# CHAPTER II

# LITERATURE REVIEW

## Breast cancer

Cancers are a group of diseases that cause cells in the body to change and grow out of control. Most types of cancer cells form a lump or mass called a tumor. It is named after the part of the body where the tumor first starts, that is, breast cancer begins in the breast tissue.

The main components of the female breast are lobules (milk-producing glands), ducts (milk passages that connect the lobules and the nipple), and stroma (fatty tissue and ligaments surrounding the ducts and lobules, blood vessels, and lymphatic vessels) (Figure 1).

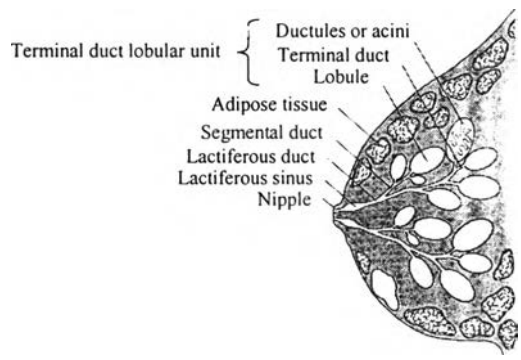


Figure 1. Anatomic structures of human breast.

Most types of tumors that form in the breast are benign, not malignant. Benign breast tumors are abnormal growths, but they do not grow and spread like malignant tumor does, and are not life-threatening. Some breast tumors are malignant, but are called *in situ*, because they have not spread beyond the area where they began. *In situ* breast cancers are confined within the ducts (ductal carcinoma *in situ*, or DCIS) or lobules (lobular carcinoma *in situ*, or LCIS) of the breast. The majority of these tumors do not progress to become an invasive tumor, and at this early stage

nearly all of these cancers can be cured. Most oncologists believe that lobular carcinoma *in situ* is not a true cancer but it is a marker of increased risk for developing invasive cancer in the future.

Other malignant breast tumors are invasive or infiltrating. These cancers start in the ducts or lobules of the breast but they have broken through the duct or gland walls to invade the surrounding fatty tissue of the breast. The seriousness of invasive breast cancer is strongly influenced by the stage of the disease, or how far the cancer has spread when it is first diagnosed. **Local stage** describes cancers confined to the breast. **Regional stage** tumors have spread to the lymph nodes. **Distant stage** cancers have metastasized (spread to distant sites) (American Cancer Society, 2001).

## The known risk factors for breast cancer

A number of factors, age, family history, age at first birth, early menarche, late menopause, consistently associated with increased risk of breast cancer are not modifiable. Other factors, alcohol consumption, use of postmenopausal hormones, and obesity after menopause, are modifiable. Some factors directly increase lifetime exposure of breast tissue to circulating sex hormones, that is early menarche and late menopause, and some factors are only correlated, e.g. higher socioeconomic status. Established risk factors for breast cancer are listed in Table 1.

Table 1. Factors correlate with the relative risk for breast cancer in women

| **Relative Risk*** | **Factor** |
|---|---|
| Relative Risk > 4.0 | – Certain inherited genetic mutations for breast cancer<br>– Two or more first-degree relatives with breast cancer diagnosed at an early age<br>– Personal history of breast cancer<br>– Age (65+ vs. <65 years, although risk increases across all ages until age 80). |
| Relative Risk 2.1-4.0 | – One first-degree relative with breast cancer<br>– Nodular densities on mammogram (> 75% of breast volume)<br>– Atypical hyperplasia<br>– High-dose ionizing radiation to the chest<br>– Ovaries not surgically removed <age 40 |
| Relative Risk 1.1-2.0 | – High socioeconomic status<br>– Urban residence<br>– Northern US residence |
| Reproductive Factors | – Early menarche (<12 years)<br>– Late menopause ( ≥ 55 years)<br>– No full-term pregnancies (for breast cancer diagnosed at age 40+ years) |
| Other factors that affect circulating hormones or genetic susceptibility | – Late age at first full-term pregnancy ( ≥ 30 years)<br>– Never breast fed a child<br>– Postmenopausal obesity<br>– Alcohol consumption<br>– Recent hormone replacement therapy<br>– Recent oral contraceptive use<br>– Tall<br>– Personal history of cancer of endometrium, ovary, or colon<br>– Jewish heritage |

(American Cancer Society, 2001)

* Relative risk means the risk of disease among people with particular exposure compare to the risk among people without that exposure.

## Estrogens and breast cancer

Early menarche (< 12 years), late menopause ( ≥ 55 years), late age at first full-term pregnancy ( ≥ 30 years), and fewer pregnancies all increase a woman risk of breast cancer by affecting endogenous reproductive hormones. Studies suggest that reproductive hormones influence breast cancer risk through effects on cell proliferation and DNA damage as well as promotion of cancer growth. Among ovarian hormones, estrogens have clearly emerged as the predominant factor involved in breast cancer. There are at least two mechanisms by which estrogens could promote breast cancer formation.

The prevailing theory is that estrogens increase the number of mutations as a result of their receptor-mediated growth promoting effect. DNA replication errors during cell division create random mutations. In the correct temporal or spatial cluster, these mutations give rise to a malignant phenotype. Equally important, the hormonal stimulus to cell division continues all along the progression pathway (Henderson and Feigelson, 2000). An alternative possibility is that estrogens are metabolized to genotoxic products which cause direct DNA damage independently of the presence of the estrogen receptor (Gruber *et al.*, 2002). Since estrogens can act by both inducing genomic damage (initiation) and increasing cellular proliferation (promotion), it is likely that estrogen function as both carcinogens and tumor promoters.

