3. Introduction

3.1. Breast Cancer- the biology of the disease

The development of breast cancer involves several types of genes that need to be activated or inactivated in order to promote malignancy. Putative precursor stages to invasive breast tumor growth are hyperplasias and carcinoma in situ. Breast cancer can also progress further to a metastatic disease, where axillary nodes are the most common sites, but distant metastasis can also occur where bone and bone marrow are among the major locations.

Classification of breast cancer

a) **Benign tumors**: These include sclerosing conditions, different types of hyperplasias, fibrocystic changes, fibroadenomas, gynecomastia, papillomas and phyllodes tumor; the most common being fibroadenomas and cysts. Fibroadenomas are simple overgrowth of the lobules. Cysts are smooth, mobile lumps and contain areas of firm fibrous fatty tissue. These benign tumors generally do not represent an important risk factor. Among males, the most common disease is gynecomastia.

b) **Malignant tumors**: There are two types of malignant tumors – viz. in situ carcinoma and invasive carcinoma. Ductal carcinoma *in situ* (DCIS) is defined as a proliferation of malignant epithelial cells within parenchymal structures of the breast while lobular carcinoma *in situ* (LCIS) defines a proliferative lesion limited to one or more mammary lobules without invasion of the basement membrane. Infiltrating ductal carcinoma (IDC) is the most common type of invasive breast cancer accounting for about 80% of all cases. There are other less common types of carcinomas such as medullary carcinomas, mucoid carcinoma etc.
3.1.1. Incidence of breast cancer-worldwide and national status

Breast cancer now ranks first among cancers affecting women throughout the world and its marked impact is not restricted to Western industrialized societies. In 1998, there were 412,000 deaths attributable to breast cancer in the world, representing 1.6% of all female deaths. The world-wide incidence and mortality rates of various cancers among women are summarised in Fig.1. In 2010, an estimated 207,090 new cases of invasive breast cancer were expected to be diagnosed in women in the U.S., along with 54,010 new cases of non-invasive (in situ) breast cancer. About 39,840 women in the U.S. were expected to die in 2010 from breast cancer, though death rates have been decreasing since 1990. In terms of absolute numbers, the greatest contribution is now from developing countries, where 2,50,000 such deaths occurred, as compared to developed countries, which account for 1,60,000 deaths. However, the proportion of deaths due to breast cancer in women remains higher in the latter countries at 2% in comparison to 0.5% in the developing countries.

The Netherlands exemplifies the high incidence of breast cancer in developed countries with an age-standardized incidence rate of 91.6 new cases per 1,00,000 woman. In contrast, low rates are found among African and Asian populations. These large geographical differences are potentially explicable on the basis of genetics or the influences of lifestyle and environment. Studies of migrant populations have revealed that when women migrate from low-risk to high risk regions, the migrant populations acquire the rates of the host country after two or three generations, indicating lifestyle as primarily determining the geographic variations in risk.

Cancer incidence in India is also rising steadily. One in every 10 women up to 75 years of age are expected to get some form of cancer in their life time. For the year 2008, 5,18,800 new cancer cases were predicted and of this, 1,15,251 were expected to be breast cancer. The mortality rates of women due to various cancers in India are summarised in Fig.2.
Fig. 1. Worldwide incidence and mortality rates of various cancers among women
(Source: GLOBOCAN 2008 (IARC))

ASR- age standardized rate

Fig. 2. Mortality rates of various cancers among women in India
3.1.2. Risk factors

The development of breast carcinoma is probably multifactorial. The major clinical risk factors for breast cancer are summarized in Table-1. The causative factors so far proposed can be divided into three main categories:

**A. Genetic factors:** They are suggested by the strong familial tendency. There is no particular pattern of inheritance, suggesting that the familial incidence is either due to the action of multiple genes or some environmental factors acting on the genes. BRCA-1 gene mutations are believed to account for 45% of families with a high incidence of breast cancer. BRCA2 gene is also reported as important to familial breast cancer. Furthermore, mutations in the gene that encodes the tumor suppressor protein p53 and activation of oncogenes such as erbB2/neu and c-ras have been reported, but are believed to be less important than BRCA1/BRCA2.

**B. Hormones:** They are widely believed to play a role in the etiology of breast cancer. Epidemiologic evidence suggests that prolonged estrogen exposure (early menarche, late menopause, nulliparity and delayed pregnancy) increases the risk for breast cancer. In a study, nulliparous women had a 2.2-fold higher risk than parous women [Rao DN, 1994]. The hormone dependency of some of the breast cancers is related to the presence of estrogen, progesterone and other steroid hormone receptors in the nuclei of breast cancer cells.

**C. Dietary and environmental factors:** A study by Fung TT et al. (2006) illustrates that diet is the most important risk factor for breast cancer. Large increases in the incidence rates of breast cancer have been reported among migrating populations, particularly in those migrating from Japan to United States (Buell, 1973). Exposure to environmental factors such as moderate to high dose ionising radiation, occupational exposures, organochlorine pesticides etc. have been linked to increased risk of breast cancer.
Table 1.
Clinical risk factors for breast cancer

Sex: Female
Race: Caucasians, Jews
Age: 30+ years, median 60 years
Family History: Mother or sister with breast cancer (fivefold)
Medical history:
  Previous breast cancer
  Fibrocystic changes with atypical ductal or lobular hyperplasia (four-to-five fold)
Menstrual history:
  Early menarche (<12 years)
  Late menopause (>50 years)
Pregnancy history:
  Nulliparous
  Late age at first pregnancy (>30 years)
  Absence of breast feeding
Others:
  Obesity
  Oral contraceptive use
  Chronic alcoholism

3.2. Metastasis in breast cancer
3.2.1. Incidence of metastasis in breast cancer

Metastasis is the leading cause of increasing mortality and treatment failure in breast cancer. Survival rates for patients with non-metastatic breast cancer has increased from 72% in
the 1940s to 96% today. However, if the cancer has spread regionally, the rate is 77% and for patients with distant metastasis, the rate is 21 percent.

About 30% of breast cancer patients with occult metastasis are cured by chemotherapy. In node negative breast cancer patients the long term survival with local therapy without systemic therapy is 70-80%. Thus if all node negative patients are treated systemically the large majority will receive therapy unnecessarily. If these patients could be identified early they could be spared systemic therapy and a more intense programme could be employed for those patients at the greatest risk, perhaps increasing their likelihood of survival.

Types of metastasis

Tumor cells disseminate by invasion of tumor cells into the blood supply (haematogenous metastasis) and the lymphatic system (lymphatic metastasis). Clinical and pathological observations suggest that transport of tumor cells via the lymphatics is the most common route of initial dissemination for many carcinomas and the cells generally spread following natural drainage routes. Sentinel lymph nodes (SLN) are the first to receive drainage from any given location and their involvement is the first sign of spread in most human cancers.

