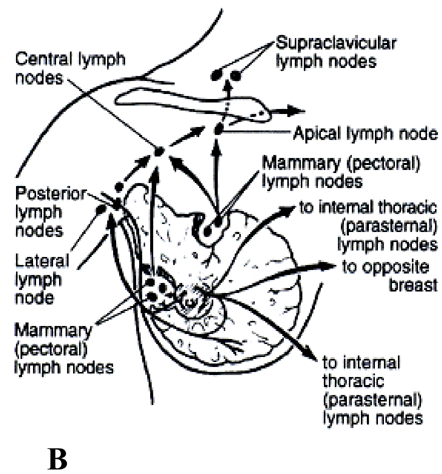
The breast is a mass of glandular, fatty and fibrous tissue. It is positioned over the pectoral muscles of the chest wall and attached to the chest wall by Cooper's ligaments. A layer of fatty tissue surrounds the breast glands and extends throughout the breast. The breast is composed of: (Fig. 2.1A)

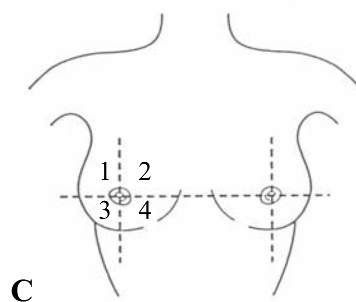
- milk glands (lobules) that produce milk
- ducts that transport milk from the milk glands to the nipple
- nipple
- areola
- fibrous tissue that surrounds the lobules and ducts
- fat

2.1.2 Breast cancer

Breast tumors may arise in the ductal epithelial cells and spread beyond the breast duct or lobule wall. While the breast tumor is increasing in size, it invades the basement membrane, mammary fat, underlying muscle, overlying skin and spreads to the blood vessels and lymph vessels of the dermis (Fig. 2.1B). Dermal lymphatic invasion causes obstruction of lymphatic drainage and therefore, commonly correlates with clinical erythema and induration of the skin of the breast, so called peau d' orange. Cancer of the breast affects the left breast slightly more often than the right. The locations of the tumors within the breast and percentage of each location are as follows in Fig. 2.1C and Table 2.1, respectively.







1 = UOQ : upper outer quadrant

2 = UIQ : upper inner quadrant

3 = LOQ : lower outer quadrant

4 = LIQ : lower inner quadrant

Fig. 2.1 Schematic illustration of breast anatomy (A), lymphatic drainage of the breast (B) and breast position (C).

Table 2.1 Locations and proportions of breast tumors

Locations	Percentage
Upper outer quadrant	50
Central portion	20
Lower outer quadrant	10
Upper inner quadrant	10
Lower inner quadrant	10

2.1.3 Classification of the breast cancer (Kumar *et al*., 1997 ; Rubin *et al*., 2005)

Breast cancer can be divided into two major groups, noninvasive (*in situ*) carcinoma and invasive carcinoma. Noninvasive carcinoma cancer cells are confined to the ducts and do not invade surrounding fatty and connective tissues of the breast. It includes ductal carcinoma *in situ* (intraductal carcinoma with or without invasion) and lobular carcinoma *in situ*. Invasive carcinoma cancer cells break through the duct and lobular walls and invade the surrounding fatty and connective tissues of the breast. It comprises invasive ductal carcinoma, invasive lobular carcinoma and uncommon types of invasive breast cancer (such as medullary carcinoma, colloid carcinoma, etc.)

2.1.3.1 Invasive carcinoma

2.1.3.1.1 Invasive ductal carcinoma (IDC) or infiltrating ductal carcinoma

IDC is the most common type of breast cancer accounting for 75% of breast carcinomas. It is formed in the milk ducts of the breast, penetrates the wall of the duct and invades the fatty tissue of the breast and other regions of the body. Clinically, it is usually manifested as a deceptively delimited mass, rarely over 3 to 4 cm in diameter, stony hard consistency. On gross examination, the tumor is typical firm and shows irregular margins. The cut surface is pale gray, gritty, flecked with

yellow and chalky streaks. Histologically, the lesion is composed of dense fibrous stroma of tumor cells, dark nuclei with few mitosis.

2.1.3.1.2 Invasive lobular carcinoma (ILC) or infiltrating lobular carcinoma

ILC is the second most common type of breast cancer. It is formed in the milk glands (lobules) of the breast but often spreads (metastatizes) to other regions of the body. It accounts for 10-15% of breast carcinomas. The clinical presentation of invasive lobular carcinoma varies from a discrete firm mass. It consists of single strands of malignant cells infiltrating between stromal fibers.

2.1.3.1.3 Intraductal carcinoma with or without invasion

This type represents about 5% of breast carcinomas. It grows within the ducts without invading the ductal basement membrane and underlying breast tissue. Histologically, the neoplastic cells may initially invade the ducts to create irregular excrescences.

2.1.3.1.4 Medullary carcinoma

It represents approximately 5% of breast carcinomas. The morphology of medullary carcinoma has a distinctive gross appearance, stony hard and often become larger up to 10 cm in diameter. Microscopically, it is composed of sheets of cells that are highly pleomorphic and high mitotic index.

2.1.3.2 Carcinoma *in situ*

2.1.3.2.1 Ductal carcinoma *in situ* (DCIS)

It is the most common type of nonvasive breast cancer. It has not spread past the milk ducts to the fatty breast tissue or any other regions of the body. It is composed of very large, pleomorphic cells that have abundant eosinophilic cytoplasm and irregular nuclei.

2.1.3.2.2 Lobular carcinoma *in situ* (LCIS)

It arises in the terminal duct lobular units. Tumor cells tend to be small and round with regular nuclei and minute nucleoli.

2.1.3.3 Cancer of the male breast

Cancer in the male breast accounts for less than 1% of all cases of breast cancer. Invasion of chest wall muscles is more frequent at the time of diagnosis in men. The prognosis for male breast cancer is similar to that for the female.

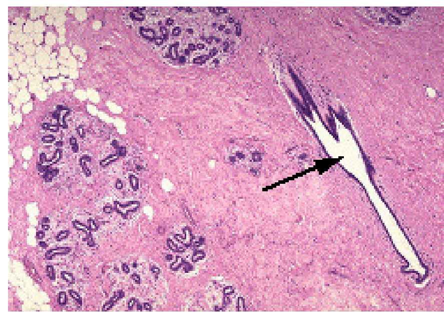


Fig. 2.2 Normal breast tissue. The arrow points to a cross-section of a duct. Lobules can be seen off to the left (Internet Pathology Laboratory, University of Utah).

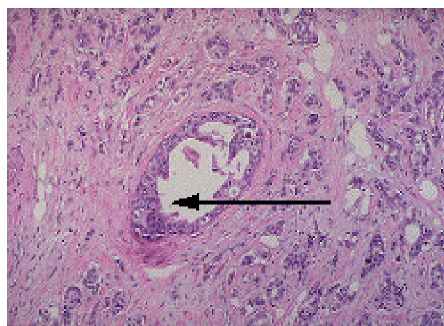


Fig. 2.3 Cancerous breast tissue. The arrow points to a duct lined by cancer cells. This type of breast cancer is called ductal carcinoma (Internet Pathology Laboratory, University of Utah).