## Estrogen receptors (ERs)

In general, estrogen induces their physiological effects through an interaction with ERs, of which two isoforms, $\alpha$ and $\beta$, have been identified. The first estrogen receptor ER$\alpha$ was cloned in 1986 (Green *et al.*, 1986). The second subtype ER$\beta$ was discovered recently (Kuiper *et al.*, 1996). ER$\beta$ is expressed in many tissues, including the central nervous system, the cardiovascular system, the immune system, the urogenital tract, the gastrointestinal tract, the kidneys and the lungs, whereas ER$\alpha$ dominates in some few specific tissues and is mainly involved in reproductive system (Gustafsson, 1999). The ERs are members of the steroid hormone, thyroid hormone, retinoeic acid receptor superfamily of nuclear transcriptional factors.

Six conserved functional domains, A, B, C, D, E and F, are common structural features of this family of proteins (Gronemeyer, 1991) (Figure 2). The A/B region in the amino-terminal end of the protein shows the highest variability in sequence and size among the family numbers. This domain was shown to contain a constitutive, hormone–independent transcription activation function (AF-1) (Gronemeyer, 1991). DNA binding domain (DBD) or C region, is the most highly conserved region of the nuclear receptor superfamily. This domain consists of two zinc finger DNA-binding motifs responsible for recognition of the *cis*-acting hormone response element, and a nuclear localization signal (NLS). The D or hinge region is less well characterized and may be involved in steroid-mediated transcriptional repression (Adler *et al.*, 1988). The E/F region at the C-terminus is a ligand binding domain (LBD), the most complex region, and contains the hormone-binding site, the region required for stable dimerization of the receptor, cofactor binding, and a second estrogen-inducible transcription activation function (AF-2) (Tora *et al.*, 1989, Gronemeyer, 1991). A schematic comparison of primary structure of ER$\alpha$ and ER$\beta$ is shown in Figure 2. There is 97% amino acid identity between the two receptors in the DBD or C region but in the LBD or E/F region, the homology is 59% and 18%. This suggests that ER$\beta$ would recognize and bind to similar estrogen response elements (EREs) as ER$\alpha$ but that each receptor would have a distinct spectrum of ligands. There is less conservation between the two receptors in N-terminal AF-1 (A/B region) and C-terminal AF-2 (E/F region). This suggests that different sets of proteins in the transcription complexes may interact with ER$\alpha$ and ER$\beta$ and direct them to specific targets (Weihua *et al.*, 2003). ER$\alpha$ is known as an estrogen-dependent transcriptional factor for modulation of target genes in target organs. It has been indicated that estrogen binds to ER$\alpha$, ER$\alpha$ undergoes conformational change, binds to EREs on unclear target genes, and recruits coactivators and general transcription factors to form an active transcriptional complex, resulting in chromatin remodelling and enhancement of target gene expression (Kurebayashi *et al.*, 2000). In addition to mediating gene transcription via the classical EREs, ER subtypes can signal from an activating protein-1 (AP-1) enhancer element that requires ligand and the AP-1 transcription factors, Fos and Jun, for transcriptional activation. ER$\alpha$ and ER$\beta$ were shown to signal in opposite ways when complexed with the natural hormone estradiol from an AP-1 site. When bound with ER$\alpha$, 17$\beta$-estradiol activated transcription, whereas with ER$\beta$, transcription is inhibited. However, when antiestrogens bind to ER$\beta$, they are potent transcriptional activators at an AP-1 site, acting as agonists rather than antagonists (Paech *et al.*,

1997). Thus, the two ERs signal in different ways depending on ligand and response element. These suggested that ER$\alpha$ and ER$\beta$ might play different roles in gene regulation.

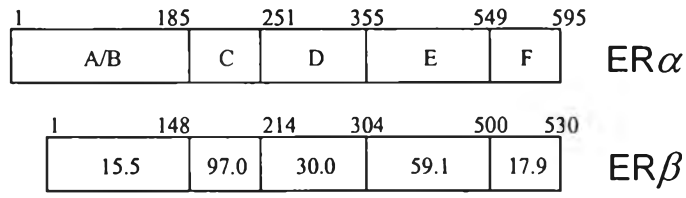


Figure 2. Comparison of the primary structures of ER$\alpha$ and ER$\beta$, respectively. The above numbers indicate the number of amino acids, with number 1 being the most N-terminal. The numbers within the ER$\beta$ receptor represent the degree of homology (%) between respective domains in the two receptors (Gustafsson, 1999, Speirs *et al.*, 2004).

## Mammary gland development in rats

The development of male and female mammary glands in rats is qualitatively similar. At about the 11$^{th}$day of intra-uterine life, two thickenings of ectoderm extend from the shoulder region tailwards to the inguinal region to form the mammary streaks. Downgrowths develop into the underlying mesenchyme and it is from these primary sprouts, twelve in number, that the mammary glands develop.

