Evaluation of the effect of Trastuzumab (Herceptin) on the development and progression of breast cancer associated skeletal metastasis

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Abstract

Breast cancer is the most commonly diagnosed cancer in women. Despite recent advances in screening and early detection, breast cancer continues to result in a high incidence of morbidity and mortality. In its late stage the majority of patients exhibit signs of destructive skeletal metastasis. This complication is promoted by the production of growth factors by tumor cells which can induce tumor cell proliferation via their interaction with their respective receptors to initiate the vicious cycle of bone resorption. Inhibition of growth factors signaling through their receptors can therefore serve as a useful therapeutic approach to block bone metastasis.

The biological characteristics of cancer cells along with the targeting properties of immune system offer a novel approach in the treatment of breast cancer. Directed against HER-2/nue oncogene, the recombinant humanized monoclonal antibody, Trastuzumab (Herceptin), has shown significant clinical benefits for the treatment of HER-2 positive metastatic breast cancer.

In the present study, the effects of Herceptin and its molecular mechanism of action in abrogating the development and progression of osteolytic bone metastasis is investigated in an experimental mouse model of skeletal metastasis using human breast cancer cells BT-474 which are known to express high levels of HER-2. Treatment of BT-474 cells with Herceptin caused a dose dependent decrease in cell proliferation. In *in vivo* studies BT-474 cells were injected by into the left ventricle of female BALB/c nu/nu mice. Intraperitoneal infusion of Herceptin from the day of tumor cell inoculation or at the time of radiologically detectable skeletal metastasis either slowed the development or prevented the progression of skeletal metastasis as compared to control groups of animals

receiving non-specific IgG. Bone histological analysis of long bones showed the ability of Herceptin to reduce the ratio of tumor volume to bone volume as well as mitotic index when Herceptin treatment was initiated from the day of tumor cell inoculation. Immunohistochemical analysis of long bones showed a significantly lower level of activated (phosphorylated) MAPK in bones of Herceptin treated animals. These studies demonstrate the ability of Herceptin to inhibit the development and abrogate the progression of skeletal metastasis associated with breast cancer by blocking the HER-2 mediated signaling pathways.

Résumé

Le cancer du sein est le cancer le plus généralement diagnostiqué chez les femmes. En dépit des avances récentes dans le criblage et le dépistage précoce, le cancer du sein continue à avoir comme conséquence une incidence élevée de la morbidité et de la mortalité. À son étape en retard la majorité de patients exhibent des signes des métastases squelettiques destructives. Cette complication est favorisée par la production des facteurs de croissance par les cellules de tumeur qui peuvent induire la prolifération de cellules de tumeur par l'intermédiaire de leur interaction avec leurs récepteurs respectifs pour lancer le cycle méchant de la résorption d'os. L'inhibition des facteurs de croissance signalant par leurs récepteurs peut donc servir une approche thérapeutique utile pour bloquer des métastases' os.

Les caractéristiques biologiques des cellules de cancer avec les propriétés d'optimisation du système immunitaire offrent une approche de roman dans le traitement du cancer du sein. Dirigé contre l'oncogène de HER-2/nue, l'anticorps monoclonal humanisé de recombinaison, Trastuzumab (Herceptin), a montré les avantages cliniques significatifs pour le traitement du cancer du sein HER-2 metastatic positif.

Dans la présente étude, les effets de Herceptin et son mécanisme moléculaire d'action en abrogeant le développement et la progression des métastases ostéolytique d'os est étudiés dans un modèle expérimental de souris des métastases squelettiques en utilisant les cellules humaines BT-474 de cancer du sein qui sont connues pour exprimer les niveaux élevés de HER-2. Le traitement des cellules BT-474 avec Herceptin a causé une diminution dépendante de la dose de la prolifération de cellules. Dans les études in vivo BT-474 des cellules ont été injectées près dans le ventricule gauche des souris de la

femelle BALB/c nu/nu. L'infusion intra péritonéale de Herceptin du jour de l'inoculation de cellules de tumeur ou à l'heure de la métastase squelettique radiologiquement discernable a ralenti le développement ou a empêché la progression de la métastase squelettique par rapport aux groupes de commande d'animaux recevant IgG non spécifique. L'analyse histologique d'os de longs os a montré la capacité de Herceptin de ramener le rapport du volume de tumeur au volume d'os aussi bien que l'index mitotique quand le traitement de Herceptin a été lancé du jour de l'inoculation de cellules de tumeur. L'analyse d'Immunohistochemical de longs os a montré de manière significative un niveau plus bas de MAPK (phosphorylé) activé dans des os des animaux traités par Herceptin. Ces études démontrent la capacité de Herceptin d'empêcher le développement et abrogent la progression des métastases squelettiques liées au cancer du sein en bloquant le HER-2 négocié signalant des voies.

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Abbreviations

ACS: American Cancer Society

bFGF: basic Fibroblast Growth Factor

BMPs: Bone Morphogenetic Proteins

BSE: Breast Self-Examination

CAMs: Cell Adhesion Molecules

CBE: Clinical Breast Examination

CT: Computed Tomography

ECM: Extra-cellular Matrix

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ER: Estrogen Receptor

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factor

HRT: Hormone Replacement Therapy

IGF: Insulin-like Growth Factor

IL: Interleukin

MAP: Mitogen Activated Protein

MMP: Matrix Metalloproteinase

MRI: Magnetic Resonance Imaging

MTX: Methotrexate

OCP: Oral Contraceptive Pill

PDGF: Platelet Derived Growth Factor

PET: Positron Emission Tomography

PLGF: Placenta-like Growth Factor

PR: Progesterone Receptor

PTHrP: Parathyroid hormone related peptide

SERM: Selective Estrogen Receptor Modulator

SPECT: Single Photon Emission Computed Tomography

TGF: Transforming Growth Factor

TNF: Tumor Necrosis factor

uPA: urokinase Plasminogen Activator

VEGF: Vascular Endothelial Growth Factor

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Chapter 1: Review of Literature

Breast Cancer: Clinical background

Female breast cancer is a major medical problem with significant health and social consequences. It is the most common form of malignant neoplasms among women and continues to be a major cause of cancer deaths (Figure 1) (1, 3, 8).

Breast cancer incident rates vary considerably worldwide, with women in North America and northern Europe at highest risk (2). It has been estimated that between 1 in 7 and 1 in 8 American women will develop breast cancer in her lifetime (1, 4).

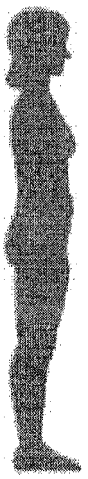
Risk Factors

The incidence of female breast cancer also varies according to the presence or absence of certain risk factors (Table 1) (1, 12):

- Age is an important predictor of breast cancer risk, so that incidence rates increase with age. However, the correlation of age and increase in incidence is not linear: the slope of the correlation curve is deepest in younger women; it flattens out and even declines during and shortly after menopause, and resumes a steeper correlation several years after menopause (1, 5).

- A positive family history is one of the strongest risk factors for the disease (9, 14). Individuals with a first-degree relative with a history of breast or ovarian cancer have a substantially increased risk of developing breast cancer compared to women without such a family history (1). A number of breast cancer susceptibility genes have been identified to date (10, 11, 13). *BRCA1* and *BRCA2* are important breast cancer susceptibility genes and mutations in these two genes leads to an increase in lifetime risk of breast cancer to up to 80% (1, 15).

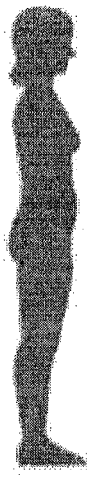
A.



Females

Breast	212,920	31%
Lung and Bronchus	81,770	12%
Colon and Rectum	75,810	11%
Uterine Corpus	41,200	6%
Non-Hodgkin Lymphoma	28,190	4%
Melanoma of the Skin	27,930	4%
Thyroid	22,590	3%
Ovary	20,180	3%
Urinary Bladder	16,730	2%
Pancreas	16,580	2%
All Sites	**679,510**	**100%**

B.