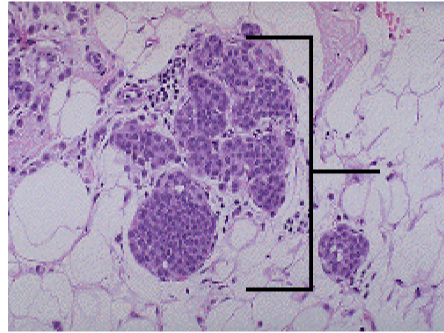


Fig. 2.4 Cancerous breast tissue. The bracket shows the area of breast tissue affected by lobular carcinoma (Internet Pathology Laboratory, University of Utah).

2.2 Risk factors

The cause of breast cancer is still unclear but research studies have shown that breast cancer is multifactorial. Among the most important risk factors are:

2.2.1 Age

Age is the most influential risk factor for developing breast cancer. Risk of breast cancer is lower in young than in older women. Women younger than age 40 accounted for only 4.7% of breast cancer diagnosis. Over 70% of all breast cancer diagnoses are made in women who are 50 or older (American Cancer Society, 2001). However, breast cancer severity and aggressiveness is greater in early-onset breast cancer. It is thought that tumors in young women may be biologically different based on the fact that in them, tumors have higher proliferation levels and are less differentiated. Usually, women suffering early onset breast cancer live a short time free of the disease, and have lower survival rates (Ruiz-Flores *et al.*, 2001). In addition, women with early onset of menarche (at or before 12 years of age) or late

menopause (after 55 years) have an increased risk of developing breast cancer (Evans and Lalloo, 2002).

2.2.2 Family history

A positive family history is one of the strongest risk factors for the development of breast cancer. The number of affected relatives and the closeness of their biologic relationship are also important factors. These risks also increase with the increasing number of affected relatives. A women with a mother, sister or daughter (first-degree relative) with breast cancer has the highest risk of developing the disease (Phillips, 2001). In addition, low-incidence families such as second-, third-, and fourth-degree relatives (grandmothers, aunts, and cousins) have low risk of breast cancer (King *et al*., 2003). So this shows that the proportion of affected relatives in families may be the most important indicator of breast cancer risk. Therefore, the important features in a family history are age at onset, bilateral disease, multiple cases in the family (particularly on one side), other related early onset tumors and number of unaffected subjects (large families are more informative) (Atri *et al*., 2002). Studies of family history suggest the presence of both breast and ovarian cancer in a family increases the likelihood that a cancer-predisposing mutation is present (Smith *et al*., 1992 ; Coliin *et al*., 1995). Family history can be used by clinicians to predict which patients are at increased genetic risk of cancer. It also can identify those who may benefit from genetic counselling and genetic testing (Emery *et al*., 2000). At present, a computer program has been developed for prediction of personal risk factors in women with positive family history (Amir *et al*., 2003).

2.2.3 Hormonal and reproductive factors

Hormonal and reproductive factors have long been recognized to be important in the development of cancer. Indirect evidence suggests that early menarche, older age at menopause and older age at first full-term pregnancy are

associated with increased risk of breast cancer by affecting endogenous reproductive hormones (Chie *et al*., 2000 ; Hulka and Moorman, 2001). Reproductive factors linked to estrogen production are associated with breast cancer risk. Nulliparity is associated with an increased risk and parity reduces the risk (Nkondjock and Ghadirian, 2004). It has been shown that prolonged exposure to endogenous estrogens is a risk factor for breast cancer (Hilakivi-Clarke, 2000). Estrogen is a steroid hormone. It has a role in regulating the differentiation and proliferation of normal breast epithelial cells including ovarian, cervix and vagina. Experimental data suggest that estrogens promote the development of mammary cancer in rodents and exert both direct and indirect proliferative effects on cultured breast cancer cells from humans (Clemons and Goss, 2001). Studies suggest that estrogen influence breast cancer risk through effects on cell proliferation and genetic instability, perhaps by inducing free radical-mediated DNA damage and mutations. Genetic instability increases the normal cells are turned to the malignant pathway (Adami *et al*., 1998 ; Clemons and Goss, 2001). In previous observations, BRCA1 was recently shown to have an ability to regulate the cellular response to estrogens. In *in vitro* studies conducted using human breast cancer cells, BRCA1 protein inhibited estrogen receptor (ER)-α-mediated transcriptional pathways related to cell proliferation (Fan *et al*., 1999, 2001). This finding suggests that in addition to maintaining genomic instability during periods of rapid cellular division and multiplication, BRCA1 may also suppress signaling initiated by estrogen-induced activation of ER-α. Thus, during puberty and pregnancy, when estrogens and BRCA1 expression are both significantly increased, the function of BRCA1 may be to protect the breast from estrogen induced genetic instability by inhibiting ER-mediated pathways and repairing genetic damage. BRCA1 might also be particularly important in controlling cellular proliferation. A loss of *BRCA1* function leads to the accumulation of genetic damage, changes in the mammary gland morphology and development of breast cancer (Hilakivi-Clarke, 2000).

In addition, exogenous estrogens, either the oral contraceptive (OCP) or hormone replacement therapy (HRT), also confers increased risk of breast cancer. The estrogen element of the oral contraceptives, although suppressing ovulation, will

still stimulate the breast cells (Gram *et al*., 2002 ; Kumle *et al*., 2002). Collaborative analysis of individual data from 54 epidemiological studies showed a statistically significant increased (24%) in risk associated with current use of oral contraceptives. However, women who stopped using oral contraceptives 10 years or more in the past have the same risk as women who have never used the pill (Collaborative group on hormonal factors in breast cancer, 1996 ; Nkondjock and Ghadirian, 2004). Recent use of combination hormone replacement therapy (HRT) has been shown to increase breast cancer risk, with higher risk associated with longer use (Rossouw *et al*., 2002 ; Olsson *et al*., 2003).

2.2.4 Race / Ethnicity

Ethnic differences in breast cancer incidence and mortality have been consistently associated with differences in genetic and epidemiologic risk factors. 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), with the highest age-standardized incidence in North America (99.4 per 100,000). The incidence is more modest in South America (46 per 100,000), Eastern Europe (42.6 per 100,000), Southern Africa (33.4 per 100,000) and Western Asia (33.3 per 100,000). The rates are low (<30 per 100,000) in most of Africa (with the exception of South Africa) and in most of Asia. The lowest incidence is in Central Africa (16.5 per 100,000). The age-standardized incidence and mortality rates for breast cancer are shown in Fig. 2.5. In part, the high incidence in the more affluent world areas is likely because of the presence of screening programs to detect early invasive cancers, some of which would otherwise have been diagnosed later or not at all.

Caucasian women are more likely to be diagnosed with breast cancer than African American women. However, African American women are more likely than white women to die of breast cancer. Women of Asian, Hispanic or American Indian descent are at lower risk than Caucasian or African American women for developing breast cancer (Ries *et al*., 2001). The lifestyle factors between population can be attributed to the difference risk of breast cancer.

