INTRODUCTION
1. INTRODUCTION

Cancer is uncontrolled growth of abnormal cells in the body. The cancer spreads to more distant parts of the body through the lymphatic system or bloodstream. All the tumors are not cancerous. Benign tumors do not grow uncontrollably, do not invade neighbouring tissues and do not spread throughout the body.

There are few types of cancers common in India among both men and women. They are prostate cancer, lung cancer, colon cancer, breast cancer and cervical cancer. According to American Cancer Society (ACS) breast cancer is currently the predominant cancer among women pushing cervical cancer to the second place and also it is the second leading cause of cancer death today.

Breast cancer is the cancer that forms in tissue of the breast, the most common form of breast cancer begins in cells lining the ducts that carry milk to the nipple (ductal cancer). Other forms of breast cancer begin in the glands that produce milk (lobular cancer) or in other parts of the breast. Based upon the localization of tumor cells, breast cancer can be classified as in situ or benign breast cancer (ductal carcinoma in situ and lobular carcinoma in situ) and invasive or malignant breast cancer (invasive ductal carcinoma and invasive lobular carcinoma).

It occurs both in men and women although breast cancer in male is rare. According to ACS the estimated new cases of breast cancer is 226,879 (female) and 2,190 (male) in United States in 2012 and death from breast cancer is 39,510 (female) and 410 (male). In India, the incidence of breast cancer is increasing, with an estimated 80,000 new cases diagnosed annually. The incidence of breast cancer increasing by approximately 1-2% every year (Chow and Loo, 2003).
Fig. 1: Breast cancer is the most common cancer among the women in Chennai and also in India (according to PBCR report, 2008)

Fig. 2: In recent years, the average age of developing breast cancer has been shifted from 50-70 years to 30-50 years according to the most recent PBCR 2006-2008 data
The age shift may be due to the hormones involved in the younger women. Estrogen plays a major role in developing breast cancer; hence the study includes the analysis of estrogen receptor involvement in breast cancer.

It occurs in women between the ages of 50-70 years, after menopause has occurred. According to Population Base Cancer Registry (PBCR) report, in recent years the average age of developing breast cancer has shifted from 50 – 70 years to 30-50 years.

There are certain risk factors for breast cancer: genetic risk factors, family history, lifestyle and habits like smoking, alcohol intake, being overweight and lack of exercise, not having children or having them later in life, use of birth control pills, etc. This study deals with a gene involved in developing breast cancer.

**Types of breast cancer**

There are many types of breast cancer. Some are more common than others, and there are also combinations of cancers. Some of the most common types of cancer are as follows:

**Ductal carcinoma in situ:** The most common type of noninvasive breast cancer is Ductal Carcinoma In Situ (DCIS). Ductal means that the cancer starts inside the milk ducts, carcinoma refers to any cancer that begins in the skin or other tissues (including breast tissue) that cover or line the internal organs, and in situ means "in its original place." DCIS is called "non-invasive" because it does not spread beyond the milk duct into any normal surrounding breast tissue. DCIS is not life-threatening, but having DCIS can increase the risk of developing an invasive breast cancer later on. This type of cancer does not spread and therefore usually has a very high cure rate.
Invasive ductal carcinoma (IDC): This cancer which starts in a duct of the breast and grows into the surrounding tissue sometimes called infiltrating ductal carcinoma, is the most common type of breast cancer. About 80% of all breast cancers are invasive ductal carcinomas. “Invasive ductal carcinoma” refers to cancer that has broken through the wall of the milk duct and begun to invade the tissues of the breast. Over time, invasive ductal carcinoma can spread to the lymph nodes and possibly to other areas of the body.

Invasive lobular carcinoma (ILC) sometimes called infiltrating lobular carcinoma, is the second most common type of breast cancer after invasive ductal carcinoma (cancer that begins in the milk-carrying ducts and spreads beyond it). According to the American Cancer Society, more than 1,80,000 women in the United States have invasive breast cancer each year. About 10% of all invasive breast cancers are invasive lobular carcinomas. (About 80% are invasive ductal carcinomas.)

Inflammatory breast cancer (IBC) is a rare and aggressive form of breast cancer. According to the National Cancer Institute, about 1-5% of all breast cancer cases in the United States are inflammatory breast cancers.

Inflammatory breast cancer usually starts with the reddening and swelling of the breast (are due to the blockage of lymph vessels by cancer cells) instead of a distinct lump. IBC tends to grow and spread quickly, with symptoms worsening within days or even hours. It’s important to recognize symptoms and seek prompt treatment.