Females

Lung and Bronchus	72,130	26%
Breast	40,970	15%
Colon and Rectum	27,300	10%
Pancreas	16,210	6%
Ovary	15,310	6%
Leukemia	9,810	4%
Non-Hodgkin Lymphoma	8,840	3%
Uterine Corpus	7,350	3%
Multiple Myeloma	5,630	2%
Brain and Other Nervous System	5,560	2%
All Sites	**273,560**	**100%**

Figure 1. Ten leading cancer types for the estimated new cancer cases (A) and deaths (B). (Adapted from: CA Cancer J Clin. 2006; 56:106-130)

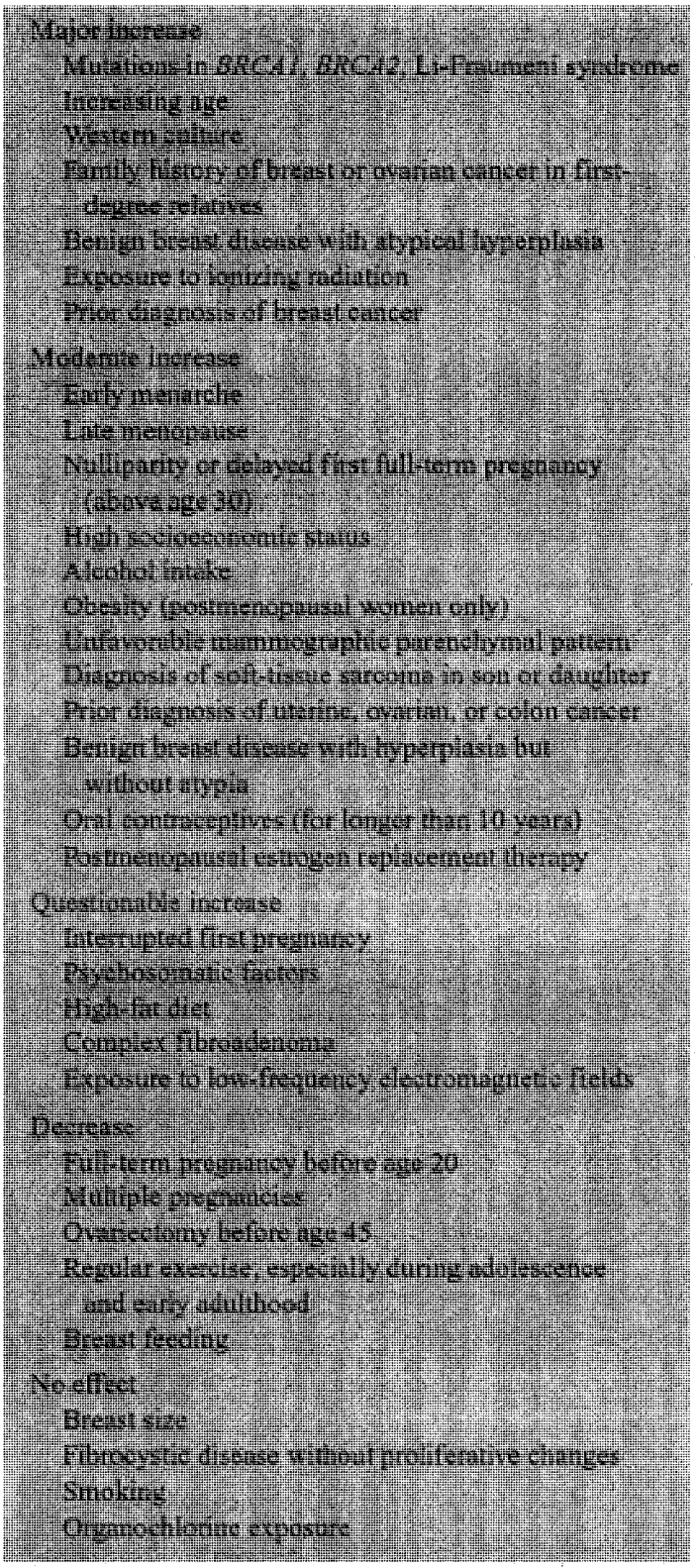


Table 1. Risk factors associated with the development of breast cancer (1).

- Breast cancer is a hormone-related cancer and exposure to women's reproductive hormones (Estrogen and Progesterone) is strongly implicated in developing the disease (5). These hormones influence normal breast cell growth and development. Endogenous as well as exogenous formulations of these hormones drive cellular proliferation in the breast, which can lead to breast tumorigenesis (1, 5). A majority of risk factors for breast cancer can be understood in light of their effects on a woman's lifetime exposure to these ovarian hormones (5): according to many studies ovarian ablation has a protective effect for mammary carcinomas, while grater number of years with ovulatory cycles (early onset of menarche, late onset of menopause, nulliparity) associates with increased risk of developing breast cancer (1).

- Environmental factors might have a major influence on the risk of developing breast cancer (1, 16). Epidemiologic studies have indicated that immigrant groups from low- to high-risk regions of the world, show increasing incidence rates that approach those of the higher risk host region (1). This finding is probably related to lifestyle factors and behavioral modification associated to changing role of women in western societies such as use of OCP (Oral Contraceptive Pill) and HRT (Hormone Replacement Therapy), age at first child-birth, and alcohol consumption (6, 7).

Screening

Historically, the primary presenting symptom of breast cancer was a palpable mass, often detected by the patient. Today, the increasing use of mammography, especially in screening programs, results in many cancers being found at a preclinical stage (1). Breast cancer screening, especially with mammography has been recommended for many decades, and has gained wide acceptance by women and physicians (17, 20). However,

according to the ACS (American Cancer Society guidelines) for early breast cancer detection, women should be informed both about the benefits and limitations of screening, and the possibility of harms associated with false-positive findings (1, 17).

Although the efficacy of mammography has been demonstrated in randomized controlled trials and observational studies, it does not achieve perfect sensitivity or specificity (17). Mammography's imperfection is due to both host characteristics (age, risk, breast density, and tumor growth rates) and provider factors (technical limitations and quality assurance failures). The consequences of these limitations include missed cancers as well as anxiety and harms associated with interventions for benign or non-progressive precursor lesions (17). Even good-quality mammography facilities generally interpret 5% to 10% of all screening examinations as abnormal; however about 90% of women with abnormal results do not have breast cancer (20). Therefore an efficient evaluation of women with an abnormal mammography result is crucial (19). The American college of Radiology recommends one of six assessments for interpretation of a mammographic screening examination (19). Abnormal screening mammography assessments are evaluated with diagnostic mammography, ultrasonography, and biopsy (19).

The logic for earlier detection of clinical findings in the breast through CBE (Clinical Breast Examination) and BSE (Breast Self Examination) is the same as the logic for detecting breast cancer before it is palpable: After breast cancers become palpable, the likelihood of regional and distant metastasis increases and long-term survival is poorer with increase in tumor size (17, 18). Although the evidence supporting the value of CBE and BSE as methods of reducing breast cancer mortality is limited, earlier detection of

palpable tumors with CBE or BSE can lead to earlier therapy and a greater possibility for a breast conserving approach (17).

Diagnosis

Palpable breast abnormalities are usually described as lumps or breast thickenings (19). The most valid criteria in deciding whether a reported palpable breast abnormality is significant and needs evaluation is whether the abnormality is "dominant" on clinical breast examination (19). A "dominant" breast abnormality is defined as a change in breast texture that is discrete and distinctly different from the rest of the surrounding breast tissue and the corresponding area in the contralateral breast (19). Diagnostic mammography, ultrasonography, and biopsy are used to evaluate palpable breast abnormalities (Figure 3) (19).

Prognosis

The natural history and prognosis for primary breast cancer vary considerably from patient to patient. Some patients present with very indolent disease and are cured by local therapy, while in other patients the disease takes an aggressive, rapidly progressive course that is refractory to treatment (1). The heterogeneity of the natural history of breast cancer complicates patient management, therefore identification of prognostic factors that would predict patient's outcome can help to select the proper intervention (1). Although the ideal prognostic factor does not yet exist, a number of variables have been identified which attempt to measure and quantify the degree of tumor aggressiveness, metastatic potential, rate of growth, and sensitivity/resistance to planned treatment (Table 4) (1).

The most widely used prognostic factor is the stage grouping used in the TNM staging system, in which information about local extent of disease (T category) is combined with the information about lymph node status (N category) and evidence of metastatic disease (M category) (21). Stage grouping provides a single statement of outcome (a single prognostic massage) since each patient is assigned a stage based on the TNM variables (21).

Several Histological variables have been reported to have prognostic significance. Among the different histological variants of breast cancer Mucinous and Medullary carcinomas have better prognosis compared to infiltrating ductal and lobular carcinomas (1).

Histological grade is another important determinant of prognosis within a given tumor stage (22). In general, tumors expressing features that indicate a high degree of tumor differentiation have the most favorable prognosis (1).

Measurement of the proliferation rates of malignant tissues has strong prognostic value for several types of cancer, including breast cancer (1). Several techniques are used to evaluate the proliferative capacity of the malignant cell, including mitotic indices, thymidine-labeling indices, S-phase fraction, and the measurement of proteins expressed preferentially during active phases of the cell cycle (1). Mitotic index, defined as the number of mitotic figures in a given area of tumor, is an accurate means of estimating tumor cell proliferation and represents an integral part of histological grade (1, 22).

ER (Estrogen Receptor) and PR (Progesterone Receptor) determinations are established procedures in the routine management of patients with breast cancer (22). Although its prognostic value has been clearly shown in extensive studies, receptor data may be of

grater value when combined with other prognostic factors (1, 22). So, the best use of steroid hormone receptors is in the prediction of response to systemic endocrine therapy and therefore the selection of optimal adjuvant systemic treatments (1).

It is well accepted that malignant tumors develop as a consequence of gene abnormalities such as oncogene activation, loss of function of tumor suppressor genes, or alteration of other genes critical for cell control mechanisms (1). Two members of the type I growth factor receptor family genes, the EGFR (HER-1) and HER-2 , are frequently amplified and/or overexpressed in breast cancer (1, 22). Overexpression of proteins encoded by these genes is associated with a more aggressive clinical course, including a higher risk of developing metastasis and more rapid tumor growth and progression (1).