In females at birth, gland areas are seen as lighter coloured patches on the skin and rudimentary nipples are present. At birth the subcutaneous fat is uniformly distributed, but during the first few weeks of post-natal life it tends to accumulate in the cervical, thoracic, abdominal and inguinal areas to form the mammary fat pads that surround and support the growing and developing ducts. After birth the ducts elongate and undergo branching. The ducts grow slowly and divide by simple branching until about the end of the third week. Gland growth in female then increases and exceeds the rate of body weight growth. In virgin animals, this positive allometric growth continues until about 90-110 days. Adult rats possess six pairs of mammary glands. One pair lies in the cervical region, two pairs on the thoracic region, two pairs in the abdominal region and one pair in the inguinal region. The glands differ from each other in

surface area as well as in thickness. Where the 2nd and 3rd glands and again where the 4th and 5th glands adjoin, there is overlapping of the ducts and their branches (Young and Hollowes, 1973).

## Normal mammary gland structure

In adult animals that are neither pregnant nor lactating, the histological structure of the mammary glands is essentially similar in both sexes. In females the nipple is canalized by a single duct that branches repeatedly. The ducts are lined by one or two layer of cuboidal or columnar secretory epithelium. Myoepithelial cells lie between the secretory epithelium and the basement membrane. They have flattened nuclei whose long axes lie parallel to the basement membrane. Outside the basement membrane the ducts are surrounded by loose connective tissue that runs in fibrous septa supported by fat cells (Figure 3). These septa also contain blood vessels and small numbers of various types of wandering cells. During phases of rapid growth the ducts end in thickened blunt-ended club-shaped structures. Linear growth of the ducts occurs by penetration of these clubs into the surrounding fat pats. When linear growth slows the clubs become hollowed out by desquamation into the lumen and rearrangement of cells. Gradually, they become differentiated into the acini. Myoepithelial cells and basement membrane appear in and around the acini concurrently with differentiation. The connective tissue stroma surrounding the alveoli differ from that of the ducts in its finer, more delicate collagen fibres and more abundant gland substance (Young and Hollowes, 1973) (Figure 4).

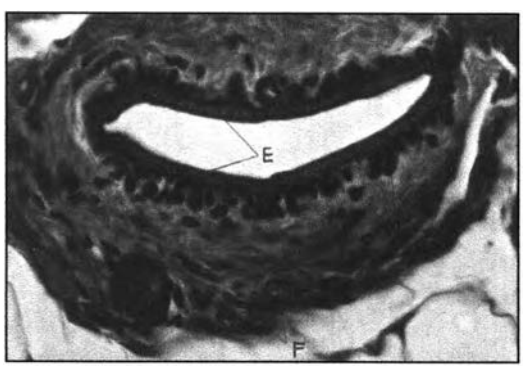


Figure 3. Transverse section of normal duct from 100-day-old female rat. Duct lined by cuboidal epithelium(E) surrounded by myoepithelial cells (M) and a layer of mesenchyma, fibroblast(F). Crystal violet staining, x200

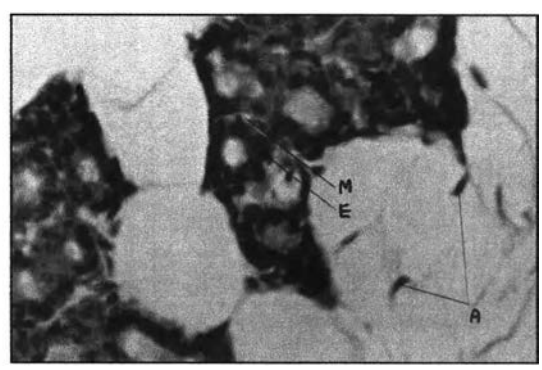


Figure 4. Group of mature acini. Glandular acinar showed single layer of low cuboidal epithelium(E) surrounded by myoepithelial cells (M) embedded in small amount of loose connective tissue, surrounded by a large fat pat, adipocyte(A). Crystal violet staining, x200

## NMU-induced tumorigenesis

N-nitrosomethylurea (NMU) is a potent mammary carcinogen in the rats (Gullino *et al.*, 1975). Experimental mammary tumors induced in rats by administration of NMU have been described to be hormone dependent. Tumor regression has been reported to occur after ovariectomy or tamoxifen administration (Lemay and Kelly, 1980). In addition, the majority (87%) of NMU-induced rat mammary tumors regressed within 1 week after hypophysectomy and administration of $E_2$ or PRL after hypophysectomy resulted in stabilization of tumor growth (Arafah *et al.*, 1982). Gullino *et al.* (1975) reported that NMU-induced tumors were adenocarcinoma or papillary carcinoma types. The tumors also appeared to metastasize to the bone marrow and spleen. Thus, this tumor model system more closely resembles the human breast cancer.

## Phytoestrogens

Phytoestrogens are plant compounds with estrogen-like biological activity. They have 2-phenylnaphthalene-type chemical structures similar to those of estrogens and have been found to bind to estrogen receptors. The rapidly growing body of literature in this area indicates that phytoestrogens may exert both estrogenic and antiestrogenic effects on metabolism, depending on several factors, including their concentration, the concentrations of endogenous estrogen and the individual characteristics, such as gender and menopausal status (Tham *et al.*, 1998).