Tumor associated lymphatics constitute the main channel for the early spread of tumor cells into the lymph nodes and this spread may occur through pre-existing vessels. An alternative hypothesis is that it involves newly formed lymphatics, which are produced by a process called lymphangiogenesis. Hartveit suggested, "tumor cells lying free in the periductal lymphatic spaces will be washed with the tide of tissue fluid into the labyrinth through the sinuses and on into the lymphatic drainage channels. There is no need to postulate active lymphatic invasion." Evidence in favour of this hypothesis or the alternative one of active tumor cell intravasation is still an area of active research.

Cancer patients can be grouped into three categories clinically: (a) those with overt clinical evidence of metastasis at diagnosis (b) those with covert or obscure metastatic disease (c) those with no metastases. With no treatment, there is most likely to be a gradual progression from no metastases to covert and then to overt metastases (Bloom HJG, 1962, Koscielny S, 1984, Heimann R, 1998). The concept of malignant progression is well accepted but the supporters of the systemic hypothesis strongly believe that by the time of clinical detectability tumors have acquired all of their malignant capacity. The concept of progression during the
clinical phase of cancer has important implications because it suggests that smaller tumors are more curable. A greater proportion of the smaller tumors are able to metastasise only to lymph nodes since they have not yet acquired the capacity to metastasise to distant sites (Heimann R, 2000).

Two clinical characteristics of metastatic predisposition can be isolated- metastagenicity (the ultimate likelihood of metastases) and virulence (the rate at which metastases become clinically apparent). Both virulence and metastagenicity increase with the size of the tumor and number of lymph nodes involved (Heimann R, 2000). The metastagenicity in patients with 1-3 positive nodes is 0.62 while in patients with ≥ 4 nodes it is 0.86 but even in the most advanced group, about 14% of the patients is cured by local therapy only. This underscores the significant heterogeneity within the clinical groups.

3.2.2. The metastatic cascade

The ability of malignant tumors to metastasise largely is responsible for their lethality. Despite the advent of better local treatment in the form of surgery and radiotherapy and systemic chemotherapy, the clinical challenge in oncology remains that of combating metastatic spread. There is, therefore, a pressing need to understand the underlying molecular and cellular mechanisms of tumor dissemination so as to develop novel therapies based on this knowledge.

Having disengaged from its primary site the metastatic tumor cell must invade the surrounding stroma, enter the vasculature or lymphatic system, survive and arrest at a distant site. From there it extravasates into the tissue and after elaboration of a blood supply, grows and develops into a secondary mass. Key processes involved include changes in cellular adhesion, production of proteolytic enzymes capable of degrading the stroma and the secretion of a variety of cytokines which attract and activate stromal cells and endothelial cells during invasion and angiogenesis. A simplistic tale of sequence of events leading to dissemination of tumor cells would begin with the escape of cells from
their local environment. For this to occur, the first step in the life of a tumor cell which has just become motile is to break all stable physical contacts with neighbouring cells. Thereafter, cells move towards through the blood vessels surrounding the basement membrane.

Fig.3. Simplified schematic representation of the steps of hemotagenous metastasis.
Tumor cell-secreted proteases are causative in the degradation of basement membranes around epithelial cells. Infiltration of newly formed blood vessels, on the other hand, might not require basement membrane degradation since the latter are frequently fenestrated. Tumor cell migration is a complex process involving a balance of adhesive and anti-adhesive matrix properties. The saga of a tumor cell establishing a colony at a distant site is visualised in Fig.3.

3.2.3. Crucial proteins of the metastatic cascade

3.2.3.1. Cell adhesion molecules

The initial escape of a tumor cell from its primary site requires the loss of cell-cell attachment which, in epithelial tumors, is mediated largely by the members of the cadherin family and in particular by E-cadherin. Alterations also occur in the nature of adhesion events between the released tumor cells and the extracellular matrix, which allow the motile neoplastic cells to migrate over underlying substrates. Integrins are of prime importance in these cell-substrate interactions. Other adhesion molecules, such as those of the immunoglobulin superfamily and/or the selectin family, are involved in several heterophilic cell-cell interactions including the adherence of tumor cells to the endothelium.

Cadherins

The cadherins are calcium-dependent adhesion molecules. The three most common cadherins are neural (N)-cadherin, placental (P)-cadherin, and epithelial (E)-cadherin. All three belong to the classical cadherin subfamily. There are also desmosomal cadherins and proto-cadherins. Cadherins are intimately involved in embryonic development and tissue organization. They exhibit homophilic adhesion. The extracellular domain consists of several cadherin repeats; each is capable of binding a calcium ion. When calcium is bound, the extracellular domain has a rigid, rod-like structure. Following the transmembrane domain, the intracellular domain is highly conserved. The intracellular domain is capable of binding the α, β, and γ catenins. The adhesive properties of the cadherins have been shown to be dependent upon the ability of the intracellular domain to interact with cytoplasmic proteins such as the catenins. In the present study we tried to look at the expression of E-cadherin and P-cadherin in
different types of breast cancer and to see whether their expression is related to node positivity and distant metastasis. Another objective of the study was the characterisation of the prognostic significance of cadherins in breast cancer. Since both the cadherins are functionally interrelated, the imbalance in the co-expression status of the two cadherins and their potential to be used as prognostic markers in breast cancer was also included in the study.

3.2.3.1. a. E-cadherin

Adjacent epithelial cells are bound together by adherens junctions that are formed by a complex of molecules that includes alpha-, beta- and gamma-catenins and actin cytoskeleton filaments (Kemler R, 1993; Steinberg MS, 1999). E-cadherin is a calcium dependent cell-cell adhesion molecule that forms the key functional component of adherens junctions of all epithelial cells. It interacts with E-cadherin molecules on the adjacent epithelial cells forming an adhesive structure, which has been likened to an intercellular zipper (Shapiro, 1995). Translation of intercellular contact signals into cellular organization is thought to be mediated by the catenins, which are key regulatory molecules in this mechanism. The implication of E-cadherin in carcinogenesis is supported by the fact that alterations in its expression are frequently seen in various carcinomas including breast cancer (Siitonen SM, 1996).