HER-2 overexpressing breast cancer is associated with high histological grade, reduced survival, lower responsiveness to Methotrexate-based treatment regimens and hormone receptor modulators, and higher responsiveness to Doxorubicin-based regimens (22). Thus HER-2 analysis is requested to obtain prognostic (outcome independent of treatment) and predictive (outcome dependent on treatment) data (22). Currently the most important clinical application of HER-2 testing is to identify candidates for HER-2 directed therapy with Trastuzumab (Herceptin) (1). The prognostic value of EGFR (HER-1) overexpression is less well established and there is no FDA (Food and Drug Administration) approved diagnostic test or therapeutic intervention that targets this receptor (1).

P53 gene is frequently mutated in breast cancers (1). Although some correlations have been shown between this mutation and response or resistance to cytotoxic or endocrine

agents, there is no compelling evidence to support the routine testing for *p53* gene mutation or abnormal protein expression as either prognostic or predictive factor (1).

Growth and risk of metastasis for some breast cancers appears to depend on the growth of new blood vessels adjacent to the tumor (angiogenesis) (22). Counting the number of tumor capillaries immunohistochemically after staining for factor VIII has been shown to have prognostic value (1). Tumors with fewer capillaries have a lower metastatic potential and better prognosis (1).

Other factors are not sufficiently studied to demonstrate their prognostic value (22).

Treatment

The expectation about breast cancer treatment is not only survival itself but also improvement of the quality of life of the patient (23).

The stage at which breast cancer is at serves as the primary factor to choose an effective treatment modality. With the introduction of mammography screening, the detection of breast cancer in early stages is increasing (1, 23). Currently, breast-conserving surgery has become the most popular treatment for early breast cancer. Lumpectomy with axillary node dissection followed by radiotherapy is the current standard of care for patients with primary breast cancer. Annual mammography after the surgery and radiotherapy is recommended in order to detect recurrent cancer (23).

Breast cancer is considered a systemic disease. Information from various sources indicates that many patients with breast cancer have disseminated disease by the time a clinical diagnosis is established (1, 23). Currently, the primary aim of oncologic surgery in establishing local control seems to be effected by reducing the tumor burden of the patient to a number of viable cells that are destroyable by host immunologic factors and

systemically administered anti-cancer agents (1). The combination of local treatment and systemic treatment is the key to success in the treatment of breast cancer; therefore postoperative adjuvant treatments are usually offered to patients to attempt eradication of micrometastatic disease with the aim of reducing the risk of relapse and increasing disease-free survival (24).

Selection of adjuvant treatments is based on the distinction between endocrine-responsive and endocrine-unresponsive breast cancer (1, 24). Patients with endocrine-unresponsive breast cancer, characterized by no expression of estrogen and progesterone receptors, are offered chemotherapy. Premenopausal women with endocrine-responsive disease are usually offered tamoxifen with or without suppression of ovarian function. Use of cytotoxic drugs before endocrine therapy is recommended if a high risk of relapse exists (metastatic lymph nodes in the operated axilla or vascular invasion). Postmenopausal women with endocrine-responsive disease are offered endocrine therapy with tamoxifen or aromatase inhibitors preceded by chemotherapy (24).

Patients who remain free of disease after adjuvant therapy compared with those needing chronic care to constantly control disease progression is the main difference between adjuvant and metastatic treatment approaches.

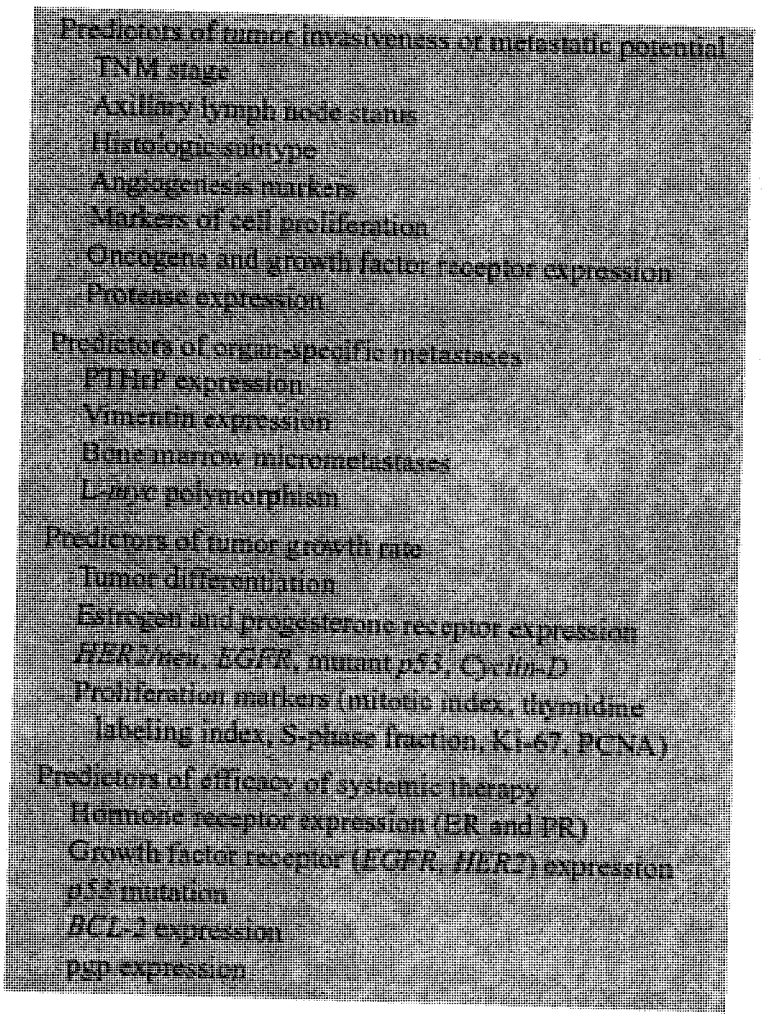


Table 2. Prognostic factors in Breast Cancer (1).

Metastatic Breast Cancer

Metastatic breast cancer is the most advanced stage of breast cancer. Breast cancer has the potential to spread to many organs of the body. Metastasis from breast cancer are usually multiple and frequently involve more than one organ site. The most common organs breast cancer spreads to is bone, followed by lung and liver (1). Current guidelines do not recommend routine use at regular intervals of any instrumental or laboratory test for early detection of metastasis in breast cancer patients; therefore, patients with a previous diagnosis of primary breast cancer who present with findings suspicious for metastatic disease should be strongly considered for diagnostic procedures directed by the organ sites most frequently involved and by patient's sign and symptoms (1, 25).

Overt metastasis usually indicates chronic, incurable disease. Although there is significant variability in the survival of patients with metastatic breast cancer, the median survival is around 2 to 3 years (26). There are many factors that predict response to treatment, time to progression, and overall survival in patients with metastatic breast cancer. Some of the predictive factors of a poor prognosis are: disease free interval of less than 2 years, evidence of bone and liver metastasis, higher number of metastatic sites, and HER-2/*neu* overexpression in the tumor (26).

Current therapies are aimed at improving the quality of life, symptom control, and prolongation of survival in the patients (24, 26). Over the past four decades, systemic therapy with endocrine therapy or cytotoxic chemotherapy became the fundamental treatment approach in the management of metastatic breast cancer (1). Recently however, advances in understanding the molecular pathways that underlie breast cancer growth and progression resulted in defining the tumor-associated growth factor receptor "HER-2" as

a potential target for therapeutic intervention (27). Consequently, the humanized anti-HER-2 mAb (Trastuzumab, Herceptin) was developed and approved by FDA in 1998 for the treatment of metastatic breast cancer (27). Due to improvement in survival, this drug has become a standard of care for women with HER-2 overexpressing tumors. Metastatic breast cancer patients with HER-2 overexpressing tumors are candidates for receiving Herceptin in combination with chemotherapy as a first line treatment or Herceptin alone for certain patients who have received chemotherapy with little success (25).

Bone metastasis is present in 80% of patients with advanced breast cancer and cause significant morbidity. In approximately 20% of these cases, the bone metastasis is isolated. Bone is also the first site of breast cancer metastasis in 26-50% of cases (52). Intractable bone pain is the most common clinical consequence of cancer metastasis to bone and may be the initial presenting symptom. Other clinical consequences of bone metastasis include pathologic fractures (fracture after trivial injury often in load-bearing bones), bone deformity, nerve compression syndromes such as spinal cord compression, bone marrow suppression leading to leukopenia and anemia, and hypercalcemia (50, 51, 52). X-ray is the most common imaging technique used to detect bone metastasis. CT (Computed Tomography), MRI (Magnetic Resonance Imaging), PET (Positron Emission Tomography), and SPECT (Single Photon Emission Computed Tomography) are also used to detect but less frequently due to expense and availability. Serum biochemical markers of bone resorption are often increased in these patients and can be used to monitor response to treatment (59, 60). Bisphosphonates are FDA-approved antiresorptive agents commonly prescribed for breast cancer bone metastasis. Although bisphosphonates are considered an effective supportive therapy to reduce pain in patients

with osteolytic cancer, there is no significant improvement in patients' life expectancy with bisphosphonate treatment according to the American Society of Clinical Oncology guidelines (61). In addition to bisphosphonates, steroidal antiestrogen therapy and adjuvant therapy with aromatase inhibitors show promise in repressing tumor progression to the bone in ER-positive breast cancer patients (61).