### *Classification and Metabolism of the Major Phytoestrogens*

There are three main classes of phytoestrogens: isoflavones, lignans and coumestans, which occur in either plants or their seeds. A single plant often contains more than one class of phytoestrogen. **Isoflavones** are found in highest amounts in soy beans and soy foods. The major isoflavones, genistein and diadzein, commonly exist as inactive glucosides. They are also derived from precursors, biochanin A and formononetin, which are converted to genistein and diadzein respectively, after break down by intestinal glucosidase (Figure 5). **Lignans**, enterodiol and enterolactone, are derived from the compounds secoisolariciresinol found in plants. Lignans are found widely in cereals, fruit, and vegetables. They occur in high concentration in flaxseed. The major **coumestan**, coumestrol is found in the soy sprout (Murkies *et al.*, 1998).

In human, after consumption of plant lignans and isoflavones, complex enzymatic metabolic conversions occur in the gastrointestinal tract, resulting in the formation of heterocyclic phenols with a close similarity in structure to estrogens (Figure 5.). Absorbed phytoestrogen metabolites undergo enterohepatic circulation and may be excreted in the bile, reabsorbed, reconjugated by the liver, and excreted in the urine (Murkies *et al.*, 1998).

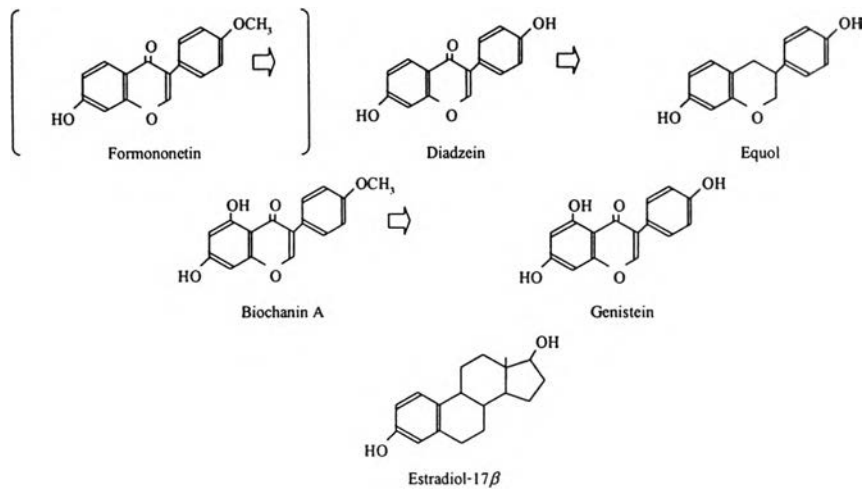


Figure 5. Metabolic pathways of isoflavones and a comparison of chemical structure of the isoflavones and estradiol-17$\beta$

## Genistein

Genistein has been the phytoestrogen of greatest interest at present. Relative potencies of genistein compared with estradiol ($E_2$) (value 100) established the concentration-dependent response curves of 0.084 (Tham *et al.*, 1998). It exhibits several biological activities, including antiestrogenic, anticarcinogenic and estrogenic activities as follow;

### *Antiestrogenic activity of genistein*

Genistein is considered as weak estrogens, presenting an activity of 100 to 1000 times lower than that of 17$\beta$-estradiol ($E_2$), depending on the system studied (Martin *et al.*, 1978, Zava and Duwe, 1997). It has been suggested that they would act as antiestrogen by competing with the more potent endogenous estrogen for binding to ERs.

### *Anticarcinogenic activity of genistein*

The antiproliferative effects of genistein occur in both ER positive and ER negative cell lines. It can be concluded that the effects did not mediate through the ER (Wang *et al.*, 1996). It has been proposed that genistein, and perhaps other phytoestrogens, inhibit tumor cell growth by interfering with the tyrosine kinase activity of activated growth factor receptors and cytoplasmic

tyrosine kinases, which are essential for the transduction of mitotic signals (Akiyama *et al.*, 1987, Wang and Kurzer, 1998). The antioxidant effects of genistein may be partially responsible for its anticarcinogenic effects. Wei *et al.* (1995) reported that genistein suppressed $H_2O_2$ production by 12-0-tetradecanoylphorbol-13-acetate (TPA) stimulated HL-60 cell and superoxide anion ($O_2^{\bullet -}$) generation by xanthine/xanthine oxidase. *In vivo* dietary administration of 250 ppm genistein for 30 days significantly enhanced the activities of antioxidant enzymes in the skin and small intestine of mice (catalase, GSSG-R, SOD). Recently, genistein was shown *in vitro* to inhibit bovine microvascular endothelial cell angiogenesis induced by human recombinant basic fibroblast growth factor (Fotsis *et al.*, 1993). However, the half-maximal effect of inhibition of angiogenesis required a concentration of approximately 150 $\mu$M, which is higher than the magnitude required for cancer cell growth inhibition.

Although there have been many interesting studies on the effects of isoflavones on biochemical targets in tissue culture experiments, in most cases the concentration used by investigators have exceeded 10 $\mu$M. It is still questionable whether such concentrations are ever reached *in vivo*. A high soy diet consumption in human produced an approximate plasma level of 0.5-4 $\mu$M of genistein (Barnes, 1995), these concentration are much lower than those required to inhibit the growth of most cultured cancers cells.

### *Estrogenic activity of genistein*

In spite of the large number of studies supporting cancer chemoprevention of genistein, some studies have suggested a potential opposite effect. It must be kept in mind that phytoestrogen are weak estrogen and under certain experimental conditions will always stimulate cell proliferation and estrogen-dependent gene expression.