There are three ways in which cancer spreads in the body. They are

Through tissue; Cancer invades the surrounding normal tissue.

Through the lymph system; Cancer invades the lymph system and travels through the lymph vessels to other places in the body.
Through the blood; Cancer invades the veins and capillaries and travels through the blood to other places in the body.

When cancer cells break away from the primary (original) tumor and travel through the lymph or blood to other places in the body, another (secondary) tumor may form. This process is called metastasis. The secondary (metastatic) tumor is the same type of cancer as the primary tumor. For example, if breast cancer spreads to the bones, the cancer cells in the bones are actually breast cancer cells. The disease is metastatic breast cancer, not bone cancer.

**Stages of breast cancer:** The stages are classified according to TNM (Tumor, Node and Metastasis) staging system. The TNM is based on three characterisation namely,

1. Size of the tumor  
2. Lymph node involvement  
3. Metastasis (whether the cancer had moved beyond the breast to the other parts of the body)

**Stage 0:** (carcinoma in situ)

There are 2 types of breast carcinoma in situ:

Ductal carcinoma in situ (DCIS) is a noninvasive condition in which abnormal cells are found in the lining of a breast duct. The abnormal cells have not spread outside the duct to other tissues in the breast. In some cases, DCIS may become invasive cancer and spread to other tissues, although it is not known at this time how to predict which lesions will become invasive.
Lobular carcinoma in situ (LCIS) is a condition in which abnormal cells are found in the lobules of the breast. This condition seldom becomes invasive cancer; however, having lobular carcinoma in situ in one breast increases the risk of developing breast cancer in either breast.

**Fig. 3: Pea, peanut, walnut and lime show tumor sizes**

**Stage I:** In stage I, cancer has formed. Stage I is divided into stages IA and IB.

In stage IA, the tumor is 2 cm or smaller and has not spread outside the breast.

In stage IB, either:

- no tumor is found in the breast, but small clusters of cancer cells (larger than 0.2 mm but not larger than 2 mm) are found in the lymph nodes; or

- The tumor is 2 cm or smaller and small clusters of cancer cells (larger than 0.2 mm but not larger than 2 mm) are found in the lymph nodes.
Stage II: Stage II is divided into stages IIA and IIB.

In stage IIA:

- no tumor is found in the breast, but cancer is found in the axillary lymph nodes (lymph nodes under the arm); or

- the tumor is 2 cm or smaller and has spread to the axillary lymph nodes; or

- the tumor is larger than 2 cm but not larger than 5 cm and has not spread to the axillary lymph nodes.

In stage IIB, the tumor is either:

- larger than 2 cm but not larger than 5 cm and has spread to the axillary lymph nodes; or

- larger than 5 cm but has not spread to the axillary lymph nodes.

Stage IIIA: In stage IIIA, no tumor is found in the breast. Cancer is found in axillary lymph nodes that are attached to each other or to other structures, or cancer may be found in lymph nodes near the breastbone; or

the tumor is 2 cm or smaller. Cancer has spread to axillary lymph nodes that are attached to each other or to other structures, or cancer may have spread to lymph nodes near the breastbone; or

the tumor is larger than 2 cm but not larger than 5 cm. Cancer has spread to axillary lymph nodes that are attached to each
other or to other structures, or cancer may have spread to lymph nodes near the breastbone; or

the tumor is larger than 5 cm. Cancer has spread to axillary lymph nodes that may be attached to each other or to other structures, or cancer may have spread to lymph nodes near the breastbone.

**Stage III B:** In stage III B, the tumor may be any size and cancer:

- has spread to the chest wall and/or the skin of the breast; and
- may have spread to axillary lymph nodes that may be attached to each other or to other structures, or cancer may have spread to lymph nodes near the breastbone.

**Stage III C:** In stage III C, there may be no sign of cancer in the breast or the tumor may be any size and may have spread to the chest wall and/or the skin of the breast. Also, cancer:

- has spread to lymph nodes above or below the collarbone; and
- may have spread to axillary lymph nodes or to lymph nodes near the breastbone.

Stage III C breast cancer is divided into operable and inoperable stage III C.

In operable stage III C, the cancer:
is found in ten or more axillary lymph nodes; or
is found in lymph nodes below the collarbone; or
is found in axillary lymph nodes and in lymph nodes near the breastbone.

In inoperable stage IIIC breast cancer, the cancer has spread to the lymph nodes above the collarbone.

Stage IV: In stage IV, the cancer has spread to other organs of the body, most often the bones, lungs, liver, or brain.

Symptoms of breast cancer: The first sign of breast cancer is a new lump or mass in the breast that can be felt. A lump that is painless, hard and has uneven edges is more likely to be cancer.