Biology of Breast Cancer

The adult female breast is composed of characteristic units, called lobules, embedded in a fat and fibrous tissue framework. Lobules which consist of tubulo-alveolar tissue, connect to each other through terminal interlobular ducts and drain into nipple via excretory lactiferous ducts (28). The lining of the ducts is formed of a simple cuboidal epithelium from which most of the breast carcinomas arise (Figure 2) (28).

Normal breast growth and development are regulated by the complex interaction of many hormones and growth factors in autocrine, paracrine and endocrine fashions. These hormones include estrogens (E), progesterone (Pg), prolactin (P), thyroid hormones ($T_{3,4}$), insulin (I) and insulin-like growth factors (IGF-1 and IGF-2), epidermal growth factor (EGF), and transforming growth factors (TGF-α and TGF-β) (29). The interaction of these growth factors, cytokines and hormones with their specific receptors, located on the cell membrane, interior of the cell, or within the cell nucleus, triggers a cascade of intracellular biochemical signals resulting in activation or repression of various subsets of genes (1). Since normal breast tissue is regulated by these factors and their receptors, it is not surprising that malignant cells, arising from breast tissue, might also express receptors for many of these factors and are controlled by their interaction (Figure 3) (1, 30).

As with other hormone dependent malignancies, the initiation and progression of breast cancer is a multi-step process involving both genetic alterations and changes in tissue microenvironment that ultimately leads to the development of highly advanced, metastatic breast cancer (1) (Figure 4).

Carcinoma of the breast develops when the normal ductal epithelium of the mammary gland transforms into a cancer cell as a result of genetic aberrations leading to disturbance of normal growth control mechanisms. Such cellular transformation occurs through the loss or mutation of tumor suppressor genes, activation of oncogenes, or both (31).

Alterations in two known suppressor genes, the retinoblastoma gene (*RB1*) and the human *p53* gene, have been identified in human breast cancer cells. Mutations in the *p53* gene have been found in families with the Li-Fraumeni syndrome, who have a markedly increased incidence of breast cancer and other neoplasms. Moreover, 50% of sporadic breast cancers have been shown to have mutations in *p53* gene (32). The two mutated genes associated with familial breast cancer, *BRCA1* and *BRCA2*, also function as tumor suppressor genes under normal conditions and mutations in these two genes accounts for 2-4% of all breast cancers worldwide (10,15). Since the normal function of the protein products of these genes is to control cell proliferation (*RB1* and *p53*) or to mediate DNA repair (*p53*, *BRCA1*, *BRCA2*), mutations lead to dysregulated transit of cells through the cell cycle and thus to the development of cancer cells (1).

Breast cancer cells have been shown to express several oncogenes, that is, genes involved in normal regulatory processes, that when overexpressed , can induce or promote the malignant phenotype via activation of multiple intracellular signal transduction pathways leading to unregulated cell growth (1). The products of oncogenes are frequently growth factors or their receptors. Oncogenes often found to be overexpressed in human breast cancer tissue include members of *myc* and *ras* family (c- *myc*, Ha-*ras*-1) and the members of the EGF receptor (EGFR, *erb*B) family, including *erb*B-2 (or HER-2 or *neu*),

HER-3, and HER-4 (30, 33). Overexpression and mutation of growth factor receptors often leads to their constitutive activation and hence constant growth promoting signaling in the absence of their cognate ligands (30).

Cancer is a complex tissue resulting from disrupted normal tissue homeostasis maintained between epithelial cells and their microenvironment (29). During malignant transformation and progression, there are deregulated reciprocal interactions between the neoplastic cells and the adjacent stromal cells (29). Recent studies suggest that breast cancer cells can synthesize and secrete their own growth factors that could autostimulate breast cancer cells or adjacent stromal tissue (34). Stromal tissues also secrete growth factors such as EGF, TGF-α, IGF-1 and IGF-2 that can stimulate breast cancer cells (35-37). Epithelial growth inhibitors, such as TGF-β and mammastatin, are also secreted by human breast cancer cells; therefore the malignant potential of breast cancer depends on the balance between growth stimulators and growth inhibitors produced by the tumor and its surrounding tissue (Figure 3) (38, 39).

In the mammary gland, estrogens regulate the expression of several genes corresponding to peptides and proteins involved in growth and development. Estrogens exert their effects through high-affinity binding to estrogen receptor (ER) in breast cells (1). A substantial body of experimental and clinical evidence has shown that the effects of estrogens on the growth of breast epithelium influence breast cancer risk (42). Breast cancer has two subgroups according to ER status: ER-positive and ER-negative. Each subgroup has different biological and clinical attributes. In ER-positive breast cancer cells, expression and secretion of certain growth factors are stimulated by estrogen and

inhibited by anti-estrogens whereas in ER-negative breast cancer cells secretion of these factors is not estrogen regulated (40).

Although the natural history and prognosis for primary breast cancer vary considerably from patient to patient, in the majority of cases, the disease follows an aggressive, rapidly progressive course. Tumor progression is the phenomenon where by cancer cells become more aggressive with time. The key requirement in the process of tumor progression is the ability of tumor cells to produce certain factors within the tumor cell environment in order to facilitate their growth and spread (43).

Figure 2. Structure of the female breast (28).

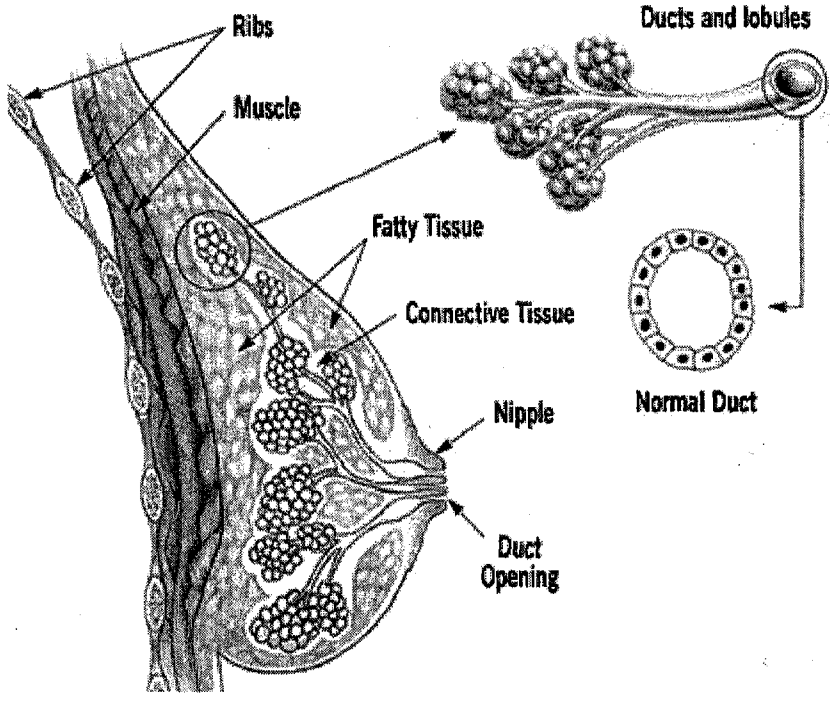


Figure 3. Growth regulation of breast cancer by hormones and growth factors (1).