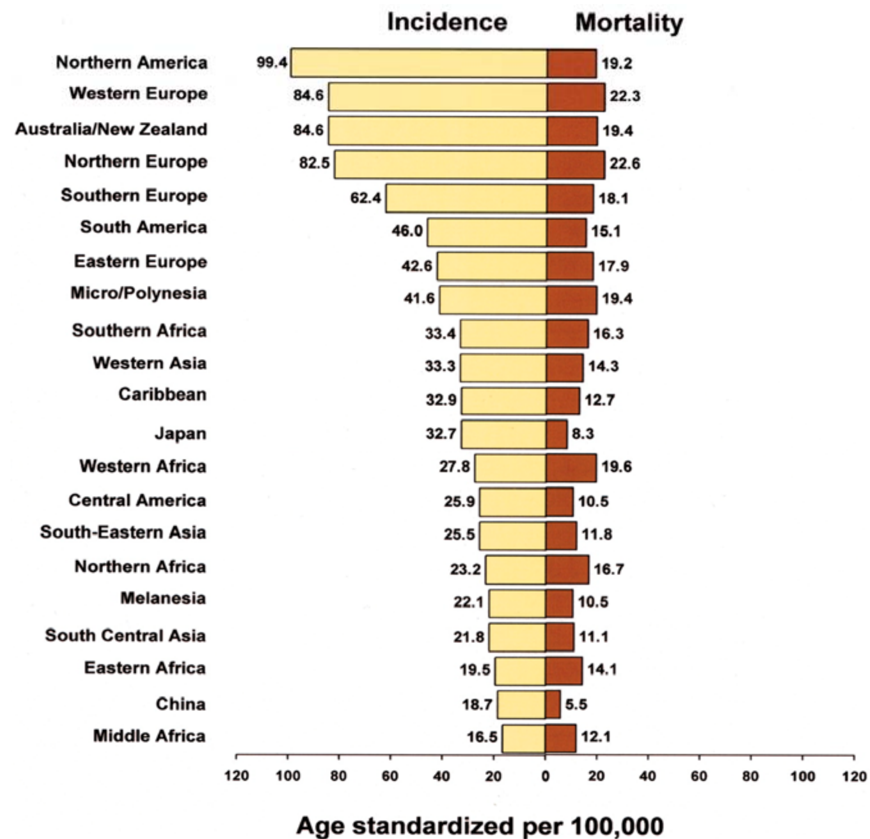


Fig. 2.5 Age-standardized incidence and mortality rates for breast cancer (Parkin *et al*., 2005.)

2.2.5 Smoking

Evidence for the association between smoking and breast cancer risk remains inconsistent. Some studies have suggested a positive link in the general population, while others have found no relationship and a few have reported that smoking has a protective effect. A recent study suggested that women who were exposed to cigarette smoke (both active and passive smoking) were indeed at higher risk of breast cancer (Kropp and Chang-Claude**,** 2002). Other study proposed that smoking of long duration, smoking before a first pregnancy and passive smoking probably increased breast cancer risk (Terry and Rohan, 2002). The carcinogenic effects of different compounds found in tobacco smoke have been hypothesized to be stronger or weaker according to genotypes. The individual human body differently

activated or detoxified those compounds (Nkondjock and Ghadirian, 2004). A positive association was evident between the duration of smoking and breast cancer risk among African-Americans with mutations of another gene related to DNA repair (*XRCC1* codon 399 Arg/Arg genotype), although no relationship was apparent among white women. It is possible that the direction and magnitude of the association of breast cancer observed with cigarette smoking vary with the characteristics of the studied population (Duell *et al*., 2001).

2.2.6 Breastfeeding

It is found that the longer women breastfeed the more they are protected against breast cancer (Collaborative group on hormonal factors in breast cancer, 2002). This effect is also accounted for by several hypotheses: first, direct pituitary action on ovarian activity, changes in postpartum hormonal state, decreasing estrogen levels through an ovulation. Therefore, accumulated exposure to estrogen is reduced. Second, breast-feeding favors carcinogen expulsion through milk. Experiments evaluating induction of breast tumors in mice, demonstrated more tumors in glands with excised nipples than in normal mice suggesting that nipple obstruction causes a prolonged carcinogen exposure (Ruiz-Flores *et al*., 2001).

2.2.7 Food

Studies have not shown a clear association between food and increased risk of breast cancer. But experimental evidence in animals demonstrates that a high fat intake increases the rate of mammary tumor formation. However, prospective epidemiologic studies generally have not found a relationship between fat intake and breast cancer. It is well known that measurement of dietary intake is inexact and prone to misclassification, which would tend to obscure a true relationship (Hulka and Moorman, 2001 ; Nkondjock and Ghadirian, 2004). Other dietary components that have been investigated including micronutrients such as vitamins A, C, E and selenium. Vitamins A, C, E and selenium are thought to reduce cancer risk through their antioxidant properties (Hulka and Moorman, 2001). In addition, women who

take physical exercise and maintain a healthy weight in early life are protected against breast cancer after menopause (King *et al*., 2003).

2.2.8 Benign breast disease

Benign breast disease has 2 types, namely proliferative and nonproliferative disease. Either proliferative or nonproliferative disease indicates the growth of abnormal cells and risk of increased developing breast cancer. Proliferative disease with atypical hyperplasia features increases in breast cancer risk some three- to fourfold; without atypical hyperplasia features (Hulka and Moorman, 2001).

2.2.9 Environmental factor

Environment might contribute to breast cancer development. Studies are focused on a possible link between environmental pollutants, such as pesticides or ionizing radiation and an increased risk of breast cancer, but no clear link has been established. It is suggested that women having long time exposure to pesticides such as chlorophenothane, dichlorodiphenyl trichloroethane (DDT) and polychlorinated biphenyls (PCBs) or radiation exposure have an increased risk of breast cancer (Hulka and Moorman, 2001).

2.2.10 Genetic factor

Approximately 5-10% of all breast cancer cases are thought to be caused by genetic mutations in the cancer susceptibility genes, *BRCA1* and *BRCA2*. Several other genes known to be responsible for inherited susceptibility to breast cancer include *TP53* in Li-Fraumeni syndrome, *PTEN* in Cowden's disease and *ATM* in Ataxia Telangiectasia (Ruiz-Flores *et al*., 2001). Of these, *BRCA1* is the most common breast cancer susceptibility gene. It accounts for nearly 50% of hereditary breast cancer (Hofmann *et al*., 2000 ; American Medical Association, 2001). Germ-line mutation in *BRCA1* puts women at high risk for developing breast cancer at early age, although the disease may develop at any age. Risks of breast cancer to relatives with *BRCA1* mutation was 20% by age 40, 55% by age 60 and >80% by

age 80 (Table 2.2) (King *et al*., 2003). In addition, lifetime risk of breast cancer and ovarian cancer in women with *BRCA1* mutation is listed in Table 2.3.

Table 2.2 Cumulative risks (standard error) of breast cancer among relatives with *BRCA1* mutation (King *et al*., 2003).