Any of the following changes in the breast can be a symptom of breast cancer:

- swelling of all or part of the breast
- skin irritation or dimpling
- breast pain
- nipple pain or the nipple turning inward
- redness, scaliness, or thickening of the nipple or breast skin
- a nipple discharge other than breast milk
- a lump in the underarm area

Genes involved in breast cancer: There is an observation that breast cancer develops later in life and it has been suggested that more than one genetic event is involved in its development (Schmidt, 2002). There are quite a
number of genes involved in the development of breast cancer. Few genes which are involved in cancer development has already been discovered and few more are yet to be identified.

The popularly known genes that increase the risk of developing breast cancer are BRCA1, BRCA2, CDH1, PTEN, STK11, and TP53 (Rebbeck et al, 1996; Greene et al, 1997). Apart from these well-defined, high penetrance genes, there may be other genes that also increase the susceptibility to breast cancer. Candidates are proto-oncogenes and genes involved in metabolic, estrogen, and immunomodulatory pathways (Martin et al, 2000). Proto-oncogenes are involved in the regulation of normal cell growth and differentiation. Mutations in proto-oncogenes lead to disturbances in the cell cycle and can result in abnormal growth or proliferation (Weber et al, 2000). Well known proto-oncogenes are the ras gene, the HER2 gene, and the myc gene.

Twist as an oncogene: Twist was originally discovered as a Drosophila gene, whose mutation causes the characteristic 'twisted' phenotype in embryos (Thisse et al, 1988). Later, mouse, human, frog and chicken homologs were cloned and characterized (Castanon and Baylies, 2002). In human, twist germline mutations, resulting in a reduced Twist protein level, are supposed to be responsible for some Saethre–Chotzen syndrome (SCS) cases (Ghouzzi et al, 1997; Howard et al, 1997).

Twist protein features: The Twist1 and Twist2 (formerly Dermo-1) proteins belong to the basic helix–loop–helix (bHLH) family of transcription factors. They differ in their N-terminal but their C-terminal halves are sequentially very close, encompassing a conserved bHLH motif as well as an interaction domain named “Twist Box”. Through their bHLH motif, the twist proteins are able to recognise E-box responsive element (CANNTG) and behave either as transcription repressors or activators, depending on the cellular context
(Hamamori et al., 1999; Gong and Li, 2002; Pan et al., 2009). Twist proteins are known to directly interact with a large set of transcription factors and to modulate their activity. Twist proteins have the ability to either form homo- or heterodimers that display distinct, sometimes even antagonistic activities (Castanon et al., 2001; Firulli et al., 2005; Connerney et al., 2006; Connerney et al., 2008).

Twist is mainly active in early embryonic development where it enables the cells to move from one part of the embryo to another and allocates the cells to different tissues. As an embryo develops, Twist expression is no longer necessary and soon it becomes dormant in most of the tissues for the rest of the organism’s life (Yang et al., 2004). Twist has been shown to block terminal differentiation of mesodermal cells and to inhibit p53-dependent apoptosis (Maestro et al., 1999). A number of Twist-regulated genes were discovered in humans as well as in Drosophila and C. elegans, and several protein partners directly binding to the Twist protein in vivo. (Castanon and Baylies, 2002).

A possible participation of twist in cell transformation has also been suggested. Approximately 50% of rhabdomyosarcoma samples display abnormally high levels of the Twist protein (Maestro et al., 1999). In addition, the methylation status of the twist promoter has also been used as a sensitive marker for the detection of breast cancer cells in ductal lavage fluid (Evron et al., 2001).

Twist encodes a protein that contains a basic helix-loop-helix (b-HLH) motif and, therefore, most likely functions as a transcription factor (Thisse et al. 1988; Murre et al. 1989). Twist is expressed initially at the cellular blastoderm stage, in aband of cells on the ventral midline that is destined to form mesoderm and mesectoderm (Thisse et al. 1987, 1988).
Twist functions during embryogenesis and in adult tissues:

In *Drosophila*, the ancestral Twist gene twi is essential for proper gastrulation as well as for the generation of neural crest cells (Thisse *et al.*, 1987; Leptin, 1991). Molecularly distinct, Twist1 and Twist2 expression patterns overlap (Li *et al.*, 1995). Twist1 is not needed for gastrulation in mammals, Twist1 -/- homozygous mice die due to multiple defects including failure of neural tube closure, abnormal limb buds and increased apoptosis in the somites (Chen and Behringer, 1995). Twist2 expression in mice increases in somites and limb buds during embryogenesis and restricts, with time, to the perichondrium, which becomes the connective tissue bordering cartilage and bones (Li *et al.*, 1995). On the other hand, its expression is progressively increased in the dermis until birth and it is maintained in neonates but downregulated in adult tissues (Li *et al.*, 1995). Twist2 knockout mice develop normally but die shortly after birth due to an overexpression of proinflammatory cytokines resulting in severe cachexia (Sosic *et al.*, 2003). Thus, despite their sequence homology, Twist1 and Twist2 display distinct embryonic functions.