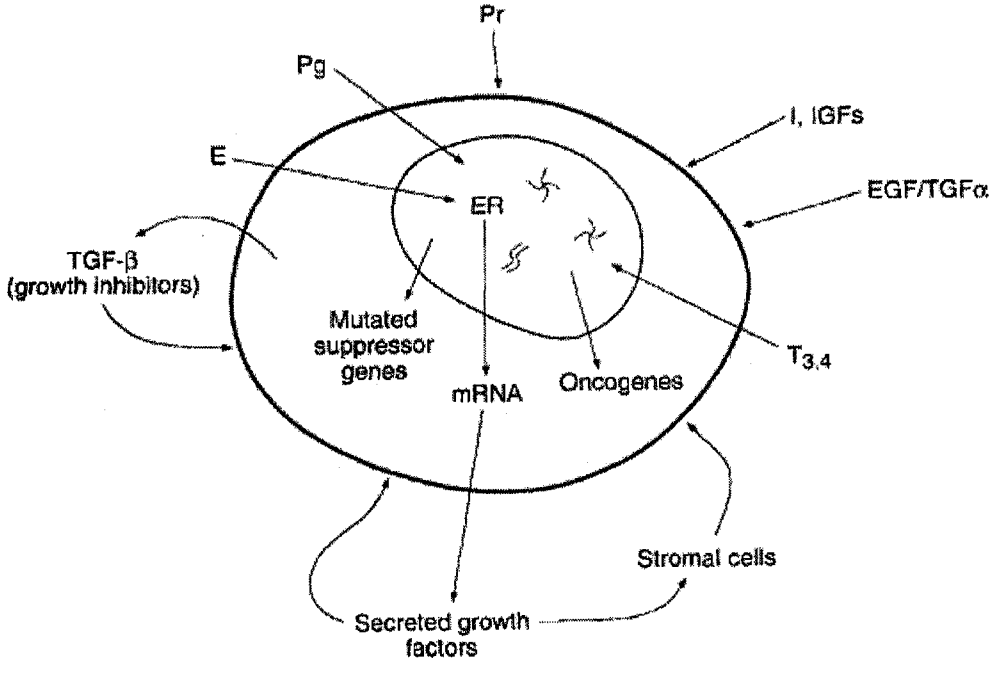
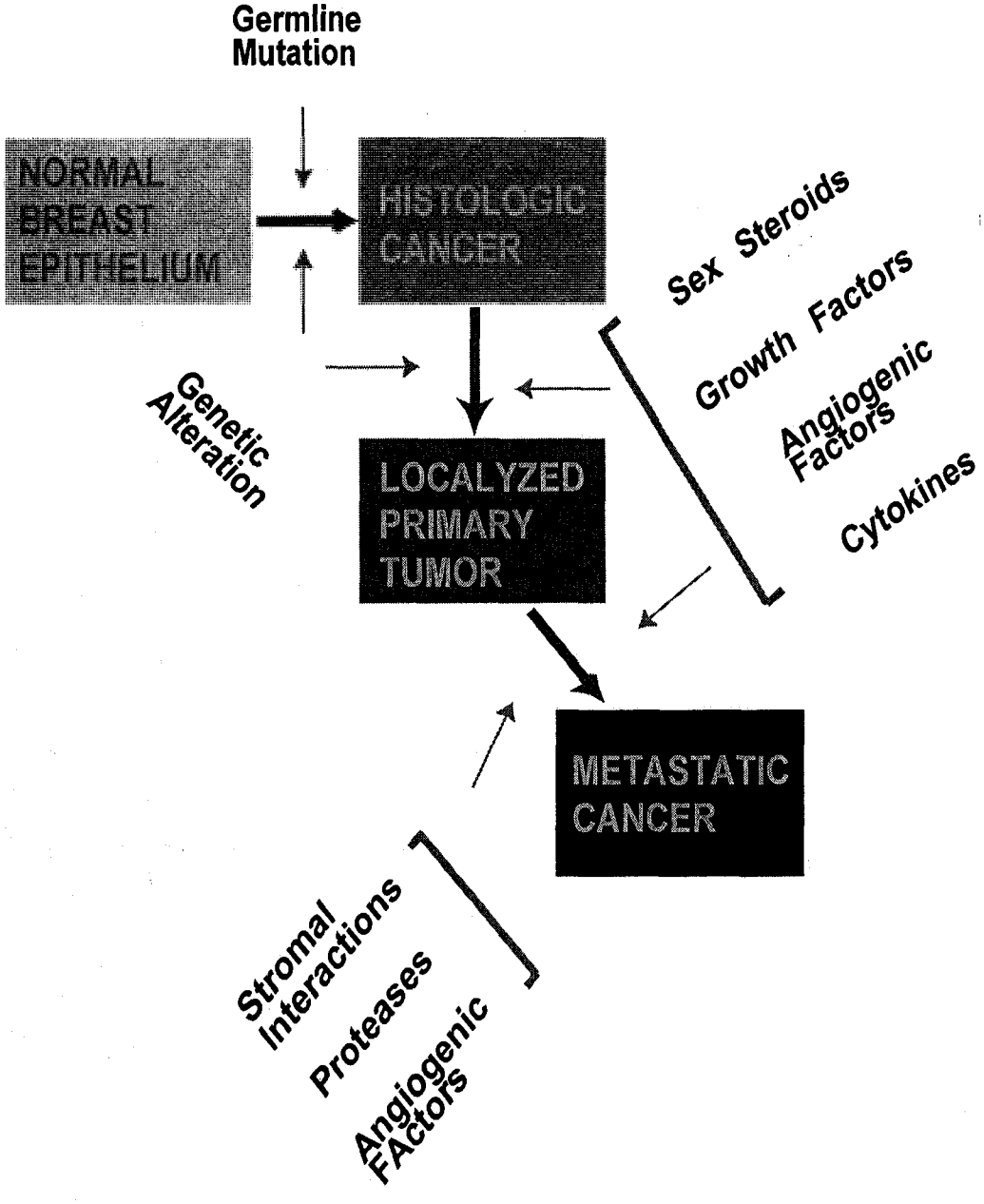


Figure 4. Biological progression of Breast Cancer



The process of metastasis

Metastasis is the single most catastrophic complication of malignancies. Localized primary tumors are rarely associated with mortality. Rather, the high mortality rates associated with cancer are caused by the metastatic spread of the tumor cells from the site of tumor origin (43).

The classical metastatic cascade includes intravasation by tumor cells, their circulation in lymph and blood vascular system, arrest in distant organs, extravasation, and growth in the metastatic foci (44) (Figure 5). This process involves angiogenesis, altered cell adhesion, proteolysis, migration, survival, proliferation and homing in target organs (43).

To begin the process of metastasis, the primary malignant neoplasm promotes new blood vessel formation through the process of tumor-mediated angiogenesis. This "angiogenic switch" is characterized by tumor expression of pro-angiogenic proteins such as VEGF (Vascular Endothelial Growth Factor), bFGF (basic Fibroblast Growth Factor), PDGF (Platelet Derived Growth Factor), IL-8, PLGF (Placenta-Like Growth Factor), and TGF-β (41, 43). It also involves downregulation of angiogenesis suppressor proteins, such as thrombospondin (41).

The change in the adhesive properties of the cancer cells, as well as their reciprocal interactions with the ECM (Extra-cellular Matrix) and neighboring stromal cells lies at the core of the metastatic process (43). Three main groups of cell adhesion molecules, the integrins, the immunoglobulin superfamily, and the cadherins, have all been implicated in metastasis (45, 46). These molecules do not simply act as molecular glue mediating physical interactions. Instead, they direct the interchange of information between the cells and the ECM by transducing signals into the cells (43). At the molecular level the

signaling pathways through these molecules are linked to gene activation, apoptosis inhibition, increased cellular proliferation and migration (43, 46).

Degradation and remodeling of the ECM and the basement membrane by proteolytic enzymes are essential steps in local invasion, intra- and extravasation, and colonization at distant sites. Among the principal classes of enzymes that degrade the ECM are MMPs (Matrix Metalloproteinase), cathepsins, heparanase, and tissue serine proteinases including uPA (urokinase Plasminogen Activator), thrombin, and plasmin (43). Breakdown of the ECM allows tumor cells to traverse the walls of small blood vessels present in the primary organs or the newly recruited ones and enter the circulation, enabling them to reach distant sites. Many circulating tumor cells do not survive the host anti-tumor defense mechanisms such as the innate immunity effectors. The few cells that manage to survive and reach the secondary organ, extravasate from the blood vessels into the stroma of the organ (43).

The locations of distant secondary tumors are non random. The high proportion of certain metastasis in certain cancers, such as bone metastasis in breast carcinomas, is an example of selective homing of cancer cells to a specific organ (43, 44). Three major types of homing mechanisms have been proposed (44). The first is selective growth; that is tumor cells extravasate ubiquitously but grow only in organs that have the appropriate growth factors or ECM microenvironment. The initial delivery and arrest of cancer cells to the metastatic organs seems to be primarily mechanical. Molecular factors present in individual organs then influence whether cancer cells will grow there (47). The second mechanism is selective adhesion of tumor cells to particular molecules on the surface of vascular endothelial cells only at the site of homing organ. The third major mechanism is

selective chemotaxis of circulating tumor cells to the organ producing specific attraction factors or chemokines (48). All of these mechanisms have been found to play a role in experimental metastasis (44).