In human, McMichael-Phillips *et al.* (1998) examined the effects of dietary soy supplementation on the proliferation rate of premenopause, histologically normal breast epithelium and the expression of progesterone receptor. A total of 48 women with benign or malignant breast disease were randomly assigned to receive their normal diet either alone or with a 60 g soy supplement (containing 45 mg isoflavones) taken daily for 14 days. Biopsy samples of normal breasts were labelled with [$^3$H] thymidine to detect the number of cells in S phase and were immunocytochemically stained for the Ki67, proliferation antigen. They found that the

proliferation rate of breast lobular epithelium and progesterone receptor expression significantly increase after 14 days of soy supplementation, suggesting that dietary soy protein may have estrogenic effect on the breast and stimulate breast proliferation.

Wang *et al.* (1996) reported that genistein characterized the estrogen-like property by stimulating pS2 mRNA expression. The stimulation of pS2 expression by genistein ($10^{-7}$M) could be blocked by the addition of tamoxifen ($10^{-5}$M). These results supported that genistein acts through an ER-mediated transcriptional event. In addition, they found that the effect on the proliferation of MCF-7 cells was biphasic. Genistein concentration between $10^{-8}$ and $10^{-5}$M stimulated the growth of MCF-7 cells, however, at concentration of higher than $10^{-5}$ M, genistein appeared to inhibit the proliferation. When they used the ER-negative MDA-MB231 cells, genistein did not stimulate the growth, but the inhibitory effects of genistein remained. These results led them to conclude that the stimulation of cell proliferation by genistein was mediated through the ER, but the antiproliferative effect was not. The biphasic effect of genistein on growth of ER-dependent cell line, such as MCF-7 and T47D, was determined by several investigators (Zava and Duwe, 1997, Wang and Kurzer, 1998, Hsieh *et al.*, 1998).

In 1998, Hsieh *et al.* studied the estrogenic and proliferative inducing activity of genistein *in vitro*. They found that genistein elicited dual threshold effects. Low concentrations of genistein (0.01 – 10 $\mu$M) enhanced the proliferation of estrogen – dependent human breast cancer cells (MCF-7) and increased expression of the estrogen responsive gene pS2. At higher concentrations (>10 $\mu$M), genistein inhibited MCF-7 cell growth. *In vivo*, they found that dietary treatment with genistein (750 ppm) for 5 days enhanced mammary gland growth in 28-day-old ovariectomized athymic mice. It indicated that genistein acted as an estrogen agonist in normal mammary gland. When MCF-7 cells were subcutaneously implanted in ovariectomized athymic mice, tumors were larger in the genistein-treated group than the negative control group. These results demonstrated that dietary genistein was able to enhance the growth of MCF-7 cell tumors *in vivo*. The plasma concentration of genistein in athymic mice fed genistein (750 ppm) is 0.14 - 2.1 $\mu$M. It is within the range of concentration that enhanced the proliferation of MCF *in vitro* and is similar to what is observed in human consuming a soy product (0.74 – 6 $\mu$M) (Xu *et al.*, 1995).

In 2001, Ju *et al.* studied physiologically achievable concentrations of dietary genistein of 125-1,000 $\mu$g/g which produced a plasma genistein level of 0.39 – 3.36 $\mu$M on the growth of estrogen – dependent human breast cancer cells implanted in athymic nude mice. They found that dietary genistein ($\geq$ 250 $\mu$g/g) increased tumor size in a dose – dependent manner. The percentage of proliferating cells was significantly increased by genistein at and above 250 $\mu$g/g. Expression of pS2 mRNA was also significantly increased with increasing dietary genistein levels.

From these results, it is suggested that genistein can act as an estrogen agonist both *in vitro* and *in vivo* and resulting in the growth of MCF-7 cell. Thus, there is the potential for daily genistein consumption to stimulate the growth of estrogen - dependent tumors in postmenopausal women who had low circulating endogenous estrogen levels.

### *Animal studies on the mammary cancer*

Table 2. shows the experiments in which the effects of soy or soy isoflavones intake on the mammary cancer were studied. The evaluations were considered protective effects when at least one of the following parameters of carcinogenesis, tumor number, incidence, metastasis, and latency, was favourably altered.

Table 2. Effects of soy or soy isoflavone on chemically induced mammary tumorigenesis

| Author (year) | Animals (stage of exposure) | Carcinogen | Phytoestrogens | Results |
|---|---|---|---|---|
| Hawrylewicz (1991) | SD rats, adult | NMU | Soy protein isolate | Protect, ↓ tumor incidence ↓ tumor number |
| Lamartiniere (1995) | SD rats, neonatal | DMBA | Genistein, 5mg SC. | Protect, ↓ tumor multiplicity ↑ latency period |
| Murrill (1996) | SD rats, prepubertal | DMBA | Genistein, 500 mg/BW SC. | Protect, ↓ tumor multiplicity ↓ tumor incidence |
| Fritz (1998) | SD rats, perinatal | DMBA | Genistein in diet (250 mg/kg diet) | Protect, ↓ tumor multiplicity |
| Hilakivi-Clarke (1999) | SD rats, prepubertal | DMBA | Genistein, 1 mg/kg BW SC. | Protect, ↓ tumor multiplicity |
| Cohen (2000) | F344, adult | NMU | Soy protein isolate | No effect |
| Appelt (1999) | SD rats, adult | DMBA | Diet containing isoflavone (0.81 mg/g diet) | No effect |
| Day (2001) | Mice, adult | DMBA | Diet containing genistein (1g/kg diet) | Stimulate, ↑ tumor multiplicity ↑ malignant |