The mature form of E-cadherin is a 12 KDa protein that consists of an extracellular portion of five tandem repeats, the most distal of which is required for calcium mediated homotypic binding (Gumbiner B, 1988). E-cadherin also contains an intracellular portion that is responsible for linking E-cadherin to the actin cytoskeleton through α-, β- and γ-catenin that is necessary for the adhesive function of E-cadherin (Miller JR, 1996). The posttranslational cleavage of the native E-cad results in a novel membrane bound 97 KDa fragment E-cad in cells destined to undergo apoptosis (Vallorosi CJ, 2000). This cleavage effectively removes the β-catenin binding domain, rendering the E-cadherin molecule functionless.

Loss of E-cadherin expression has been found to be associated with tumor cell dissemination and metastasis in many carcinomas (Perl AK, 1998; Vleminckx K, 1991; Mareel M, 1995). Germline inactivating mutations in the E-cadherin gene have been found in families with inherited predisposition to gastric carcinomas, particularly those with diffuse type
histology and perhaps breast carcinomas (Guilford P, 1998; Gayther SA, 1998). Somatic mutations in E-cadherin are the most prevalent in lobular breast carcinomas and in diffuse type gastric carcinomas with about 50% of the cancers of each of these types displaying somatic mutations inactivating both E-cadherin alleles (Hirohashi S, 1998; Becker KF, 1994; Berx G, 1995). However, in most cancers with reduced or absent E-cadherin gene and protein expression, mutations in E-cadherin are rarely detected and proposed mechanisms of E-cadherin inactivation include promoter hypermethylation, changes in chromatin structure (Hennig G, 1996), and alterations of specific transcription factor pathways regulating E-cadherin gene expression (Ji X, 1997; Hajra KM, 1999).

Studies on the human E-cadherin promoter have led to the characterization of several positive regulatory elements in the 5' proximal promoter, including a CCAAT-box, a GC-rich region and an enhancer element (Behrens J, 1991; Hennig G, 1996). The factors interacting with the proximal GC-rich region and the enhancer element have been identified as AP2 and SP1 transcription factors. Inactivation of E-cadherin is most likely associated with aberrant CpG island hypermethylation (Yoshiura K, 1995; Kanai Y, 1997). The binding of certain transcription factors has been reported to be inhibited by CpG methylation in their binding sites (Gray SC, 1999; Ohtani-Fujita N, 1993). Furthermore, methylation of specific CpG sites in Hpa II and FnuDII methylation enzyme recognition sites were found to decrease the activity of retinoblastoma promoter. It has been documented that methylation of CpG inhibits the binding of transcription factor, thus resulting in transcriptional inactivation (Chan MF, 2000). One possible mechanism through which DNA methylation can regulate gene expression is the prevention of binding of transcription factors by the addition of 5-methyl cytosine, which protrudes into the major groove of the DNA helix (Kass SU, 1997).

3.2.3.1.6. **P-cadherin**

Although E-cadherin is expressed in all epithelial tissues, the expression of P-cadherin is only restricted to the basal or lower layers of stratified epithelia, including prostate and skin and also the breast myoepithelial cells (Nose A, 1986). This unique distribution of P-cadherin suggests that in addition to maintaining cellular adhesion, this molecule may also have other
unknown functions, which can be important in cell differentiation and proliferation (Daniel CW, 1995).

The expression pattern of P-cadherin has been linked to a more dedifferentiated stem-cell-related population of tumor cells, although the importance of P-cadherin in different tumors is not well understood. The localization of human P-cadherin gene is at 32 kb upstream of the human E-cadherin gene, also mapping to chromosome 16q22.1, showing the evolutionary conservation of the tandem arrangement of two genes encoding cell adhesion molecule, suggesting that the close proximity of these genes may be important for their regulation (Bussemakers MJ, 1994).

Upregulation of P-cadherin has been shown in several lesions, including breast cancer, in which there is usually downregulation of E-cadherin (Palacios J, 1995). Breast carcinomas show aberrant P-cadherin expression in about 30% of the cases and have been reported as a prognostic marker of poor outcome in patients (Gamallo C, 2001). The differential pattern of P-cadherin expression in breast cancer development coupled with its possible prognostic value, prompted us to investigate its expression in a series of invasive breast carcinomas.

Physical loss of chromosome 16q, on which P-cadherin is located is much more common in low-grade, nonbasal cancers than in high grade cancers. If chromosome 16q alterations are found in these latter cancers, then the mechanism observed is mitotic recombination (Cleton-Jansen AM, 2002). This would affect the dosage of P-cadherin, thus, high grade breast cancers are likely to have two copies of chromosome 16q, even if loss of heterozygosity is observed.

3.2.3.2. **Signaling molecules of the metastatic cascade**

Interactions between cells responsible for cell to cell adhesion also can communicate signals into the cellular interior, often involving interactions with cytoskeletal elements to produce changes in cell motility, migration, proliferation and shape. The cadherins are cell surface adhesion molecules that help form tight junctions between cells such as formation of epithelial cell layers. In addition to mediating adhesion with other cells, cadherins transduce
signals into cells through interactions with the catenins. Catenins probably affect actin cytoskeletal function through interactions with proteins like actinin and vinculin. Catenins also probably trigger changes in cell shape and motility with signals through the Rho small GTPases. Another important cell adhesion molecule is CD-31, or PECAM-1, involved in the formation of junctions between endothelial cells, cell migration, migration of lymphocytes, and regulation of lymphocyte activation. Src phosphorylates PECAM-1 on tyrosine residues causing SHP-2 association with PECAM-1. Paxillin acts as an adaptor protein between proteins involved in adhesion signaling like FAK and src and cytoskeletal elements. In addition to signals created by adhesion molecules to alter cellular responses, other signaling pathways can alter adhesion through components of the focal adhesion complex. Thus in the present study, we tried to focus on two important signal transducing molecules of the metastatic cascade-namely, beta catenin and Focal adhesion kinase (FAK).

3.2.3.2.a. Beta-catenin

Catenins were discovered as proteins with cytoplasmic domain of transmembrane proteins. Cadherins, components of adherens junction, interact with catenins which link E-cadherin to actin cytoskeleton and thus the cadherin-catenin interaction is important in stabilizing cell-cell adhesion. Although β-catenin was first isolated in association with E-cadherin cytoplasmic domain, this protein is an essential component of the Wnt/wingless pathway. In the downstream of Wnt pathway, β-catenin was recognized as another oncogene. It is believed that the accumulation of β-catenin induces uncontrolled activation of downstream genes such as c-myc and cyclin D1, which are involved in tumorigenesis. Mutations in β-catenin are also responsible for the upregulation of oncogenes such as c-myc and cyclin D1.