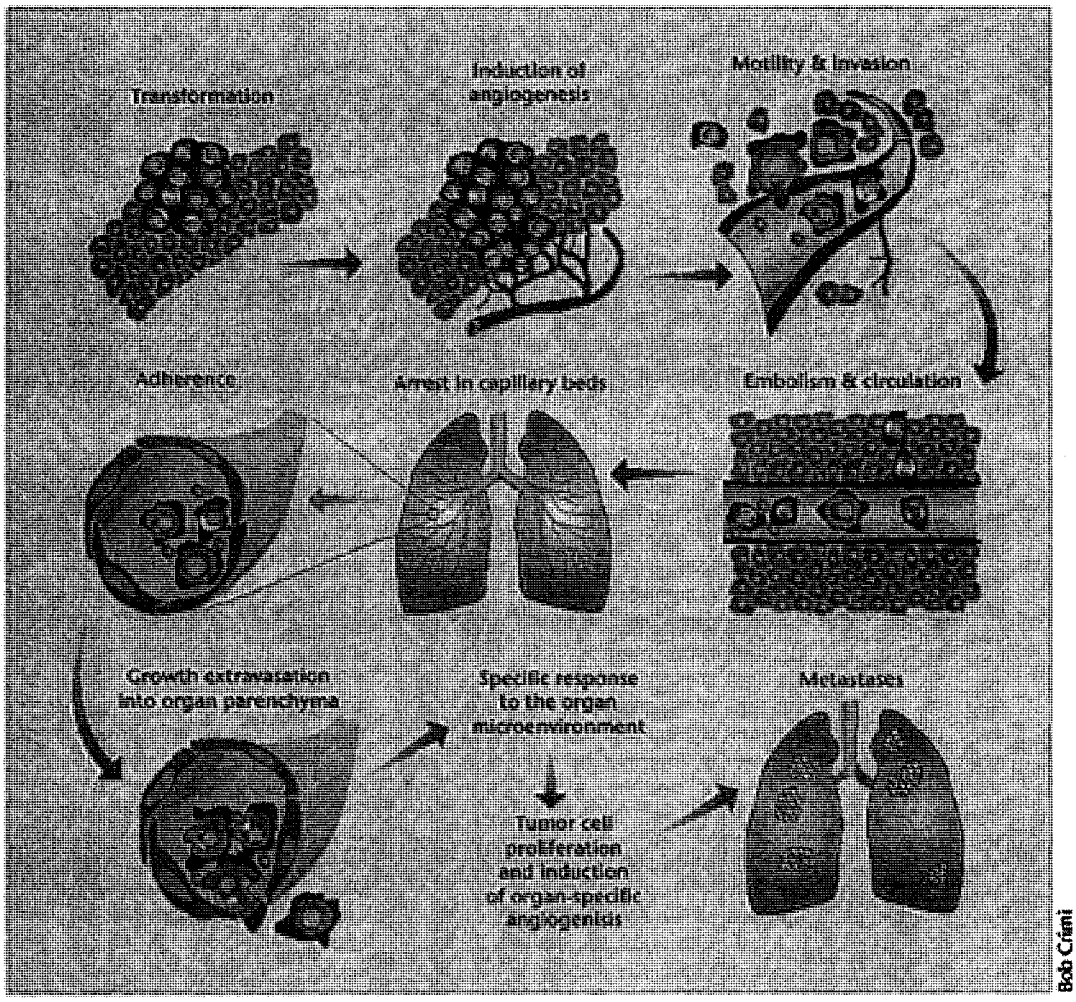


Figure 5. The process of metastasis
(From: Nat Med. 2000; 6: 500-502)

Metastasis to the bone

Bone is one of the three most common sites of tumor metastasis. The most common human cancers (lung, breast, and prostate) have a great avidity for bone, and in many patients with advanced disease the skeleton is the site of the most significant tumor burden (49, 50). When solid tumors metastasize to the skeleton, they cause a variety of alterations in bone cell function. Cancer cells are capable of having stimulatory effects on osteoblasts to form new bone, represented as osteoblastic or osteosclerotic lesions, or on osteoclasts that cause bone resorption, represented as osteolytic metastasis (51).

Advanced breast cancer is almost always associated with destructive osteolytic skeletal metastasis. Many cell-cell and cell-matrix interactions are involved in the pathogenesis of osteolytic lesions. Studies on the pathophysiology of osteolytic bone metastasis have shown that tumor cells, osteoblasts, osteoclasts, and bone matrix are the four components of a vicious cycle necessary for the development and progression of skeletal metastasis (52). Cancer cells secrete various factors which directly or indirectly activate bone resorption by osteoclasts. Tumor production of factors such as PTHrP (Parathyroid hormone related peptide), IL-6, IL-8 and IL-11 stimulate production of receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) by osteoblasts and stromal cells. PTHrP also decreases the production of osteoprotegerin, a decoy receptor that prevents RANKL from binding to its receptor on osteoclast progenitor cells. Signaling through RANK in osteoclast progenitors activates transcription factors, leading to the differentiation of osteoclast progenitors into mature osteoclasts. IL-1 and TNFα (Tumor Necrosis Factor) have RANK-independent effects on osteoclastogenesis. These osteoclasts mediate bone resorption (52, 55). In metastatic human breast cancer, PTHrP is the main mediator of

osteoclast activation. Human breast cancer cells with the property to form osteolytic metastasis have been shown to express PTHrP *in vivo* (50). Immunohistochemistry and in situ hybridization experiments have shown that breast cancer metastasis in bone express higher levels of PTHrP than cells that have metastasized to soft tissue or that are present in the primary site. This indicates that PTHrP is a specific mediator of osteolysis in metastatic breast cancer (53, 54).

The mineralized matrix of bone is abundant in growth factors released during bone resorption. These factors reach high concentrations within the bone microenvironment, hence attract tumor cells to bone and promote metastatic growth through activation of their receptors (51, 52).

Candidate bone-derived growth factors include IGFs, PDGF, FGFs (Fibroblast Growth Factor), BMPs (Bone Morphogenetic Proteins), and TGFβ (52). It has been shown that IGF-2 and then IGF-1 are the most abundant growth factors in bone matrix. The next most abundant growth factor is TGFβ, whereas BMPs, FGFs, and PDGF are found at lower concentrations (56). Although all of these factors that are made available in active form by local bone resorption may be responsible for aggressive behavior of tumor cells in bone, only TGFβ has been shown to play a direct role in stimulating tumor cells (51, 52). TGFβ is growth-inhibitory in the early stages of tumorigenesis. However, advanced cancers lose TGFβ-mediated growth inhibition but retain TGFβ regulation of their metastasis-promoting genes (57). Studies have documented that TGFβ is a critical growth factor in breast cancer metastasis through stimulating the expression of necessary factors such as IL-11 and PTHrP (58). A model for the osteolytic bone metastasis caused by breast cancer is summarized in Figure 6.

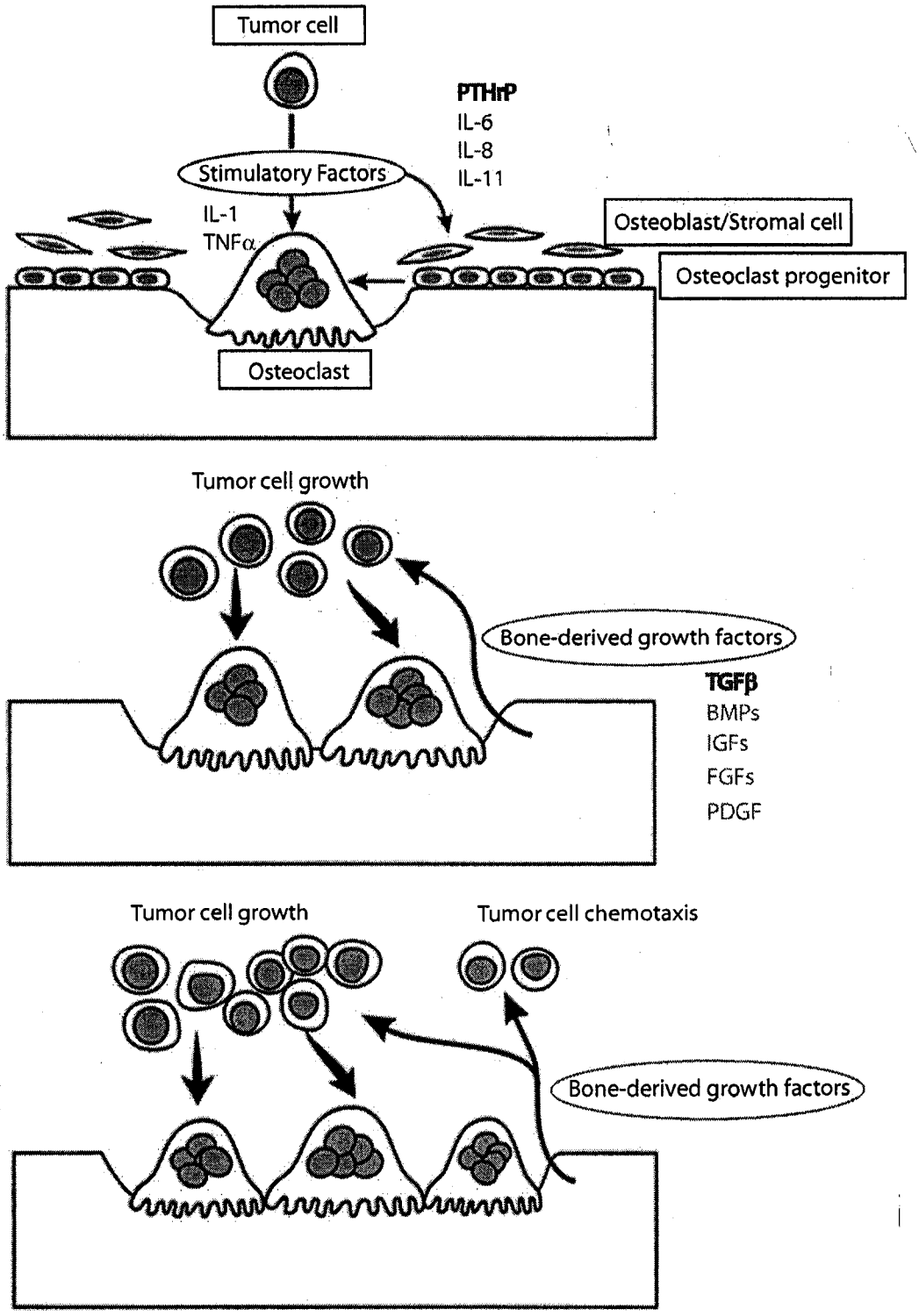


Figure 6. Pathophysiology of osteolytic bone metastasis

(Adapted from: Clin Orthop Relat Res. 1995; 312: 34-44)

HER-2/nue Oncoprotein

HER-2/neu is a transmembrane 185 KD protein with its proto-oncogene located on the short arm of chromosome 17. It is the second member of the human epidermal growth factor receptor (HER) family. This family is composed of four homologous receptors: EGFR (HER-1), HER-2/neu, HER-3, and HER-4. These receptors are located at the cell membrane and share a similar structure, comprising an extracellular ligand-binding domain, a lipophilic transmembrane segment, and an intracellular tyrosine kinase domain. HER proteins exist as monomers in the plasma membrane. Their activation is usually dependent on the presence of ligands and other receptors of the HER family. Ligand binding results in homo or heterodimerization of the receptors, leading to the activation of their intrinsic tyrosine kinase activity and tyrosine autophosphorylation. These events lead to the recruitment and phosphorylation of different intracellular substrates. Distinct signaling pathways are initiated from different substrates. A major signaling rout of the HER family is the mitogen-activated protein kinases (MAPK) pathway. The MAPK belongs to the large family of serine-threonine kinases. It is one of the most important and intensively studied signaling pathways which is at the heart of the molecular signaling network that governs the growth, proliferation, differentiation, and survival of many, if not all cell types. This pathway is deregulated in various diseases ranging from cancer to immunological, inflammatory, and degenerative syndromes (67). Members of the HER family and their ligands have been of considerable interest in the cancer area due to their potential to induce tumorigenesis when their signaling functions are deregulated (62). In particular, HER-2 protein has been shown to be overexpressed in a wide variety of human tumors including lung, gastric, breast and ovarian cancers (63).