Risk by age (year)	Breast cancer
	BRCA1
30	0.03 (0.01)
40	0.21 (0.03)
50	0.39 (0.04)
60	0.58 (0.05)
70	0.69 (0.05)
80	0.81 (0.06)

Table 2.3 Estimated cancer risks in *BRCA1* mutation carriers (White *et al*., 2002)

	Lifetime risk	
Cancer type	*BRCA1* mutation carriers	General population (non-carrier)
Breast	56%-85%	12.5%
Ovarian	15%-60%	1.4%
Contralateral Breast cancer	Up to 65%	15%-20%
Ovarian cancer secondary to breast cancer	30%-60%	2%-3%

2.3 Breast cancer susceptibility gene 1 or *BRCA1*

Breast cancer susceptibility gene 1 or *BRCA1* is a tumor suppressor gene. The concept of tumor suppressor genes arose from Knudson's observation that the inactivation of two alleles of the retinoblastoma gene (*Rb*). All patients with hereditary retinoblastoma carry a germ-line mutation in one allele of the *Rb* gene, leaving them with only one functional copy. Loss of the remaining functional allele results in the inactivation of both *Rb* alleles: the first one by the inherited germ-line mutation and the second by a somatic event. Thus, he proposed a two-hit hypothesis (Knudson, 1971) (Fig. 2.6). In familial cancers, the first mutation (germ-line mutation) is inherited, and present in all cells of an individual; the second hit is somatic resulting frequently from loss of the wild type allele. In the sporadic cancers both hits are somatic. Within familial cancers, the loss of the remaining functional gene can often be detected in tumor samples by the loss of genetic markers in the chromosomal region of interest, this is termed loss of heterozygosity (LOH). Consistence LOH for a genetic marker at a given locus in tumors from multiple patients has been considered strong evidence of the presence of a tumor suppressor gene in that region.

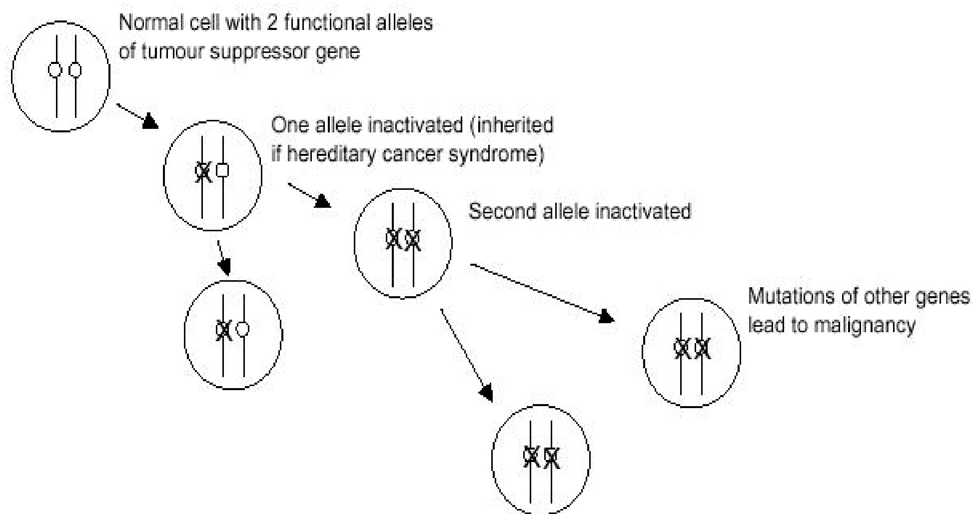


Fig. 2.6 Two-hit hypothesis of tumor suppressor gene (Carter, 2001)

Following the concept, *BRCA1* is regarded as tumor suppressor gene for two reasons (Hofmann and Schlag, 2000). First, germ-line mutation in *BRCA1* (first hit) are associated with tumor development and predispose the carriers to breast cancers. Second, loss of the wild type allele (LOH) (second hit) is frequently (30-80%) observed in sporadic and familial breast cancers (Foulkes *et al.*, 1991 ; Lindblom *et al.*, 1993 ; Cropp *et al.*, 1994 ; Xu and Solomon 1996). These two events lead to inactivation of the tumor suppressor function, loss of control of cell growth, instability in repairing DNA and alterations in genes that regulate programmed cell death (Chai *et al.*, 1999 ; Deng and Scott, 2000).

2.4 Structure and function of the BRCA1 protein

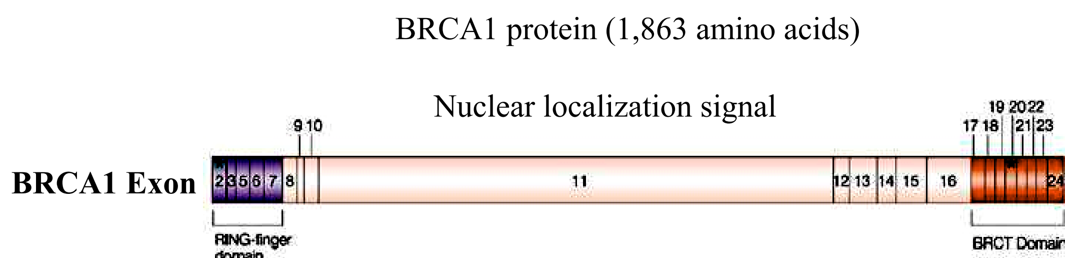
BRCA1 gene is located on chromosome 17q21. It was identified by linkage analysis in 1990 (Hall *et al.*, 1990), and subsequently cloned (Miki *et al.*, 1994). This gene encompasses 24 exons, which are 22 coding exons and two non-coding exons (exon 1, 4) and 22 introns (Smith *et al.*, 1992 ; Hofmann and Schlag, 2000 ; Phillips, 2001 ; Brankovic-majic *et al.*, 2002). It spans approximately 100 kb of genomic DNA. The gene product is a protein of 1863 amino acids and has a molecular weight of 220 kDa (Brzovic *et al.*, 1998 ; Hofmann and Schlag, 2000 ;

Phillips, 2001 ; Ruiz-Flores *et al*., 2001; Arai *et al*., 2004). The BRCA1 protein contains three regions (Deng and Brodie, 2000) (Fig. 2.7).

1. RING-finger domain at the *N*-terminal region, contains a zinc-finger domain (residues 24-64) with a conserved pattern of cysteine and histidine residues, which are found in a variety of proteins that interact with DNA directly or indirectly (Wu *et al*., 1996 ; Jin *et al*., 1997). The first 109 amino acids of BRCA1 protein constitute a protease-resistance domain that is responsible for BRCA1 homodimer and BRCA1-BARD1 (BRCA1-associated RING-domain protein) heterodimer formation (Irminger-Finger *et al*., 1999). This domain also interacts with E2F1 (Meza *et al*., 1999 ; Wang *et al*., 1997) and BAP1 (BRCA1- associated protein1) (Jensen *et al*., 1998).

2. Nuclear localization signal (NLS) domain in exon 11, which is the largest exon coding over 60% of the protein and contains two nuclear localization signals for targeting BRCA1 to the nucleus. It has been shown that BRCA1 interacts with the importin-α subunit of nuclear transporting signal receptor (Chen *et al*., 1996). Proteins that interact directly or indirectly with BRCA1 exon11 include RAD50 (Zhong *et al*., 1999), RAD51 (Scully *et al*., 1997a ; Scully *et al*., 1997b), Rb (Aprelikova *et al.,* 1999) and c-Myc (Wang *et al*., 1998), etc.