β-catenin is a ubiquitous intracellular protein important in both intercellular adhesion and signal transduction. β-catenin plays a pivotal role in cell-cell adhesion by linking the cytoplasmic domain of cadherins to α-catenin which anchors the adhesion complex to the cytoskeleton (Gumbiner BM, 1996). β-catenin mediates the interactions between cadherins and other transmembrane receptor proteins, such as the EGFR. Through this latter interaction,
the cadherin-catenin complex becomes a target for regulatory signals that govern cellular adhesiveness and motility (Kinch MS, 1995).

β-catenin is also a signalling molecule and can activate gene transcription by forming a heterodimer with T-cell factor/lymphoid enhancer-binding factor family of DNA binding proteins (Behrens J, 1996). In the absence of growth or differentiation signals, cytoplasmic β-catenin is rapidly turned over, under the control of the APC protein and the GSK-3 β. Protein turnover occurs in the proteasome, where β-catenin is degraded after targeted phosphorylation of highly conserved serine and threonine residues and ubiquitination in the aminoterminus. Deletion of the amino terminus or mutation of one or more of the phosphorylation sites inhibits the ubiquitination and degradation of β-catenin, enhancing its availability as a transcriptional activator (Morin PJ, 1997). Loss of control of intracellular β-catenin levels through mutation of either β-catenin or APC has been proposed as an important oncogenic step in colorectal carcinogenesis.

The two functions of β-catenin, E-cadherin binding and signal transduction are interrelated. E-cadherin can modulate the cytoplasmic pools of β-catenin available for signalling, because ectopic expression of high levels of E-cadherin in Xenopus embryos antagonizes the axis-duplicating activity of β-catenin. Expression of E-cadherin is often abnormal in carcinomas including breast cancer (Pierceall WE, 1995).

Hence we thought that while dissecting the role of cadherins in breast cancer metastasis, it would be inappropriate if the role of β-catenin was left unanalyzed. While looking at the expression status of β-catenin in different subsets of breast cancer, the mutation status of β-catenin and its potential in the development of breast cancer metastasis was also looked at. Since exon 3 is the hotspot of mutations in β-catenin, we sought to look into the mutation spectrum of exon 3 of β-catenin in breast cancer.

3.2.3.2.6. **Focal adhesion kinase (FAK)**

Focal adhesions are points of close apposition between the cell membrane and the extracellular matrix which is comprised of proteins such as collagen, fibronectin or vitronectin.
(Burridge, 1988). Integrins physically link the ECM to the cytoplasmic actin cytoskeleton and may function to transmit signals from the ECM to the cytoplasm (Schwartz, 1992). Engagement of integrin receptors with extracellular ligands gives rise to the formation of complex multiprotein structures that link the ECM to the cytoplasmic actin cytoskeleton. These adhesive complexes are dynamic, often heterogeneous structures, varying in size and organization. In motile cells, sites of adhesion within filopodia and lamellipodia are relatively small and transient and are referred to as focal complexes whereas adhesions underlying the body of the cell and localized to the ends of actin stress fibers are referred to as focal adhesions. The formation and remodeling of focal contacts is a dynamic process under the regulation of protein tyrosine kinases and small GTPases of the Rho family.

One of the molecules that may be involved in the regulation of focal adhesion integrity is FAK. The focal adhesion kinase, FAK or pp125 FAK is a 125 KDa protein tyrosine kinase whose name is derived from its subcellular localization (Schaller, 1992). Unlike other nonreceptor tyrosine kinases FAK does not contain SH2 or SH3 domains. It is a highly conserved protein comprising a central catalytic domain flanked by NH2 and C-terminal noncatalytic domains. The C-terminal 150 residues of FAK contain the focal adhesion targeting (FAT) sequence which is responsible for directing and/or anchoring FAK to cellular focal adhesions (Hildebrand, 1993). Alternative splicing of the FAK gene can lead to independent expression of the C-terminus of FAK. This protein has been termed FRNK (FAK-related nonkinase) (Schaller MD, 1993). FAK is now the prototypical member of a small family of PTKs comprised of two members, FAK and PYK2/CAK (Lev et al, 1995).

FAK regulates cell spreading and migration and has been proposed to function in the assembly of cellular focal adhesions and cell spreading on ECM proteins since inhibitors of PTKs inhibit both events (Burridge, 1992). Fibroblasts derived from dominant negative variant of FAK embryos exhibit an increased number of focal adhesions but a decrease in cell migration in invitro assays (Ilic, 1995). The increase in focal adhesion expression was somewhat surprising, but it suggests that FAK may regulate the cycles of focal adhesion assembly and disassembly rather than assembly per se. These evidences implicate FAK as a regulatory protein in the basic cellular processes of cell spreading and migration.
FAK was originally isolated as a candidate substrate for pp60src and subsequently shown to be a pp60src binding protein (Cobb, 1994). FAK is thus a candidate for a mediator of some of the effects of src transformation in cells in tissue culture. Irby et al (2002) provide evidence for the role of src, Ras and FAK in the regulation of homotypic adhesion which may in turn regulate the metastatic potential of human cancer cells. Changes in cell surface expression of integrins can modify the tumorigenic and metastatic properties of cells (Albelda, 1990) which could conceivably be mediated by altered signaling through FAK. FAK expression levels are elevated in some tumors (Owens, 1995). Indeed as a regulator of basic cellular responses such as migration, FAK may also contribute to the pathology of other human diseases like vascular diseases involving hyperproliferation and migration of vascular smooth muscle cells.

FAK gets phosphorylated concomitantly becoming activated following antibody crosslinking of cell surface integrins or upon integrin dependent cell adhesion (Burridge, 1992). Apart from integrins, many other stimuli have been shown to induce the tyrosine phosphorylation of FAK including PDGF (Rankin, 1994), bombesin (Zachary, 1992), hyaluronic acid (Hall, 1994). The integrity of the actin cytoskeleton is crucial for signaling to FAK. The tyrosine phosphorylation of FAK regulates complex formation with signaling proteins that contain SH2 domain like GRB2. The association of FAK with GRB2 implicates p21ras as a downstream component of FAK signaling (Cohen, 1995).

It has been proposed that the major substrate of FAK is FAK itself (Richardson A, 1996). When cells adhere to fibronectin, FAK becomes autophosphorylated which causes a second tyrosine kinase called src to become associated with FAK (Calalb MB, 1995). Then src phosphorylates FAK which leads to the recruitment of Grb-2 and SOS to the FAK signaling complex (Schlaepfer DD, 1994). Ras is known to activate MAPKs which are involved with the initiation of DNA synthesis. Therefore, these studies have linked FAK to pathways that are associated with mitogenesis. The substrates of FAK include paxillin, p130cas (Burridge, 1992), tensin (Lo, 1994), PI3K (Chen, 1994). However, these proteins exhibit tyrosine phosphorylation in FAK deficient fibroblasts. This observation may indicate the existence of multiple mechanisms of phosphorylation of these substrates, only one of which is FAK dependent.
Focal adhesion kinase is a protein tyrosine kinase linked to signaling events between cells and the extracellular matrix and has been implicated in the regulation of cell migration. (Schaller MD, 2001). Several studies point to a correlation between the overexpression of FAK and carcinogenesis in many cancers, including breast cancer. Very few studies are available looking into the role of FAK in metastatic breast cancer and its putative role in lymphatic and hematogenous metastasis. Hence we decided to probe the importance of FAK in metastatic cancer and its value as a prognostic marker in breast cancer.