Unlike the other members of this family, a direct ligand for the HER-2 receptor has not been identified yet. However, HER-2 acts as a co-receptor, enabling it to heterodimerize and participate in signal transduction in the absence of a specific ligand (64). HER ligands are bivalent molecules. They have two binding sites for HER receptors: a high-affinity, narrow-specificity site that binds directly to the receptor HER-1, HER-3, or HER-4 and a low-affinity, broad-specificity type that recruits a homo or heterodimerization partner (65). As a low-affinity receptor, HER-2 binds to and is transactivated by many different HER ligands through functioning as a co-receptor. Indeed, research has shown that interactions within the HER family do not occur at random. Rather, there is a clear preference for HER-2 as a dimerization partner. Overexpression of HER-2 in human cancer cells enhances this preferential binding of the low-affinity arm of ligands to HER-2 (63, 64, 65). It has been shown that heterodimers containing HER-2 have a particularly high ligand binding and signaling potency compared with non-HER-2 containing dimers. HER-2 undergoes a slower rate of ligand-induced endocytosis compared with other HER receptors, causing a prolonged signaling by the HER ligands. Therefore, signaling by HER-2 heterodimers is relatively prolonged and results in enhanced activation of signaling pathways such as the MAPK rout (63).

In turn, the activation of MAPK pathway generates many responses in breast cancer tumors and cell lines. In addition to expected effects on cell growth, proliferation, differentiation and transformation, it has been reported to effect breast cancer hormone sensitivity and development of hormone independence. This can lead to antiestrogen resistance that remains a problem during endocrine treatments. Further, MAPK activation

in breast cancer generates aggressive phenotypes characteristic of highly metastatic malignancies (67, 68).

HER-2 and Breast Cancer

Overexpression of HER-2 is associated with cancer of the breast (72). HER-2 gene amplification is the most common mechanism leading to increased HER-2 protein expression. Amplification of the HER-2 gene leads to overexpression of the receptor and disrupts normal control mechanisms, leading to the formation of aggressive tumor cells (66). The HER-2 gene is approximately amplified in 20-30% of breast cancers and is associated with aggressive tumor behavior. HER-2 protein levels in cancerous cells may be 100 times those seen in normal breast epithelium. HER-2 overexpression occurs in all stages of breast carcinoma and has not been identified in benign breast disease. Overexpression is maintained in metastatic lesions, suggesting a continuous function for HER-2 (63).

With regard to the HER-2 receptor and breast cancer, the main areas of experimentation and interest are the utility of HER-2 as a prognostic indicator and predictive factor and the use of HER-2 as a therapeutic target.

At present, the general consensus is that HER-2 overexpression is a negative prognostic factor for breast cancer. It correlates with a number of recognized prognostic factors including nodal status, tumor grade, mitotic index and ER/PR negative status. In addition, it predicts a more aggressive course of the disease, reduced disease free and overall survival, and increased recurrence risk (69). Several studies have shown that HER-2 overexpression predicts a lack of response to both hormonal therapies, especially tamoxifen and CMF (cyclophophamide-methotrexa-5-fluorouracil) chemotherapy.

However, there has been some evidence suggesting that these tumors have a relative increased sensitivity to anthracycline-based regimens (70, 71).

Apart from the clinical utility of HER-2 in obtaining prognostic and predictive information to guide patient management and treatment selection, the overexpressed HER-2 receptor represents an ideal target for specific anticancer therapies. Owing to the elevated levels of HER-2 in malignant cells and low levels in normal tissue, its accessible location on the cell surface and its role in carcinogenesis, HER-2 serves as a favorable antigen for antibody-targeted immunotherapy of breast cancer (62).

Targeting HER-2 protein by means of directing a monoclonal antibody at this receptor, has shown encouraging results of anti-tumor activity in advanced clinical trials and has led to approval of Trastuzumab (trade-name Herceptin) in 1998 for use alone or in combination with chemotherapy in treatment of patients with advanced and metastatic breast cancer (72,74).

Trastuzumab (Herceptin)

Herceptin (Genentech Inc., San Francisco, CA) is a bioengineered human IgG1 containing murine elements in the complementarity determining regions. The main mechanisms through which this antibody inhibits tumor growth after binding to the extracellular domain of HER-2 receptor, will be discussed here.

- Herceptin mediated receptor downmodulation: Downmodulation of receptor-ligand complexes is thought to be a major weakening mechanism for receptor-induced signaling. Significant removal of HER-2 protein from the plasma membrane occurs with Herceptin treatment. Herceptin obligates HER-2 homodimerization, particularly in cells that overexpress HER-2. These homodimers are capable of receptor transphophorylation.

However, antibody-induced receptor activation does not induce effective downstream signaling. Instead, Herceptin leads to downmodulation of HER-2 from the tumor cell surface. Removal of HER-2 from the plasma membrane results in less receptor being available for dimerization with itself or other HER family members, which in turn diminishes HER-2 initiated growth signaling (72).

- Antibody Dependent Cell-mediated Cytotoxicity (ADCC): Herceptin-mediated ADCC is assumed to be very effective against tumor cells that overexpress HER-2. Herceptin contains an IgG1 Fc, and its ADCC is mediated through interaction with type III receptors (Fc RIII) which are expressed primarily on Natural Killer cells, some monocytes and activated T-cells. According to many experiments, HER-2 overexpressing tumor cells are preferentially targeted for ADCC rather than tissues that express normal levels of HER-2 (72).

The benefits of Herceptin were shown in two separate clinical trials before approval by the FDA. In both clinical trials, the patients who responded best to Herceptin had the highest level of HER-2 protein as assessed by immunohistochemistry. So testing of tumors from women with breast cancer is critical for the identification of patients who overexpress HER-2 and who can potentially benefit from treatment with Herceptin. Herceptin is administered intravenously to these breast cancer patients as a first-line treatment in combination with chemotherapy (74).

Chapter 2: Effect of Herceptin on the development and progression of skeletal metastasis in a xenograft model of human breast cancer

Aims of this study

The discovery of HER-2 over-expression in a group of human breast cancers and its significance as an independent adverse prognostic factor prompted investigators to use HER-2 as a target for treatment. In this regard, Genentech researchers developed a monoclonal antibody targeting the extra-cellular domain of HER-2 receptor which was found markedly to inhibit proliferation of cell lines that over-expressed HER-2 but had little or no effect on cells without elevated levels of HER-2. This antibody was also found to be a potent inhibitor of growth of human breast cancer xenografts and was therefore selected for further clinical development (62).

The humanized form of this monoclonal antibody (Herceptin) has shown encouraging results of anti-tumor activity and is currently being used alone or in combination with chemotherapy in treatment of patients with advanced breast cancer. The benefits of Herceptin were shown in two clinical trials before approval by FDA. In a randomized clinical trial, 469 patients with metastatic disease who over-expressed HER-2 received chemotherapy alone or chemotherapy with Herceptin. As a group, the women who received chemotherapy plus Herceptin had less rapid tumor growth, more tumors were reduced 50% or more in size, and one year survival rates were higher (79% in the Herceptin combination group versus 68% for chemotherapy alone). Specifically, the median time to disease progression was 7.2 months for those receiving Herceptin and chemotherapy and 4.5 months for patients receiving chemotherapy alone. In a second clinical trial with 222 patients, Herceptin was found effective when used alone for a

group of breast cancer patients who had relapsed following previous chemotherapy for metastatic disease (74).

According to the previous studies, the mechanism through which Herceptin inhibits tumor growth mainly involves diminishing of HER-2 initiated pathways which promote tumor cell growth (72).

In the present study, we investigated the effects of Herceptin and its molecular mechanism of action in abrogating the development and progression of osteolytic bone metastasis in an experimental mouse model of human breast cancer.

Characterization of various human breast cancer cell lines for their level of HER-2 expression and *in vitro* effect of Herceptin on cell proliferation was examined.

We utilized the *in vivo* model of metastatic breast cancer by inoculation of a human breast cancer cell line into the left ventricle of nude mice which resulted in the development of osteolytic skeletal metastasis.

Specificity of the effect of Herceptin was established by monitoring the effect of Herceptin in animals inoculated with BT-474 cells which express high levels of HER-2, in non-tumor bearing animals and in animals inoculated with MDA-MB-231 cells which do not overexpress HER-2.