Twist proteins in adult humans are mainly expressed in precursor cells including the myogenic, osteoblastic, chondroblastic, odontoblastic and myelomonocytic lineages, maintaining their undifferentiated state. More recently, twist proteins were found to perform important roles in lymphocyte function and maturation. Twist1 and Twist2 are key regulators of B cell activation in an inflammatory environment such as autoimmune disease (Doreau *et al.*, 2009). Twist1 expression is induced following B cell stimulation with IL-17 and BAFF cytokines, and is essential for naive or memory B cell survival and proliferation as well as their differentiation into immunoglobulin producing cells.
Twist1 also functions downstream NF-κB in T helper 1 lymphocytes and is induced following repeated T cell Receptor binding. Twist1 inhibits the production of IFN-γ, IL-2 and INF-α, thus preventing their pro-inflammatory action (Niesner et al, 2008). In both B and T lymphocytes, Twist1 behaves as an early response gene, suggesting common regulation mechanisms (Niesner et al, 2008; Doreau et al, 2009). Similarly, in macrophages, IFN-induced Twist1 expression was shown to prevent TNF-α production (Sharif et al, 2006), contributing in down-modulating the inflammatory response.

In adult mice, Twist2 expression is restricted to the dermis where it remains barely detectable (Li et al, 1995). Its expression in adult human tissues has not been directly investigated. Twist2 transcripts are undetectable in normal colon, esophagus, lung, kidney and diseased tissues of melanocytic controls (Ansieau et al, 2008). Twist2 is transcriptionally active in a handful of mesenchymal tissues including myelomonocytic progenitors, where it inhibits their proliferation and differentiation, and in mature myeloid cells, where it inhibits the production of various cytokines including IL-4, IL-10 and IL-12 (Sharabi et al, 2008).

**Twist involvement in metastasis:** Twist pro-metastatic potential depends on their ability to induce an Epithelial to Mesenchymal Transition (EMT), a process that converts joined and polarized epithelial cells into isolated and motile mesenchymal ones, able to bypass the basement membrane and infiltrate into the surrounding extracellular matrix (Thiery and Sleeman, 2006; Nakaya and Sheng, 2008; Yang and Weinberg, 2008).

This process involves the loss of cellular junctions (tight and adherent junctions as well as desmosomes) and reorganizes the cytoskeleton. EMT contributes to several human pathologies, including renal, hepatic and lung
fibrosis, by turning epithelial cells into collagen-producing mesenchymal ones (Iwano et al, 2002; Boutet et al, 2006; Kim et al, 2006; Zeisberg et al, 2007).

Twist proteins promote EMT by turning-down the expression of epithelial specific proteins, such as E-Cadherin (cell adhesion protein) and by upregulating the expression of mesenchymal markers such as the N-cadherin, the vimentin and the smooth-muscle actin. When E-cadherin is down regulated, it loses the cell to cell adhering capability and becomes isolated from the tissues and mixes into blood stream thereby causing metastasis.

Although, there are few reports known about Twist, its expression and function varies in concern to estrogen receptor (Vesuna et al, 2011). The reason for the varied gene expression level with the receptor presence and absence is yet to be known. This study deals with the role of twist expression with respect to the estrogen receptor.

**Estrogen Receptor**

Estrogens are primary female sex hormones. Natural estrogens are steroid hormones whereas synthetic ones are non-steroid. Estrogens are synthesized in all vertebrates and in some insects. All steroid hormones including estrogen diffuse across the cell membrane and in the cytosol they bind to and activate estrogen receptors which in turn modulate the expression of a number of genes.

**Fig. 4:** Twist expression in benign tumors favours metastatic conversion (Figure adapted from Bastid et al, 2009)
The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2) and estriol (E3). They make up 10-20%, 10-30%, and 60-80% of circulating estrogens. Though estriol is the most plentiful of the three estrogens it is also the weakest, whereas estradiol is the strongest with a potency of approximately 80x that of estriol. Thus, estradiol is the most important estrogen in non-pregnant females. Another type of estrogen called estetrol (E4) is produced during pregnancy.
There are two different forms of estrogen receptors, referred as α and β, each encoded by separate gene. The ER's helix 12 domains plays a crucial role in determining interactions with coactivators and corepressors and, therefore, the respective agonist or antagonist effect of the ligand (Bourguet et al 2000; Ascenzi et al 2006).