3. BRCA1 *C*-terminal domain (BRCT), which consists of two BRCT regions in BRCA1 protein and binds several specific proteins responsible for multiple functions, including roles in DNA repair, transcriptional transactivation and cell-cycle check points. The domain also interacts directly or indirectly with p53 (Ouchi *et al*., 1998 ; Zhang *et al*., 1998 ; Chai *et al*., 1999), RNA polymerase II (Scully *et al*., 1997b), RNA helicase A (Anderson *et al*., 1998), p300 and CBP (CREB binding protein) (Neish *et al*., 1998), histone deacetylase complex (Yarden *et al*., 1999), CtIP (Yu *et al*., 1998), BRCA2 (Chen *et al*., 1998), BACH1 (Caldecott, 2003 ; Clapperton *et al*., 2004) and phosphopeptide (Williams *et al*., 2004).



Region of

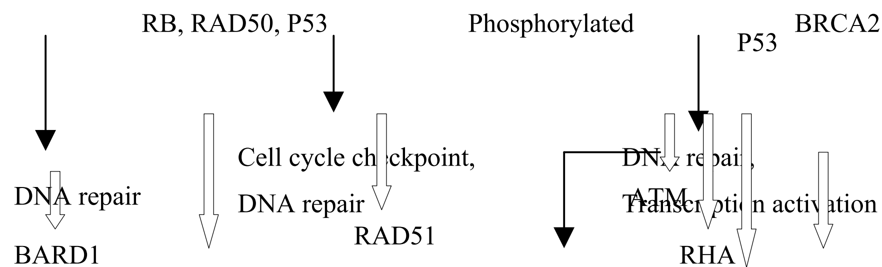


Fig. 2.7 Schematic diagram of the BRCA1 protein and sites of its interaction with other proteins (Deng and Brodie, 2000).

2.5 Pathobiology of hereditary breast cancer

The most pathobiologic characteristic of *BRCA1* related hereditary breast cancers is invasive ductal carcinomas similar to that of sporadic breast cancer. The characteristics of cell are aneuploid, estrogen and progesterone receptor negative, high grade carcinomas with an abundant lymphocyte infiltration and strikingly higher proliferation rate. Invasive ductal histology can show the specific prognostic marker approach to *BRCA1*-related breast cancer. Some also reported that *BRCA1*-associated tumors were more likely to be of meduallary type, to be a higher grade, aneuploid and higher tumor cell proliferation rate (Lakhani, 2003). Thus, it indicated that the relation between *BRCA1*-associated breast cancer data still have not clear. There is increasing need for accurate identification of *BRCA1*-associated breast cancer with immunophenotype of BRCA1 such as estrogen receptor (ER), HER-2/neu. It is an important prognosis and predictive marker for breast cancer (Lakhani, 2003).

2.6 Mutation spectrum of *BRCA1*

The Breast Cancer Information Core (BIC, 2004) database shows mutation data indicating that there are more than 1,500 mutations in *BRCA1* (Table 2.4). The mutations identified are distributed through out the whole coding and noncoding region of the *BRCA1* gene. Approximately 60% of mutations are found in exon11, which is the largest exon in *BRCA1* gene. Most of the known mutations are germ-line mutations and are predicted to result in the truncation of the proteins, caused by frameshift, nonsense or splice site mutation (Evans and Lalloo, 2002).

Frameshift mutation approximately 50% is the most common in *BRCA1*. Nonsense mutation is found in 11% of *BRCA1*, which are C to T transitions occurring at CpG islands and based on the deamination of 5-methylcytosines. Splice site mutation occurrs at intron-exon boundaries. It alters the splicing signal necessary for the proper excision of an intron. Splice site mutation resulting in frameshift mutation was found in 8% of *BRCA1*. Additionally missense mutation was found in 29% of *BRCA1*. Polymorphism, resulting in missense mutation, refers to base change at DNA level that does not lead to a change in the amino acid composition of the protein product. Usually, polymorphism is not associated with disease. Type of mutation is regulatory mutation, which is involved in regulating *BRCA1* expression and affects mRNA production or stability changes in the defined regulatory regions of the *BRCA1* gene (promoters, enhancers or repressors) (Xu and Solomon, 1996). Regulatory mutation was found only 2% in *BRCA1*.

Table 2.4 Total number of mutation, polymorphism and variants of *BRCA1* from Breast Cancer Information Core (BIC) database (Breast Cancer Information Core, 2004)

Exon type	Total number of entries	Distinct mutations, Polymorphism and variants	Alternation report only once
1	2	2	2
2	1713	42	24
3	121	40	25
4	1	1	1
5	301	37	15
6	146	29	17
7	111	28	18
8	108	29	18
9	108	20	12
10	30	13	9
11	45	33	25
11a	814	184	99
11b	820	182	104
11c	1180	215	126
11d	1160	181	98
12	86	36	24
13	319	46	28
14	83	25	15
15	174	37	17
16	430	69	40
17	115	49	30

18	182	51	28
19	88	27	15
20	1030	51	26
21	66	23	15
22	132	26	11
23	49	24	15
24	142	39	21
totals	**9556**	**1539**	**878**

Patmasiriwat and colleagues (2002) reported the study on *BRCA1* mutations in Thai patients from the central region of the country including Bangkok. This study included a total of 23 patients with breast and/or ovarian cancer. Seventeen patients were from 12 families having at least two affected cases of breast or ovarian cancer diagnosed among first degree relatives. The other six out of 23 patients were isolated cases without family history. Of these isolated cases, four were breast cancer patients diagnosed before age 32 years and two had both breast and ovarian cancers. DNA was extracted from genomic DNA, which was separated from peripheral blood leukocytes by standard phenol-chloroform procedure (Sambrook *et al.,* 1989) and screened *BRCA1* variants by PCR-based heteroduplex analysis and DNA sequencing. To study, they found 5 types of mutations in *BRCA1* gene and whose frequencies of *BRCA1* mutation types are shown in Table 2.5.

Table 2.5 Frequencies of *BRCA1* mutation types

Types	**Frequencies**
T320G	3/23 = 0.13
744ins20	1/23 = 0.04
3300delA	3/23 = 0.13
C3271G	2/23 = 0.08
IVS20+78 G>A	1/23 = 0.04

- T320G is a conservative missense mutation in exon 5, which nucleotide changes thymine to guanine at nucleotide 320. This mutation causes a substitution of glutamic acid for aspartic acid at residue 67.

- 744ins20 is a frameshift mutation in exon 10, as a result of 20 base insertions (agggatgaaatcaggagcca). This mutation creates a stop codon at nucleotide 839, leading to premature translational termination at codon 240.

- 3300delA is frameshift mutation in exon 11, whose adenine base at nucleotide 3300 is deleted. This mutation introduces a stop codon at position 1061, resulting in truncated 1060 amino acids BRCA1 protein product.

- C3271G is a conservative missense mutation in exon11, of which cytosine is replaced by guanine at nucleotide 3271. This mutation leads a substitution of serine for threonine at residue 1051.