3.2.3.3. Proteolytic enzymes

One requisite for neoplastic cell invasion during tumorigenic processes is the remodeling events that occur within the stroma or ECM. Excess matrix degradation is one of the hallmarks of cancer and is an important component of the tumor progression process. Many proteinases are capable of degrading ECM components, the most important being serine proteases, cathepsins and matrix metalloproteinases (MMP). The principal proteinases involved in the degradation process are matrix metalloproteinases (MMPs) and plasminogen activators.

3.2.3.3.a. Matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP)

MMPs are a family of zinc- and calcium-dependent endopeptidases. Fourteen human MMPs have been identified and can be grouped into at least five groups: the collagenases, gelatinases, stromelysins, membrane type MMPs and others (Baramova and Foidart, 1995). MMPs exist in latent and active form and complexed with and without their specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). MMPs have the following functional characteristics:

(a) they are proteinases that degrade at least one component of the extracellular matrix and contain a zinc ion and are inhibited by chelating agents
(b) they are secreted in a latent form, requiring activation for proteolytic activity
(d) they are inhibited by tissue inhibitors of metalloproteinases (TIMPs)
(e) they share common amino acid sequences

Overexpression of MMPs is now known to be a characteristic of most malignant tumors. The constitutive expression level of MMPs is normally low but increased expression is observed in many disease states such as arthritis and neoplasia. Some of the MMP genes are known to be inducible and AP-1 binding sites are found within the promoter regions of these genes. AP-1 transcription factors are proteins, composed of Jun and Fos subunits. Binding of Fos/Jun complexes to the AP-1 site is associated with transcriptional activation of MMP genes. Aberrant methylation of the promoter region of the TIMP-3 gene was identified in a variety of solid tumors including breast (Bachman KE, 1999). A single nucleotide polymorphism that creates an Ets binding site has been described in the MMP1 promoter. This polymorphism is associated with increased levels of transcription of MMP-1 in both normal and malignant cells (Rutter JL, 1998). The MMP gene has also been shown to be a p53 target gene. Wild type p53 downregulates the promoter activity of MMP-1 in a dose dependent fashion while mutant p53 does not display this repressive activity. Apart from these regulatory mechanisms, a number of compounds like thyroid hormone, retinoic acid etc. are known to inhibit the synthesis of MMPs through binding to members of the nuclear receptor subfamily (Schroen DJ, 1996).

The TIMPs are a family of low molecular weight proteins, currently numbering four in total, which function as the major physiological inhibitors of the activated forms of the MMPs. The TIMPs do not simply act as inhibitors of MMPs, but they also play important functions in their activation. For example, the activation of MMP-2 requires the formation of a ternary complex comprising MT1-MMP, proMMP-2 and TIMP-2.

The expression of MMPs can be increased in vitro by a variety of growth factors and cytokines and their conversion to active enzymes can be induced by plasmin or trypsin. Equally the activity of the inhibitors of MMPs, TIMPs can also be regulated by local cytokines and growth factors. It is the overall balance between the levels of activated MMPs and free TIMPs that determines the overall MMP activity and ECM degradation.
There are a number of points of intersection of the coagulation and fibrinolytic pathways with the MMP activation cascade. Plasmin is an efficient activator of MMP-3 and MMP-13 while activated MMP-3 is a potent activator of MMP-9 and MMP-1. MMPs participate in both altered matrix degradation and cell adhesion via multiple interactions with constituents of the cell adhesion apparatus. β-catenin, a member of intracellular attachment proteins acts as a transcriptional regulator of specific genes through the Tcf-4 pathway. Recent evidence suggests that the MMP-7 (matrilysin) gene is also a target of the β-catenin/TCF (Crawford HC, 1999). Integrins can also affect the transcription of MMP genes (Jones JL, 1997). The possibility of blocking matrix degradation as a therapeutic strategy is currently being explored. The MMP inhibitor Batimastat, has been shown to inhibit human tumor growth and spread in nude mice (Wang X, 1994) and its orally administered derivative, Marimastat, is undergoing clinical evaluation.

MMP-9 was selected for the present study since it can degrade the main components of the extracellular matrix, type IV and V collagen and gelatin and thus its activities are closely related to the ability of the invasiveness and metastasis of tumor cells. By forming a 1:1 complex with MMP-9 and inhibiting its enzymatic activity, TIMP-1 plays a negative role in the invasion and metastasis of tumor cells. Therefore, attentions have been paid to the role of MMP-9 and TIMP-1 in the progression of tumor and it has been reported that the expression of MMP-9 and TIMP-1 was correlated to prognosis in many studies. Hence we decided to analyse the expression status of both MMP-9 and TIMP-1 and to observe the relationship of their expression with nodal and distant metastasis and the survival of breast cancer patients. Since imbalance in the expression of MMP-9 and TIMP-1 is a prominent factor in their role in invasion and metastasis, that was also looked into.

3.2.3.3.6. **Urokinase plasminogen activator (uPA)**

Apart from matrix metalloproteinases, another serine protease that plays a central role in tumor invasion and metastasis is urokinase plasminogen activator (uPA). The action of uPA is conversion of plasminogen to plasmin, which can then activate type IV procollagenase that then degrades collagen and proteins of the basement membrane. The proteolysis may ultimately lead to tumor cell invasion and metastasis.
Plasminogen activators (PAs) are members of the serine proteinase family and two PAs exist: urokinase type (uPA) and tissue type (tPA). PAs cleave plasminogen to the active proteinase plamin, which can catalyse the degradation of a variety of proteins including fibrin and laminin (Rickli E E, 1975), as well as activating other proteinases by cleaving the pro-form. The uPA/uPAR system has been shown to play a key role in many physiological processes including embryogenesis, angiogenesis and wound healing.

uPA is a serine proteinase with a molecular weight of approximately 50,000. The active form consists of two polypeptide chains while the zymogen form pro-uPA consists of only one chain. The conversion of pro-uPA to uPA is catalysed by plasmin. The catalytic activity of this plasminogen-plasmin system is modulated by PAs and by inhibitors of both plasmin and PAs. Plasmin inhibitors include α- 2 antiplasmin and α -2 macroglobulin and PA inhibitors include the type 1 and 2 plasminogen activator inhibitors (PAIs).