Different ligands may differ in their affinity for alpha and beta isoforms of the estrogen receptor

17-beta-estradiol binds equally well to both receptors

Estrone and raloxifene bind preferentially to the alpha receptor

Estriol and genistein bind to the beta receptor

The different estrogen receptor combinations may respond differently to various ligands, which may translate into tissue selective agonistic and antagonistic effects (Kansra et al 2005). The ratio of α- to β- subtype concentration has been proposed to play a role in certain diseases (Bakas et al 2007).

The same ligand may be an agonist in some tissue (where coactivators predominate) while antagonistic in other tissues (where corepressors dominate). Tamoxifen, for example, is an antagonist in breast and is, therefore, used as a breast cancer treatment (Derro et al 2006) but an ER agonist in bone (thereby preventing osteoporosis) and a partial agonist in the endometrium.

Fig. 5: Figure represents the triggering of gene activation by Estrogen receptors. (adapted from Kelly et al, 2010)
Estrogens are hormones that stimulate the development and maintenance of female characteristics and sexual reproduction. Estrogens function as signalling molecules by travelling through the bloodstream and interact with the cells in the target tissues. The breast and uterus are the main targets of estrogen, they act on target tissues by binding to the cell through the specific receptor called Estrogen Receptor (ER) which are present inside the target cells. Estrogen binds to the target tissues only when the cells contain estrogen receptor, it will not exert its effect on the cells which do not contain the receptor. In majority of the cases estrogen receptor resides inside the nucleus along with the ERE on DNA. Unless and until the presence of estrogen molecule, the estrogen receptor is inactive and does not influence the target genes (DNA). On binding of estrogen to the receptor which causes to change the shape of the receptor to form estrogen receptor complex in turn bind to the specific DNA sites called estrogen response elements which are located near genes that are controlled by estrogen. After the estrogen-receptor complex attached to the estrogen response elements in DNA, it also binds to the coactivator proteins and activates more nearby genes. These active genes
produce molecules of messenger RNA and synthesis protein which can influence cell behaviour (Jeanne Kelly et al, 2010).

The main function of the estrogen receptor is as a DNA-binding transcription factor that regulates gene expression. It regulates number of genes which are involved in breast cancer progression. One such gene is twist. Twist protein is a transcription factor which binds to the E-Box of estrogen receptor and suppresses the ER expression to promote breast cancer (Vesuna et al 2011).

**Quercetin - a bioflavonoid**

Herbal therapies are currently the most effective treatments due to three main reasons. Herbs boost the immune system, activate the body’s natural healing process and support the body during chemotherapy or radiotherapy. The World Health Organization recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines. There is a large, global market for extracts from herbs that have medicinal properties. The extracts from these herbs are commonly termed nutraceuticals, and are sold as ‘alternative’ or ‘natural’ health care remedies. Herbal extracts are easier to dose. The efficiency of herbal extracts depends on many factors, such as, plant parts being used, growing environment, harvesting season, type of solvent used and extraction procedure. Several classes of natural compounds have been evaluated for their medicinal properties that can alleviate symptoms or prevent cancer. Each of these classes of plant-derived compounds or extract interacts with the host to confer a preventive benefit by regulating cellular signalling of proliferation and death (Po-Lin-Kuo et al, 2005).

**Fig. 6: Chemical structure of Quercetin**
Quercetin is one such plant-derived flavonoid, which has effective antioxidant and anti-inflammatory properties (Laura et al, 2008: Davis et al, 2009). Quercetin helps prevent cancer by blocking the flow of nutrients and oxygen to cancerous cells, effectively cutting off their food supply. Quercetin is also a phytoestrogen, or a plant hormone that mimics the effects of estrogen in the human body. Quercetin binds to estrogen-receptor sites in place of estrogen, so that breast cancers that need estrogen to flourish are no longer stimulated to grow. Laboratory studies have shown that quercetin from citrus fruits can reduce the growth rate of breast cancer cells by as much as 50 percent. In addition, quercetin has been shown in animal and in vitro studies to inhibit the growth of colon, prostate, breast, lung cancer cells and as anti-depressant (Davis et al, 2000, Anjaneyulu et al, 2003). Thus, the present study involves the study of Twist gene expression correlated with estrogen receptor and whether the flavonoid quercetin has any role in controlling the twist expression or the estrogen receptor expression.