- IVS20+78G>A is a rare intronic variant, of which guanine is replaced by adenine at position 78 upstream in intron 20.

The aforementioned mutations are disease-related mutations. The *BRCA1* exon 11 3300delA mutation was detected in two out of twelve unrelated risk families and three of the affected cases had experienced ovarian cancer. So 3300delA mutation might be an ovarian cancer-related mutation found within this region. The mutated gene encodes BRCA1 protein product of 1060 amino acids residues. The 744ins20 mutation in exon 10 had been identified in patients both with breast and ovarian cancers. The mutated gene encodes BRCA1 protein product of 239 amino acids residues. Both mutations cause truncation in BRCA1 protein which abolish the integrity of molecular properties necessary for full functions. Additionally C3271G and T320G mutations are missense mutation, which may alter the molecular surface and thus destabilize the interaction with other proteins. Importantly, the *BRCA1* exon 5 T320G mutation was found in two unrelated families. This mutation produces the mutant protein with the substitution of glutamic acid for aspartic acid at position 67 (D67E), which is located in a close proximity of a RING finger domain and located in the proteolysis-resistant domain of BRCA1 protein. Atomic structure of the RING domain reveals two separate Zn^{2+}- binding sites (Brzovic *et al.,* 2001).

Invariant residues including C39, H41, C61 and C64 constitute conserved site II of Zn^2-coordination site. Coordination with Zn^{2+} ions stabilizes molecular structure of the RING domain which exerts its functions through protein-protein interactions. So, effect of the D67E mutation may be alternation of the native protein surface that could interfere native protein-protein interactions that are crucial for exerting *BRCA1* functions. However, molecular detail for this mutation in breast cancer is unknown. The last type is IVS20+78G>A mutation, which is an intronic variant that has been reported once. But the role of this mutation in breast cancer is unknown.

2.7 Genetic testing for the *BRCA1* gene

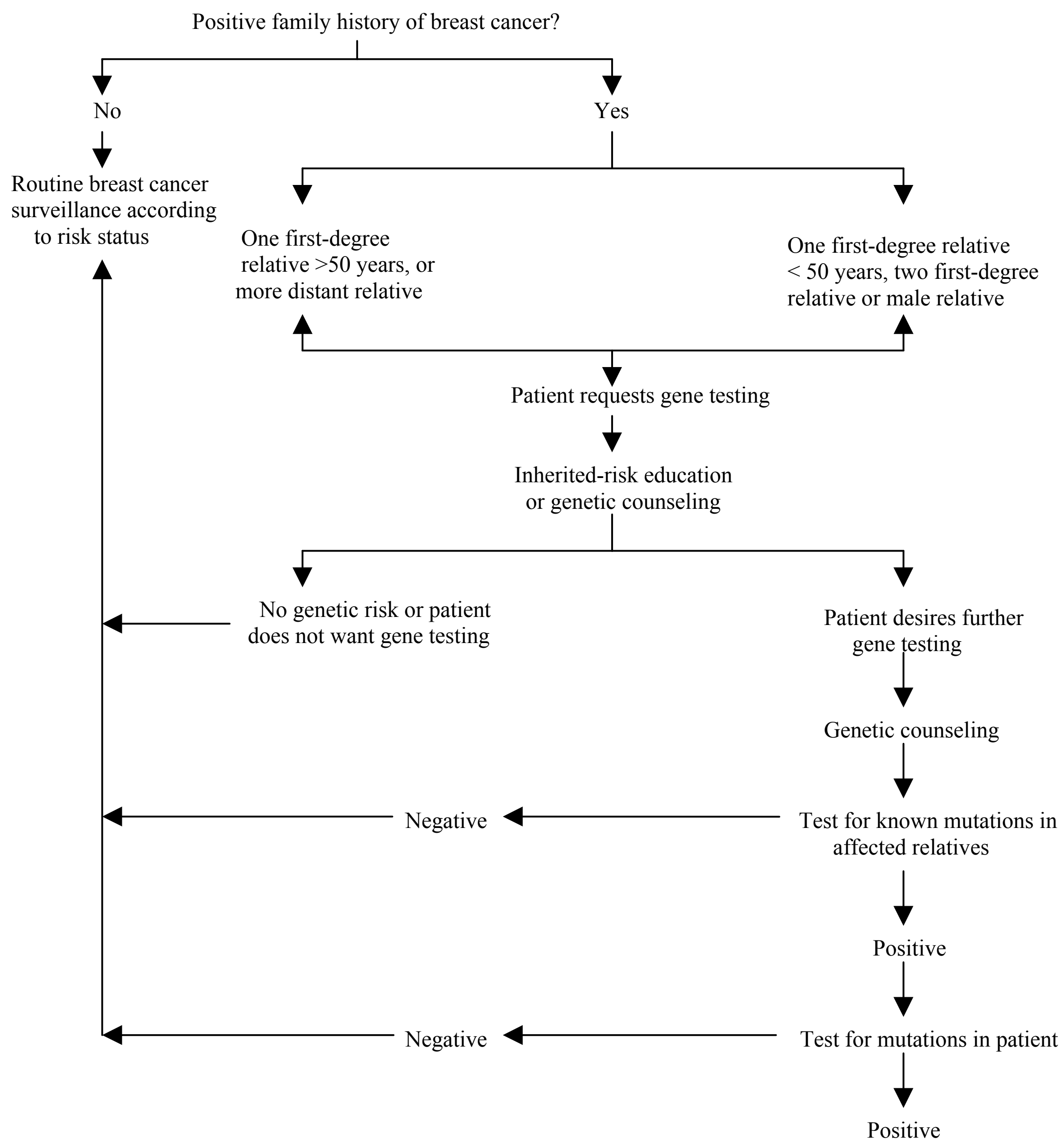
Genetic testing involves studying of gene mutation. It also provides important information for family members, especially close relatives or when they come from families with a strong family history of cancer. Carriers of the mutation therefore pass the trait to 50% of their offspring, either male or female. Thus, the testing is provides mean to reduce the risk or improve the chance of recovery from cancer, as genetic testing give clues about the risk of inherited cancers by analyzing genes for mutations. Results help making informed decisions about options for early cancer detection and risk reduction (White *et al*., 2002)

Patient selection may be the single most important aspect of *BRCA1* testing. Criteria for *BRCA1* mutation testing should be: (Fig. 2.8) (Rosenthal and Puck, 1999 ; American Medical Association, 2001)

- Strong family history of early-onset breast or ovarian cancer
- Two first-degree relatives with breast cancer
- Diagnosis of bilateral or multifocal breast cancer
- The patient has fewer than three affected relatives, but classified as one of the following:
    - The patient was diagnosed with breast and /or ovarian cancer at 45 years of age or less
    - A family member has been identified with a detectable mutation
    - There are one or more cases of ovarian cancer at any age
    - There are multiple primary or bilateral breast cancers in the patient or

one family member

- There is breast cancer in a male patient or in a male relative
- The patient is at increased risk for specific mutation due to ethnic background such as Ashkenazi Jews



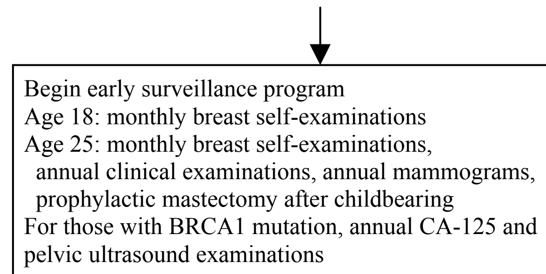


Fig. 2.8 Suggested algorithm for genetic screening in women with a family history of breast cancer (Rosenthal and Puck, 1999).