The effects of uPA on cell migration may be caused by a proteolytic as well as a non-proteolytic mechanism. A proteolytic mechanism of uPA enhancement of cell migration would imply plasmin generation at focal adhesion sites, catalyzed by uPAR bound uPA, which would lead to ECM degradation and thus facilitate detachment of the trailing edge. Binding of uPA to the uPA receptor activates the protease and catalyses the conversion of plasminogen to plasmin which subsequently activates type IV collagenase or directly degrades ECM proteins such as fibrin, laminins and proteoglycans. In the non-proteolytic mechanism, uPA would stimulate cell migration by enhancing adhesion at the leading edge, through stimulation of binding of uPAR to vitronectin, modulation of uPAR/integrin interactions and/or by initiation of signal transduction cascades. Both mechanisms of action could be operating simultaneously in individual migrating cells.

In tumors, non-malignant cells also may be migratory and invasive during processes of cancer cell-directed tissue remodeling. Components of the uPA system may be engaged in such processes and may thus contribute to tumor progression and metastasis, even if they do not participate directly in cancer cell migration and invasion. Ossowski et al (1991) reported that
antibodies against human uPA prevented local invasion of cancer cells while lung metastasis was not inhibited.

Early studies established that the level of uPA in malignant tumors is significantly higher than in the corresponding normal tissue or in benign tumors of the same tissue (Duffy, 1993, 1996). Immunohistochemical studies have revealed components of the uPA system to be located at the invasive edge of cancers and in the associated stromal cells (Pyke C, 1991). The levels of uPA, uPAR, PAI-1 and PAI-2 in malignant tumors have been found to vary considerably and to be related to patient prognosis. uPA was the first proteinase shown to be a prognostic marker in human malignancy.

Duffy et al (1988) showed that patients with breast tumors containing high levels of uPA had a significantly shorter disease free interval than patients with tumors with low levels of uPA. In a rat breast cancer model, overexpression of uPAR increased the invitro invasiveness of such cells and also led to the growth of larger tumors and the enhanced occurrence of metastasis (Xing RH, 1996). uPA antigen levels appear to be among the strongest prognostic factors so far described for breast cancer. Using univariate and multivariate analyses, uPA is at least as strong a marker as nodal status and stronger than the accepted prognostic indices such as tumor size, estrogen receptor status and cathepsin D levels (Duffy, 1990; Janicke, 1990; Janicke, 1993).

During the transition from insitu to invasive cancer, tumor cells penetrate the epithelial basement membrane and enter the underlying interstitial stroma. After the tumor cells enter the stroma, they gain access to lymphatics and blood vessels for further dissemination. During intravasation, extravasation and initiation of metastatic colonies, tumor cells must penetrate the basement membrane and interstitial stroma. Therefore, tumor-cell interaction with the extracellular matrix occurs at multiple stages in the metastatic cascade (Liotta LA, 1986).

Hence in the present study, which dissects the proteins of the metastatic cascade, uPA was included to get a clear picture of its association with lymphatic and distant metastasis. Similarly, there are conflicting reports relating to the prognostic role of uPA. That’s why the
importance of uPA in predicting the survival of breast cancer patients was also included in the current study.

3.2.3.4. **Angiogenic factors**

The formation of new blood vessels or angiogenesis permits the expression of a tumor mass in three dimensions. Angiogenesis, like tumor invasion is a cascade of processes. The angiogenic process can be divided into four distinct steps: degradation of the extracellular matrix, endothelial cell migration and proliferation and structural reorganization (Folkman J, 1992; Auerbach W, 1994; Cockerill GW, 1995). The major angiogenic factors include basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), platelet derived endothelial cell growth factor (PD-ECGF), hepatocyte growth factor (HGF) and platelet derived growth factor (PDGF). It has been suggested that local production of VEGF, which has a half-life of three minutes, is sufficient to maintain angiogenesis in the primary tumor but angiostatin, which has a half-life of 2.5 days, suppresses angiogenesis in metastatic tumors (O'Reilly MS, 1994).

3.2.3.4.a. **Vascular endothelial growth factor (VEGF)**

VEGF produces a number of biologic effects, including endothelial cell mitogenesis and migration, induction of proteinases leading to remodeling of the extracellular matrix, increased vascular permeability and vasodilation, immune modulation via inhibition of antigen presenting dendritic cells and inhibition of endothelial cell apoptosis.

As a therapeutic target, tumor angiogenesis has several attractions. Since only proliferating endothelium is targeted, toxicity would be low. Additionally the problem of drug resistance is overcome because endothelial cells lack the inherent genetic instability that facilitates the emergence of drug resistant clones in population of transformed cells. Preliminary results suggest that targeting of the tumor associated angiogenic response may provide a means of regulating cancer growth and metastatic development.

The VEGF family, currently including VEGF-A to VEGF-D and placental growth factor, play a central part in many human tumor types (Nicosia RF, 1998). They bind variably
to three high affinity endothelial cell tyrosine kinase receptors flt-1 (VEGFR1), KDR (VEGFR2) and flt-4 (VEGFR3).

There are reports in breast cancer (Toi et al., 1994) and in colon cancer (Kang et al., 1997) that the expression of VEGF is a significant independent prognostic factor. In gastric cancer a worse prognosis was found for patients expressing VEGF than those without VEGF expression (Maeda K et al., 1997). A study conducted in 362 node positive patients showed that the tumoral VEGF content was not only of prognostic value, but also it predicted the anatomical site of first recurrence (Linderholm B, 2000).

In the present study, we proposed to look at the expression of VEGF in breast cancer by immunohistochemistry and study the association of VEGF expression with invasion, nodal metastasis and distant metastasis. The prognostic value of VEGF in predicting the overall and recurrence free survival of breast cancer patients was another objective of the study.