In neonatal and prepubertal animals with genistein treatment reduced the incidence and multiplicity of DMBA-induced mammary adenocarcinomas (Larmartiniere *et al.*, 1995, Murrill *et al.*, 1996, Hilakivi-Clarke *et al.*, 1999). Study in the mammary whole mounts revealed that genistein treatment in neonatal and prepubertal rats decreased numbers of terminal end buds and increased numbers of lobular structures. According to the study of Russo *et al.* (1979) terminal end buds and terminal ducts are undifferentiated and most susceptible to chemical carcinogens, while lobules are progressively more differentiated and less susceptible to the formation of adenocarcinomas. Thus, genistein treatment in neonatal and prepubertal rats altered the ontogeny of the mammary gland and rendered the adult animals less susceptible to chemically-induced mammary cancer. These results are consistent with an epidemiological study that young women partaking of a traditional Asian diet high in soy have a low incidence of breast cancer (Shu *et al.*, 2001). Thus, it is possible that the chemoprotective action of the soy isoflavone against breast cancer depends on the timing of exposure. Early exposure to phytoestrogen (during postnatal period) may cause precocious maturation of breast terminal end buds to be more differentiated lobules and subsequently protect the breast cancer. In contrast, increment of the exposure time

during post-pubertal period may potentially increase breast cancer risks. In adult animals, however, the effects of soy isoflavones on mammary tumor development are still controversial. Some report indicates that adult rats exposed to soy protein were prevented the development of breast cancer (Hawrylewicz *et al.*, 1991). Other studies reported that the soy isoflavone did not inhibit the development of chemically induced mammary tumor (Cohen *et al.*, 2000, Appelt and Reicks, 1999). Furthermore, the study in adult mice showed that genistein increased mammary tumorigenesis (Day *et al.*, 2001). Dietary feeding of genistein in ovariectomized athymic nude mice, giving the plasma concentration similar to those in human consuming a soy product, enhanced estrogen-dependent tumor growth (Hsieh *et al.*, 1998, Ju *et al.*, 2001). From the above mention, treatment of phytoestrogens may stimulate breast tumor growth and it leads to the concerns for the safety of using soy products in women with breast cancer.

## Tamoxifen

The majority of breast cancers are estrogen dependent, and it is likely that many of women with breast cancer are being treated with tamoxifen. Tamoxifen, the most commonly used antiestrogenic drug, has been shown to provide a 26% annual reduction in recurrence and 14% annual reduction in death (American Cancer Society, 2001). It also reduces the incidence of breast cancer among high-risk women who have never been diagnosed with the diseases (Fisher *et al.*, 1998). The antitumor effects of tamoxifen are thought to be due to its antiestrogenic activity, mediated by competitive inhibition of estrogen binding to estrogen receptors, formed a receptor complex that is converted in completely to fully activated form (Allan *et al.*, 1992, McDonell *et al.*, 1995). As a result of imperfect changes in the tertiary structure of protein, the complex is only partially active in initiating the programmed series of event which is necessary to orchestrate gene activation (Metzger *et al.*, 1988). As a consequence, tamoxifen inhibits the expression of estrogen-regulated genes including growth factors and angiogenic factors which are secreted by the tumor and it may stimulate growth by autocrine or paracrine mechanisms (Arteaga and Osborne, 1991). The net result is the block of the G1 phase of the cell cycle and a slowing of cell proliferation (Taylor *et al.*, 1983, Osborne *et al.*, 1983). Tamoxifen also directly induced programmed cell death (Ellis *et al.*, 1997). Tumor may then regress because of this altered balance between cell proliferation and ongoing cell loss.

***Tamoxifen and genistein action on growth of estrogen-dependent tumors***

The majority of the breast cancers are estrogen dependent and many of female patients are being treated with tamoxifen. Treatment with tamoxifen is known to worsen symptoms such as hot flashes, depression, mood swings, sleeping disorders and vaginal dryness. Mostly those symptoms are treated with synthetic estrogen as a estrogen replacement therapy (ERT). Owing to the increment of breast cancer risk, most oncologists do not recommend ERT to estrogen-dependent cancer patients. Thus, some breast cancer patients are given an alternative treatment, a dietary supplement of phytoestrogens. However, there are some published reports of the antagonistic and agonistic effects of genistein. Schwartz *et al.* (1998) revealed that genistein in the physiologically relevant concentrations was sufficiently to mediate ER agonism and reversed the inhibitory effects of 40H-tamoxifen on ER-responsive reporter genes in cultured cells. A recent study reported that dietary genistein could negate or overwhelm the inhibitory effects of tamoxifen on estrogen-dependent tumor growth (Ju *et al.*, 2002). Six treatment groups were performed; control (C); 0.25 mg estradiol ($E_2$) implant (E); $E_2$ implant + 2.5 mg tamoxifen implant (2.5 TE); $E_2$ implant + 2.5 mg tamoxifen implant + 1000 ppm genistein (2.5 TEG); $E_2$ implant + 5 mg tamoxifen implant (5 TE), and $E_2$ implant + 5mg tamoxifen implant + 1000 ppm genistein (5 TEG). They found that treatment with tamoxifen (2.5 and 5 mg) suppressed $E_2$-stimulated MCF-7 tumor growth in ovariectomized athymic mice, but dietary genistein consumption negated the inhibitory effect of tamoxifen, lowered plasma $E_2$ level and increased expression of estrogen-responsive genes (e.g., pS2, PR. cyclin D1). Results from this study raise concerns about the consumption of isoflavone in tamoxifen-treated postmenopausal women with estrogen-dependent breast cancer.