Prior to the testing, the patient must provide informed consent. Physicians who offer the option for genetic testing provide with patient education, counseling and support. This process leads to the patient being a knowledgeable participant, understanding the disorder and implications of data. A genetic counseling team has to be composed of at least a physician, genetic counselor, psychologist and a nurse. Pretest education should include the following information: (Brankovic-Magic *et al*., 2002)

- A description of patient's risk status
- An explanation of what it means to have an inherited susceptibility to cancer
- An information about the meaning of testing outcomes, whose results may be positive, negative or uninformative
- An appraisal of the risks, benefits and limitations of genetic testing
- A discussion about cancer surveillance and the limitations of anticancer therapies
- A review of the psychosocial issues related to genetic testing
- An information about the risk of passing a mutation to children
- An explanation of the alternatives to genetic testing

2.8 The screening techniques

Screening techniques for mutation provide a more rapid approach to survey large numbers of patients for mutations. The direct sequencing is a most sensitive screening technique for genes mutation. However, sequencing of complex

genes is highly technical demanding, expensive and very time-consuming. There are pre-screening methods to detect alterations of DNA in the large gene prior to sequencing. These techniques can indirectly indicate the existence and location of a mutation, after which the DNA in the indicated region can be sequenced to identify the specific mutation. Various methods based on the use of polymerase chain reaction (PCR) amplified materials have been applied for detecting alterations of DNA sequences, for example, denaturing gradient gel electrophoresis (DGGE) (Myers *et al*., 1989), allele-specific oligonucleotide (ASO) (Lipshutz *et al*., 1995), protein truncation test (PTT) (Roest *et al*., 1993), heteroduplex analysis (HA) (White *et al*., 1992), chemical cleavage mismatch (CCM) (Cotton *et al.,* 1988), denaturing high performance liquid chromatography (DHPLC) (Oefner, 2000), single- stranded conformation polymorphism (SSCP) (Orita *et al*., 1989), etc. However, each of these methods has different advantages and disadvantages as shown in Table 2.6.

Table 2.6 The advantages and disadvantages of each method used in routine diagnosis (Strachan and Read, 1999).

Method	Advantages	Disadvantages
Single-stranded conformation analysis (SSCP)	Simple, cheap equipment	Sequence > 300 bp only Limited sensitivity Does not reveal position of change
Heteroduplex (HA)	Simple, cheap equipment	Sequence > 300 bp only Limited sensitivity Does not reveal position of change
Denaturing high-performance liquid chromatography (DHPLC)	Quick, high throughput; quantitative	Expensive equipment Does not reveal position of change
Denaturing gradient gel electrophoresis (DGGE)	High sensitivity	Choice of primer is critical Expensive primers Does not reveal position of change
Chemical cleavage of mismatch (CCM)	High sensitivity Show position of change	Toxic chemicals Experimentally difficult
Protein truncation test (PPT)	High sensitivity for chain terminating mutations Shows position of change	Chain terminating mutations only Expensive Experimentally difficult Usually needs RNA

Direct sequencing	Detects all changes Mutations fully characterized	Expensive Can be hard to interpret

The SSCP technique is one of the most frequently used pre-screening methods for the *BRCA1* gene. It is rapid, highly sensitive, efficient and economical (Markoff *et al*., 1997 ; Gross *et al*., 1999). Subsequently, all the *BRCA1* sequence variants were confirmed by direct sequencing. In addition, two other approaches are widely used to for *BRCA1* mutation screening such as HA and PTT (Nollau *et al*., 1997 ; Greenman *et al*., 1998).

2.8.1 Single-stranded conformation polymorphism (SSCP)

The sensitivity of the SSCP ranges between 60%-90% (Sarkar *et al*., 1992 ; Cotton, 1993 ; Gross *et al*., 1999). This assay is reported to resolve single-base substitution, small deletions or insertions (Orita *et al*., 1989 ; Markoff *et al*., 1997). The SSCP technique is based on the principle that under non-denaturing conditions single-stranded DNA molecules form unique secondary structures depending on their primary nucleotide sequences. Consequently, electrophoretic mobility of single-stranded DNA is related to its folded conformation, and electrophoretic mobility shifts indicate differences in the sequences of the DNA fragment examined (Orita *et al*., 1989). In the conventional SSCP protocol, a target sequence of genomic DNA is amplified by PCR using PCR primers or nucleotides, and then the amplified DNA fragments are denatured and separated in polyacrylamide gel. The sensitivity of SSCP depends on gel matrix, gel additives like glycerol, electrophoretic conditions like temperature, ionic strength, acrylamide concentration, type and position of the mutation, and length of the DNA fragment (150-300 base pairs). In general, the sensitivity decreases rapidly as the size of the DNA increases (Sheffield *et al*., 1993 ; Ravnik-Glavac *et al*., 1994). Some reports indicated that the effects of fragment length were not so dramatic (Fan *et al*., 1993 ; Highsmith *et al*., 1999). However, the location or character of the sequence change cannot be determined using SSCP technique. Thus, the detected mutations have to be identified by other established methods such as direct sequencing.

2.8.2 Heteroduplex analysis (HA)

HA is based on the observation of electrophoretic molbility shifts resulting from structural deformation in mismatch hybridization of double-stranded DNA between normal and deleted or inserted mutant DNA strands. Heteroduplex formation is carried out by mixing PCR products of mutant and normal samples then heat-denatured and cooled down slowly in room temperature to allow reannealing of the single-stranded DNA molecules. The single-stranded DNA molecules reanneal to their complementary strand are called homoduplex, while some reanneal to mutated stranded are called heteroduplex. Thus, this technique takes advantage of the formation of heteroduplexes in the PCR between different alleles from heterozygous individuals. These heteroduplexes can be detected on polyacrylamide gels because they migrate more slowly than their corresponding homoduplexes (White *et al.,* 1992 ; Ozcelik *et al*., 1996). HA is also a sensitive method and highly rapid screening for the large numbers of sample. The degree of mobility shift depends on the size of gap in the heteroduplex, thus it is highly effective in detecting frame-shift mutation in DNA fragments.

2.8.3 Protein truncation test (PTT)

The PTT is screened in the coding region of a gene in which of translation terminating mutations occur, using *de novo* protein synthesis from amplified copy. The procedure includes three important steps. The first step involves the isolation of genomic DNA and amplification of the target gene coding sequences using PCR or, alternatively, isolation of RNA and amplification of the target sequence using reverse transcription PCR (RT-PCR) with 5'-primer containing promoter sequence specific for the bacteriophage T7 RNA polymerase. The resulting PCR products are then used as a template for the *in vitro* synthesis of RNA, which is subsequently translated into protein. The final step is the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE) of the synthesized protein. The shorter protein products of mutated polypeptides are easily distinguished from the full length protein products of normal polypeptides. This approach is suitable for the

detection of large DNA fragments. PTT fails to detect very small in-frame deletions/insertions because the mobility shifts are too small to detect and affect the amount of RNA produced or which render the mutated mRNA unstable (Hogervorst, 1997).