3.2.3.5. **Endothelial adhesion molecules**

A number of cell adhesion molecules have been shown to contain one or more immunoglobulin like domains and thus are classified as members of the immunoglobulin superfamily. Recently, a new class of proteins called junctional adhesion molecules (JAMs), belonging to the immunoglobulin superfamily, have been implicated as additional mediators of leukocyte transmigration. These molecules are involved specifically in the process of leukocyte interaction with and extravasation across the endothelial wall. During inflammation, cytokines including TNF-α and IL-1 induce the expression of such immunoglobulin family members as ICAM-1, ICAM-2, VCAM and PECAM on the endothelial wall. These interact with a number of integrins expressed on the surface of leucocytes causing them to arrest prior to extravasation. Very little is known about the role of the major endothelial adhesion molecules-VCAM-1, ICAM-1 and E-selectin in the metastatic progression of breast cancer. These molecules are activated by cytokines via a NF-κB dependent pathway. They are also implicated in lymphocyte trafficking for the host immune anti-infection response.
3.2.3.5.a. **Vascular adhesion molecule-1 (VCAM-1)**

Though the major physiological role of VCAM-1 appears to be in the adhesion of leukocytes to endothelium during acute inflammation states, a role in the adhesion of malignant cells during the process of metastasis has been documented (Zetter BR, 1993). VCAM-1 is a glycoprotein with a molecular mass of approximately 110 KDa expressed on the surface of activated endothelial cells. VCAM-1 is transiently expressed on endothelial cells in response to stimulation by various cytokines including VEGF (Kim II, 2000), IL-3, IL-4 (Fukushi J, 2000) and TNF-α (Osborn L, 1989). Increased serum concentrations of VCAM-1 have been reported in most of the cancers. Hence in the present study we decided to look at the expression status of VCAM-1 in breast cancer and consequently to analyse its prognostic significance.

3.2.3.5.6. **Intercellular adhesion molecule-1 (ICAM-1)**

Intercellular adhesion molecule-1 (ICAM-1 CD54) is a single chain, 94 KDa inducible cell-surface glycoprotein and a member of the immunoglobulin supergene family which mediates many adhesion dependent cell-cell interactions. It is constitutively expressed on haemopoietic cells such as tissue macrophages, monocytes, B-cells, activated T-cells and germinal centre dendritic cells. ICAM-1 expression can also be induced by inflammatory cytokines such as IFN-α, IL1-α and TNF-α in non-haemopoietic cells including vascular endothelium, thymic epithelium and dermal fibroblasts (Springer, 1987; Springer, 1990).

Expression of ICAM-1 has previously been examined by immunohistochemistry in a variety of human neoplasms including breast cancer, lung cancer, squamous cell carcinoma, gastric cancer etc. (Vogetseder, 1989; Koyama S, 1992; Ogawa, 1998). Some studies have found a positive correlation between ICAM-1 expression and metastatic disease (Johnson, 1989; Koyama S, 1992). This is due to the fact that ICAM-1 expression on tumor cells may mediate their attachment to activated leukocytes, promote the migration of individual cells away from the primary tumor and enhance endothelial binding, extravasation and invasion into surrounding tissues. Given the role of ICAM-1 in host anti-tumor immune response and in cell-
cell interactions, we decided to look at the role of ICAM-1 in nodal and distant metastasis in breast cancer.

3.2.3.5. c. Selectins

The selectins are a family of divalent cation dependent glycoproteins. They are carbohydrate-binding proteins, binding fucosylated carbohydrates, especially, sialylated Lewis\(^x\), and mucins. (Brown JR, 2003). The three family members include: Endothelial (E)-selectin, leukocyte (L)-selectin, and platelet (P)-selectin. E- and P-selectins are endothelial adhesion molecules and their ligands are expressed on the surface of leukocytes and tumor cells. E-selectin is an endothelial membrane protein also known as endothelial leukocyte adhesion molecule-1 (ELAM-1) with a molecular weight of 97-115 kDa. Soluble E-selectin has been reported to be associated with human malignancies (Banks et al, 1993). From the study by Wenze K (1994), it is suggested that the E-selectin ligand interaction may be important in facilitating head and neck squamous cell carcinoma cells to adhere during metastasis. In the current study, the expression status of E-selectin was analysed by immunohistochemistry and its relationship to metastasis and prognosis was studied.

3.2.3.6. Alterations in Cadherin promoter methylation and beta-catenin exon 3 mutation

DNA methylation is essential for the development of mammals (Li E, 1992). DNA methylation, catalysed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide and results in the generation of methylcytosine. Methylation of cytosine to methylcytosine in DNA is a heritable genetic alteration, which occurs during cell replication in the absence of any change in the genetic sequence. CpG rich regions (CpG islands) are often found within the promoter of genes. CpG islands of various genes have been shown to be aberrantly methylated in various types of cancers. Moreover, hypermethylation of gene promoters has been shown to result in repression of gene transcription and gene silencing, thus serving as an alternative mechanism of gene inactivation. Cellular DNA methylation involves the interplay of at least three
independent DNA methyltransferases: DNMT1, DNMT3A and DNMT3B (Robertson KD, 2000).

There are two main mechanisms by which nucleotide methylation is proposed to lead to transcriptional silencing of genes. Firstly, methylation of CpGs within transcription factor binding sites in promoters may block their binding and inhibit gene expression. The second repression mode uses the CpG methyl groups as tags that via methyl binding domain (MBD) proteins such as MeCP2, recruit complexed histone deacetylases (HDACs). The targeted nucleosomal histone deacetylation mediated by HDACs leads to a condensed chromosomal architecture that is not conducive to the formation of transcription activating complexes at promoters. This suggests that methylation changes are potentially causative rather than merely a secondary consequence of tumorigenesis. At late stages of disease progression, methylation of DNA is important in a subset of tumors that have epigenetic instability caused by the simultaneous silencing of many genes- this characteristic is called the CpG island methylator phenotype (CIMP) (Issa JP, 2000).

The transcriptional silencing of E-cadherin in human breast cancer has been associated with aberrant promoter region CpG island hypermethylation. Treatment of human breast cancer cell lines lacking E-cadherin with DNA methyltransferase inhibitor elicits CpG island demethylation and re-expression of E-cadherin protein, thereby indicating that aberrant methylation of these CpG islands plays a substantial role in suppressing transcription of the gene in breast cancer cells (Graff JR, 1995).

There are several ways to study hypermethylation of promoter CpG islands including Southern blotting and hybridization after methylation-sensitive restriction enzyme digestion, bisulphite genomic sequencing and methylation specific polymerase chain reaction (MS-PCR). In MS-PCR, primers are designed to anneal to the different DNA sequences of methylated and unmethylated DNA after bisulphite conversion. Each DNA sample is amplified by methylated and unmethylated MS-PCR. The presence of methylation can be demonstrated by successful amplification using methylated MS-PCR. MS-PCR is rapid, simple and sensitive. It requires little DNA and is applicable to DNA extracted from paraffin sections. Hence, we decided to analyse the methylation pattern of E-cadherin promoter in breast cancer samples using MS-PCR.
Aberrant activation of the Wnt signaling pathway, through the stabilizing β-catenin mutations in exon 3 or inactivating APC mutations, is strongly implicated in the cause of many cancers. Accumulation of β-catenin through stabilizing mutations or other mechanisms may result in deregulated transcription of Wnt signaling target genes and promotion of oncogenic signals that lead to tumor formation. In breast cancers, investigators have reported beta catenin exon 3 mutations and there are contradicting reports regarding their significance in metastasis. Hence we decided to look into the mutation status of the exon 3 of beta catenin in breast cancers registering at RCC.