Contrary to these findings, other investigators suggest that combination of genistein and tamoxifen may exert beneficial effects. Gotoh *et al.* (1998) found that tamoxifen together with a diet containing 10% miso (fermented soybean paste) synergistically inhibited the development of N-nitroso-N-methylurea (NMU)-induced rat mammary cancer. Tumor incidences in the control, miso, tamoxifen and miso plus tamoxifen groups were 91, 77, 68 and 10%, respectively, and tumor multiplicities for these groups were 4.5, 2.4, 1.4 and 0.2, respectively. In addition the combination of miso and tamoxifen inhibited tumor growth by approximately 50% for over 6 weeks, whereas tamoxifen itself was ineffective. Constantinou *et al.* (2001) reported that DMBA-

induced mammary carcinogenesis (tumor multiplicity) was reduced 29% by tamoxifen, 37% by soy protein isolate and 62% by the combination of tamoxifen and soy and tumor latency was increased only in the combination group.

## The regulation of mammary tumor growth

Estrogen is well known as a tumor promoter of breast cancer cell growth. It stimulates breast cancer proliferation by diffusion into the cell, binding to estrogen receptor to be estrogen-receptor complex and the complex then binds to promoter regions of specific genes to activate transcription of new mRNA. Newly transcribed mRNA enters the cytoplasm, where it is translated into the specific proteins on ribosomes. Certain estrogen-induced proteins, such as progesterone receptor, are importance for specific metabolism process in the cell. Other estrogen-induced proteins regulate events leading to cell proliferation (Holland *et al.*, 1997). Some studies suggested that breast cancer cells under the estrogen control can also synthesize and secrete their own growth factors that they could stimulate their own breast cancer cells or adjacent stromal tissues through autocrine or paracrine mechanisms (Lippman *et al.*, 1987, Osborne and Arteaga, 1990). Potential autocrine and/or paracrine growth factors that have been identified include transforming growth factor-$\alpha$ (TGF-$\alpha$), insulin like growth factors (IGFs), platelet-derived growth factor (PDGF), fibroblast growth factor, and cathepsin D, a lysosomal enzyme with the mitogenic activity and influencing tumor invasiveness. In addition to polypeptide growth factors and their receptors, breast cancer cells have shown to express several oncogenes, that is, genes involved in normal regulatory processes. The products of oncogenes are the growth factors or growth-factor receptors. When the genes are overexpressed, they can induce or promote the malignant phenotype. Tissue-specific expression of myc, ras, HER2 (*neu)* and estrogen-related genes in mammary glands of transgenic mice has been shown to result in an increased incidence of both benign and malignant breast pathology (Muller *et al.*, 1988, Sinn *et al.*, 1987).

## Gene related to estrogen receptor (ER) pathway.

### *Estrogen receptors and breast cancer*

The definitive roles of ER in the development and progression of breast cancer have been elucidated. It has been suggested that the relative expression levels of ER$\beta$ versus those of ER$\alpha$ decrease during human breast tumorigenesis (Leygue *et al.*, 1998). Speirs *et al.* (1999) have compared expression of ER$\alpha$ and ER$\beta$ in normal and malignant breast. ER$\beta$ was predominantly expressed in normal tissue (22% of sample) but not in tumor tissue. Most breast tumor expressed ER$\alpha$, either alone or in combination with ER$\beta$. Interestingly, lesions that express ER$\alpha$ and ER$\beta$ were significantly associated with lymph node-positive tumors that also tended to be of higher grade. Recently, Kurebayashi *et al.* (2000) screened the expression levels of ER$\alpha$, ER$\beta$ coactivators and corepressors in normal mammary glands, intraductal carcinomas, invasive ductal carcinomas and breast cancer cell lines using a multiplex reverse transcription-PCR. They found a positive correlation of expression levels among ER$\alpha$ and cofactors and upregulation of expression levels of ER$\alpha$ and cofactors during the development of intraductal carcinomas from normal mammary gland, and a decrease in their expression levels during the progression of intraductal carcinomas to invasive ductal carcinomas. Certain changes in the expression levels of ERs and cofactors might influence this machinery and might play important roles in the development and progression of breast cancer.