Approximately 85% of known *BRCA1* mutations are frameshift or nonsense mutations that result in premature termination of the coding sequence. Thus, PTT represents an ideal screening method. PTT was used to specifically detect nonsense and frameshift mutations of large *BRCA1* exon 11 (Jugessur *et al*., 2000).

2.8.4 Direct sequencing (DS)

Direct sequencing is considered to be the gold standard for mutation analysis because of its high sensitivity for identifying mutation. It is essentially a final step for identification of uncharacterized mutations. Unfortunately, DNA sequencing is very time consuming, requires expensive apparatus and chemicals and expertise to interpret the results. With the use of an automated and non-radioactive fluorescence technology, this approach is very efficient. The most widely used protocol is Sanger's method (Lancaster *et al*., 1997). It is used the dideoxy termination reaction where *in vitro* DNA synthesis is carried out in the presence of chemically modified (dideoxy) nucleotides, of which being incorporated in the growing chain leads to termination. Four reactions are set up. Each reaction contains small amounts of only one of the four possible dideoxy nucleotides (ddATP, ddTTP, ddGTP or ddCTP), one of which carries a fluorescent dye. The dideoxy nucleotides present during sequencing reactions lack the 3' hydroxyl group necessary for chain elongation and therefore generate a series of discrete fragments on the basis of length of the newly synthesized strand then to electrophoresed under denaturing polyacrylamide gel conditions. This is followed by denaturing polyacrlamide gel electrophoresis (Medappa, 2000).

The complete analysis of *BRCA1* mutations requires additional direct sequencing. However it is very time-consuming and expensive. Therefore, there is a need for an alternative mutation pre-screening method that is both sensitive and selective and that reduces time and costs.

2.9 Recommendations of high risk women

In principle, the preferred management option for gene mutation carrier would be to offer targeted screening for tumors. However unfortunately in many cases the effects of screening are unproven. In view of this consideration there may be a very useful role for the application of breast self examination, clinical breast examination, screening ultrasound or MRI for young women with a genetic predisposition to breast cancer.

2.9.1 Breast self-examination (BSE)

BSE has been recommended as a primary technique for the early detection of breast cancer. However, the practice of BSE may not confer any benefit. Confidence and accuracy of BSE increase with training. Female or *BRCA1* mutation carrier should receive a monthly breast self-examination that begins by age 18 to 21 years (Burke *et al*., 1997 ; Teh *et al*., 2000).

2.9.2 Clinical breast examination (CBE)

It has been shown to be useful in the detection of breast cancer. The sensitivity varies from 17-89% and is influenced by the stage and size of the cancer and the experience of the examiner. However reports have suggested that at least 10% of breast cancers may be detected by clinical examination alone and may be particularly important for women at risk of early-onset breast cancer (Miller *et al*., 1991). The increased breast cancer has been recommended that women undergo annual or semiannual clinical breast examination starting at age 25-35 years or an annual mammography beginning at age 25-35 years (Burke *et al*., 1997 ; Armstrong *et al*., 2000). The CBE methods for the increased surveillance breast cancer are as follow:

- Mammography

Mammographic screening has been shown to be useful in the early detection of breast cancer. However, it is problematical in young women as young

breasts are denser than a postmenopausal breast, resulting in greater difficulties with interpretation. For example, for women under the age of 40 years, mammography has a much more limited application due to reduced sensitivity and may also be associated with an increased radiation risk. It is recognized that the risk from radiation exposure increases with decreasing age of exposure and additionally genetic susceptibility to breast cancer may confer an increased sensitivity to radiation exposure. However, the high risk of breast cancer before the age of 50 in *BRCA1* mutation carriers increases the likelihood that the benefit from mammographic screening outweighs the risks. European recommendations suggest that mammograms should be offered on an annual basis to moderate and high risk women from the age of 35 years (Eccles *et al*., 2000 ; Brankovic *et al*., 2002 ; Evans and Lalloo, 2002). While mammography is not perfect, it is currently the best tool for screening and early diagnosis avaliable.

- Ultrasound

This is useful for confirming things detected by mammography. The advent of high resolution (high frequency) ultrasound makes this method a much more attractive option. It is used for the diagnosis of a palpable lump or further delineation of a mammographic abnormality. A recent study has suggested that, in the general population, ultrasound screening has a high false positive rate (Wilson, 1998). In practice, ultrasound may be used for screening in very young women (younger than 30 years) with a very early onset family history (Brankovic *et al*., 2002). It is especially helpful in young women with dense breast tissue when a palpable mass is not visualized on a mammogram.

- Magnetic resonance imaging (MRI)

MRI does not involve radiation and may be more sensitive than mammography, especially of the dense breast. It has also been shown to have high sensitivity in the detection of early breast cancer. Recent data comparing the use of MRI, mammography, ultrasound and clinical breast examination concluded that both the sensitivity and specificity of MRI is greater than other techniques in detecting breast lesions at a higher rate in women who are *BRCA1* mutation carriers or those who have a strong family history of breast cancer (Stoutjesdijk et al., 2001 ; Warner *et al*., 2001 ; Evans and Lalloo, 2002). However, the problem of cost remains.

2.9.3 Prevention surgery

Prophylactic mastectomy is an option for women at high risk of breast cancer. There are two techniques for prophylactic mastectomy. The first technique is subcutaneous mastectomy; this procedure preserves the nipple and overlying skin and is followed by breast reconstruction. It is a wildly used procedure. The second technique is total mastectomy, in which total excision includes the breast and nipple, with preservation of the axillary tissue. It is now accepted as an option for *BRCA1* mutation carriers. In *BRCA1* mutation carriers, the risk of breast cancers was decreased by 90% after prophylactic mastectomy (Hartmann *et al*., 1999). Anyway, mutation carriers who undergo mastectomy should continue postoperative surveillance with mammograms and clinical breast examinations (Meijers-Heijboer *et al*., 2001).

2.9.4 Chemoprevention

The use of hormonal manipulation via the use of medication has been suggested as a preventive measure in breast cancer. The current chemoprevention for breast cancer involves the use of tamoxifen and raloxifene. They are anti-estrogenic compounds that induce a conformational change in the estrogen receptor, thus restricting the ability of the breast tissue to respond to the hormone (Evans and Lalloo, 2002). Study showed that tamoxifen reduced the risk of contralateral breast cancer in women with pathogenic mutation in *BRCA1* gene (Narod *et al*., 2000). However, it has side effects which includes an increased risk of endometrial cancer and thromboembolism. Raloxifene may be an alternative preventive agent without risk of endometrial cancer. A trial in postmenopausal women with osteoporosis, indicated a 76% reduction in incidence of breast cancer during 3 years of treatment with raloxifene (Emery *et al*., 2000). Although chemoprevention is ultimately shown to be effective, it is costly and requires a long term use of drug.