3.2.4. Models of the metastatic process in breast cancer

Given the complexities of the metastatic process, understanding the process is very significant and is difficult. Hence researchers have been concentrating on developing newer and newer simple models of metastatic process. Various models have been proposed which highlight the alterations at the genetic level or protein level that lead to the evolution of metastasis in breast cancer. Some of the existing accepted models of metastasis supported by experimental evidence are given in Fig. 4.

a) The traditional model of metastasis suggests that only subpopulations of tumor cells acquire metastatic capacity late in tumorigenesis.
b) But spontaneous metastasis assays indicate that all tumor cells have the capability to develop metastasis and that occurs spontaneously.
c) The dynamic heterogeneity model proposes that the primary tumor is heterogeneous and each tumor cell has a different potential to metastasise. The frequency with which metastatic variants arise within the primary tumor determines its metastatic potential.
d) The clonal dominance theory proposes that metastatic subclones develop within a primary tumor which subsequently can overgrow and dominate the tumor mass itself.
e) The genometastasis hypothesis proposes that metastasis occurs through transfection of susceptible cells in distant organs with circulating oncogenes.
Fig. 4. Models of metastasis in breast cancer
Recent models of breast cancer metastasis

Other than the metastasis models that are depicted in Fig.4, recently a few models have been proposed to explain the incessant enigma of breast cancer metastasis. The recent models are picturised in Fig.5.

Fig.5. Recently developed models of breast cancer metastasis
(a) Gene expression profiling of human primary breast tumors can predict metastasis risk ('poor prognosis' versus 'good prognosis' signature), which indicates that the capacity to metastasise might be acquired early during tumorigenesis. b) Primary tumors with metastasising capacity display the poor prognosis signature and an additional tissue specific expression profile predicting the site of metastasis (the different tissue sites given in blue, green). c) The parallel evolution model proposes that the dissemination of metastatic cancer cells occurs early in oncogenesis and independently from tumor cells at the primary site. d) According to this model, only breast cancer stem cells and not the non-tumorigenic bulk of the tumor have the ability to metastasise and form new tumors.

Theories of metastatic efficiency

What then actually modulates metastatic inefficiency? To date, metastasis research as well as cancer research, has tended to focus on single genes, or at most, small collections of genes in an experimental model. This is often driven both by traditional scientific reductionist training as well as practical limitations. For example, research using genetically engineered mice usually involves the overexpression or mutation of a single gene. Although vast amounts of information have been gleaned from these studies and increasingly sophisticated models are available, they rarely accurately represent the complex milieu of genetic interactions that occur in naturally occurring cancer. Just as there is a growing understanding of the important role of the interactions of normal surrounding stroma and infiltrating tissues such as vascular epithelia in tumor formation, complex interaction between the genes identified by these techniques and other genomic elements are likely to be important in metastatic potential. Among the theories proposed to explain metastatic efficiency are the transient compartment model and the conventional progression model. Both these theories are based on the supposition that it is a series of random events within the tumor or tumor cell that are the primary determinants of the low efficiency of secondary tumor formation. Evidence exists for both models; however none completely explain all the observations.
Fig. 6. Comparison of the progression and the transient compartment models of metastasis efficiency.

The progression and transient models of metastasis efficiency are shown in Fig. 6. In the progression model a series of heritable random mutational events occur within a tumor resulting in a small subpopulation that acquire all the necessary alterations for metastatic competency. In the transient model, all the tumor cells have the basic ability, but due to reversible epigenetic events and position within the tumor, not all cells maintain the ability at all times.
The allelic composition of the host genome is a major determinant of metastatic efficiency and predictive gene expression patterns. Low metastatic genotypes do not express the predictive pattern and are poorly metastatic. High metastatic backgrounds produce the predictive gene expression patterns in the bulk tumor tissue. Subsequently progressive events occur on this background producing metastatic capable subpopulations. The influence of the genetic background on metastatic efficiency of the tumor is depicted in Fig.7.

**Fig.7. Influence of genetic background on metastatic efficiency.**

### 3.3. Need for better prognostic markers in breast cancer

There is a growing need for additional reliable molecular markers, since the perfect marker for breast cancer may not even exist. An ideal marker should be produced solely by cancer cells or in their immediate vicinity; it should be specific and sensitive and easily measurable in a reproducible way through simple, fast and inexpensive techniques; it should allow estimation of the tumor volume and assessment of the efficacy of therapy and might itself constitute a highly tumor specific therapeutic target. The highly heterogenous nature of breast
tumors and the diversity of processes (proliferation, adhesion, proteolysis, chemoresistance, hormone sensitivity) that characterize tumor behavior makes their exhaustive description based on the expression levels of only a few genes impossible.

For a given molecular marker to earn the label of reliable clinical marker it must undergo extensive, strictly controlled and reproducible expression studies, often covering years. To date, this process has been completed for a few candidates only. For instance, the longest established breast cancer molecular indicator, estrogen receptor alpha (ER-alpha) has been accepted both as a prognostic indicator and a predictor of patient responsiveness to antiestrogen therapy (Valavaara R, 1997). c-erbB-2 is another marker whose prognostic relevance has been demonstrated by numerous studies (Menard S, 2000). Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitors-1 and 2 (SERPINE 1 and SERPINE 2) are three additional markers that have recently been introduced into the clinical routine after extensive investigation of their expression levels in breast tumors. The clinical importance of several other markers such as bcl-2, Cathepsin D, cyclin D1, epidermal growth factor receptor, p53, progesterone receptor, pS2 and urokinase receptor (Look MP, 1999), Ki-67 (Jones RL, 2009) have been established.

Since the current staging systems for breast cancer do not accurately identify those patients curable by regional treatment alone, the evaluation of additional parameters associated with the metastatic phenotype will be very important for the differentiation of patients curable by surgery alone from those requiring systemic therapy. For instance, breast cancer patients with poor prognoses can be identified by the detection of high microvessel counts concurrent with low expression of Nm23 and/or E-cadherin in the primary tumor (Heimann R, 1998). There is a critical need for markers that will distinguish accurately those histologic lesions and disseminated cells that have a high probability of causing clinically important metastatic disease from those that will remain indolent. This justifies one of the primary objectives of the current study to identify more efficient prognostic markers with the ability to identify particular categories of breast cancer patients at risk for developing nodal and distant metastasis.