### *The expression of presenelin-2 (pS2) mRNA as an indicator of estrogenic response*

The human pS2 gene was initially characterized in the breast cancer cell line MCF-7. The expression of pS2 gene is specifically controlled by estrogen (Brown *et al.*, 1984). The processes of pS2 gene expression start from the binding of estrogen to the intracellular ERs, forming a receptor dimerization and finally binding of receptor dimer to the estrogen-responsive element (ERE) in the DNA. Consequently, the ER-ERE complex modulates the transcription of presenelin-2 (pS2) (Saegusa and Okayasu, 2000). Many studies monitored the expression of pS2 mRNA as an indicator of estrogenic response both *in vitro* and *in vivo* (Sathyamoorthy *et al.*, 1994, Wang *et al.*, 1996, Hsieh *et al.*, 1998, Liu *et al.*, 2001, Ju *et al.*, 2001). The validity and specificity of this assay were confirmed by the inhibition observed in the presence of tamoxifen

whereas other steroid hormones such as dexamethasone and progesterone were ineffective in stimulating, pS2 mRNA (Sathyamoorthy *et al.*, 1994).

## Insulin-like growth factor-1 (IGF-1) system and breast cancer

IGF-1 is an endocrine factor that belongs to a family of growth factors involved in the regulation of normal and malignant cell growth, differentiation and development. IGF-1 is produced in most organs and tissues where it can function in both an autocrine and a paracrine manner to stimulate cell growth (Martin and Stoica, 2002). The IGF-1 regulates cell entry into and progression through the cell cycle by binding to IGF-1 receptor (IGF-1R), a transmembrane protein with tyrosine kinase activity. The subsequent binding of IGF-1 to its receptor activates the tyrosine kinase and initiates a cascade of phosphorylation that activates intracellular kinase and nuclear transcription factors. In addition, IGF-1 is also a survival factor and could block tumor cell apoptosis *in vivo* (Resnicoff *et al.*, 1995).

There is accumulating evidence that cross- talk exists between ER and IGF-1 receptor (IGF-1R)-mediated pathway in ER- positive breast cancer cells (Yee and Lee, 2000). Recent studies clearly indicated that, apart from the classical model, estrogen-stimulated mitogenesis in human breast cancer cells could also be mediated by the enhancement of IGF-1 signalling pathway by the upregulation of IGF-1R (Stewart *et al.*, 1990) and insulin receptor substrate-1(IRS-1) (Lee *et al.*, 1999) expression in MCF-7 on treatment with $E_2$.

## The HER2/*neu* gene and breast cancer

The rat *neu* gene identified in rat neuroblastomas in the early 1980s. The HER2/*neu* gene encodes a 185 kD transmenbrane glycoprotein that functions as a growth factor receptor. This protein is a member of the epidermal growth factor (ErbB1/HER1), HER2 (ErbB2), HER3 (ErbB3), HER4 (ErbB4) (Schnitt, 2001). These receptors are composed of an extracellular binding domain, a transmenbrane lipophilic segment, and an intracellular protein tyrosine kinase domain with a regulatory carboxyl terminal segment. The EGF- like ligands, bind to HER1,

include epidermal growth factor (EGF), transforming growth factor alpha (TGF-$\alpha$), amphiregulin, heparin binding EGF (HB-EGF), betacellulin and epiregulin (Albanell and Baselga, 1999). These ligands induce formation of HER1/HER1 homodimers and HER1/HER2 heterodimers, although HER1/HER3 and HER1/HER4 heterodimers can be occasionally detected. A second class of ligands termed neuregulin bind directly to HER3 and/or HER4 (Tzahar *et al.*, 1994). Although HER2 forms heterodimers with other member of the family on the interaction with their ligands, it has no known ligand of its own and therefore considered an orphan receptor (Albanell and Baselga, 1999). Ligand binding to one of these receptors initiates the formation of homodimers and heterodimers. Dimerization is followed by phosphorylation, which, in turn, results in a cascade of down stream signaling events that are important for cell growth and maintenance of the transformed state. One major signaling route of the ErbB family is the Ras-Ref-MAP kinase pathway (Alroy and Yarden, 1997). Several lines of evidence suggest that at least two of these receptors, HER1 and HER2, play a role in breast cancer and, therefore, are considered as target for cancer therapy. Both receptors are frequently overexpressed in breast cancer and their overexpression is association with a more aggressive clinical behaviour (Slamon *et al.*, 1987, Fox and Harris, 1997).

## GPR54 and breast cancer

GPR54 is a novel G protein-coupled receptor first cloned from the rat brain (Lee *et al.*, 1999). The rat GPR54 cDNA encode for a 396 amino acid protein that share a unique structure feature composing seven transmembrane $\alpha$–helices. These molecules act as receptors for a diverse range of extracellular signaling molecules including small molecule (amino acids), lipids, small bioactive peptides, and large polypeptides. Recently, it has been reported that metastin, a product of *Kiss-1* gene, is the endogenous ligand for GPR54. *Kiss-1* gene has been identified as a metastasis-suppressor gene, which inhibits cell migration and cell growth (Stafford *et al.*, 2002). Shirasaki *et al.* (2001) has used *in situ* hybridization to show an impressive (80%) inverse correlation of *Kiss-1* expression with melanoma metastatic potential. In addition, transfection with *Kiss-1* gene to MDA-MB-435, human breast cancer cell line, and then injection into the mammary fat pads of athymic nude mice reduced metastatic potential by 95% compared to non-transfected cells (Lee and Welch, 1997). The mechanism of action for *Kiss-1* has not yet known. Yan *et al.* (2001) recently showed that *Kiss-1* specially reduced expression of MMP9 (matrix metelloproteinase 9) in HT1080 cells.