Materials and methods

Cells and cell culture

Human breast cancer cell line BT-474 and MDA-MB-231 were obtained from American Type Tissue Culture Collection (Rockville, MD, USA) and maintained as described previously (75). Herceptin was a gift from Genentech Inc., San Francisco, CA.

Cell proliferation assay and Western blotting

MDA-MB-231 and BT-474 cells were plated in triplicate at a density of 5000 cells/well in 100 mL media in flat bottom 96 well plates. Cells were grown in 10% FBS to 20-30% confluence and changed into serum free medium overnight to bring them to a quiescent stage. Cells in triplicate wells were cultured for 5 days in the presence of 2% FBS with vehicle alone or different concentrations (1.25—20.0 μg/ml) of Herceptin. At the end of this period effect on cell proliferation were determined by semi-automated colormetric MTT dye reduction assay. Briefly, 10 μg of 5mg/ml MTT was added to each well and the plates were incubated for 2hrs at 37°C in 5% CO2. Media was then removed and replaced with 105 μL DMSO to dissolve the purple crystals. Absorbance was measured by a photometric microplate reader (BMG Fluostar Optima, Munich, Germany) at 550 nm within 30 min.

For Western blotting, MDA-MB-231 and BT-474 (1 X 10^6) cells were plated in 100 mm petri dishes for 24 h. Cells were then washed with cold PBS and lysed with 200 μl of cold lysis buffer (150 mM NaCl, 1% Triton X100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH7.2, 0.2 mM sodium vanadate, 1% PMSF, 0.2% aprotinin). After keeping them on ice for 20 min., supernatants were collected by centrifugation at 13,000 X g for 20 min., and the protein concentrations were determined. Cell lysates were

resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes. Expression of HER-2 and β-tubulin as control were determined using anti-HER-2 (Neomarkers; Lab Vision Corporation, Neomarkers Ab-15, Fremont, CA, USA; β-tubulin; BD BioSciences, Mississauga, ON, Canada). Levels of expression of HER-2 was quantified by densitometric scanning and expressed as relative density.

Animal model and experimental protocols

Five week old (15-20g) female Balb/c/nu.nu mice (Charles River, St. Constant, QC) were used throughout the course of the studies. One day before tumor cell inoculation all animals were implanted with 21 day-release 0.25 mg estradiol pellet (Innovative Research of America, Sarasota, FL) via subcutaneous route using a trocar (76). For tumor cell inoculation animals were anesthetized by xylazine/ketamine mixture and 1 X 10^5 BT-474 or MDA-MB-231 cells were injected in 0.1 ml of cell suspension into the left ventricle of heart using a 26 gauge needle (76). Following tumor cell inoculation animals were randomized and treated with 1 mg/kg non-specific purified human IgG (Sigma, St. Louis, MI) or Herceptin via intraperitoneal route twice weekly for five weeks (Figure 1, protocol A). Alternatively following tumor cell inoculation all animals were evaluated for the development of skeletal metastasis at weekly intervals by X-ray analysis. Once the evidence of skeletal metastasis was established radiologically (at week 5 post-tumor cell inoculation), animals with similar degrees of skeletal metastasis were selected which comprised 70% of total animals inoculated with tumor cells. These selected animals were randomized, and treated with 1.0 mg/kg twice weekly with non-specific IgG or Herceptin via intraperitoneal route (Figure 1, protocol B) (77). All animals were killed at week 10 post-tumor cell inoculation.

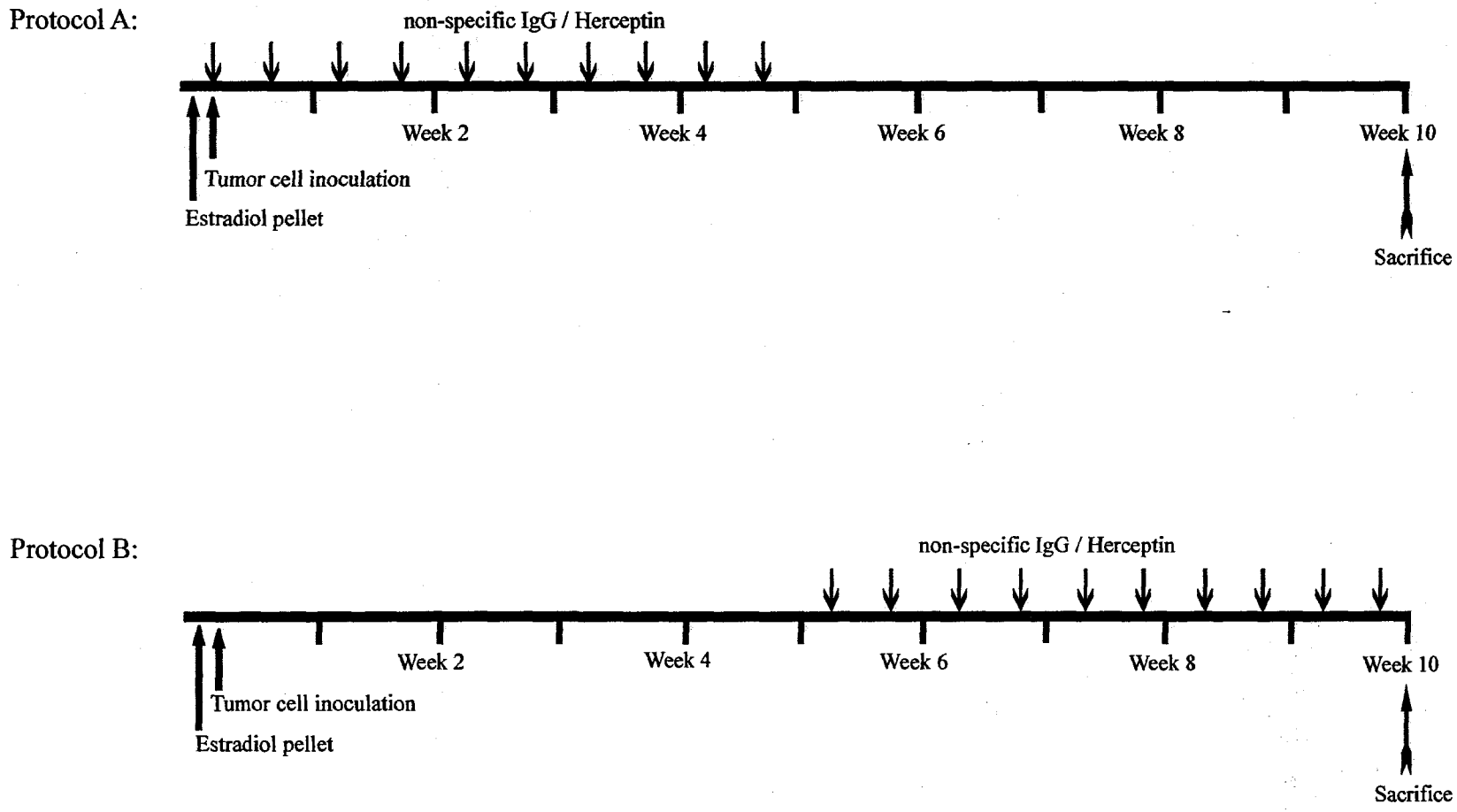


Figure 1. Xenograft model of human breast cancer skeletal metastasis and experimental protocols

X-Ray quantification and histological analysis

High resolution whole body radiographs of animals were obtained under ketamine/xylazine anesthesia with the animals placed in a prone position against films (18 x 24 cm; Mamoray Screens, AGFA, Mortsel, Belguim) and exposed to X-ray at 26 kV for 8 sec using a Mammo Diagnost UC (Philips, Hamburg, Germany). X-ray films were processed in a Curix Compact processor (AGFA). The analyses were carried out in both femora and tibia of all animals using an image analysis system following digital capture of radiographic prints. The area and number of skeletal lesions was determined in all of the control and experimental groups of animals by careful evaluation of radiographs at the end of these studies using BioQuant image analysis software, version 6.50.10 (BioQuant Image Analysis corporation, Nashville, TN, USA). Results are presented as lesions area in mm^2. All radiographs were carefully evaluated by at least three investigators, including one radiologist, who were blinded to experimental protocols.

At the end of these studies all long bones were removed and subjected to histological analysis. Tumor burden in the long bones were measured using a "stereological technique" as previously described (78). To reduce possible variation in analysis, four to six levels of each bone were cut and each level was assessed to get to the mid portion of bone with maximum tumor burden which was considered as a representative section. Histological measurement of total tumor volume was done in representative sections of both tibias of the animals treated with non-specific IgG or Herceptin in both protocol A and B. The analysis was performed on the region 1 mm below the growth plate with OsteoMeasure system (Osteometrics Inc., Atlanta, USA) using an IBM compatible

computer. The tumor volume (TuV) and tissue volume (TV) was measured and the percentage of tumor volume in total tissue volume was calculated (79).

Immunohistochemical analysis was carried out using antibodies against HER-2, P-HER-2 (Neomarkers, CA, USA), MAPK and P-MAPK (Cell Signaling Inc., Beverly, MA. USA) as previously described (80, 81).

Determination of mitotic index was achieved by manually counting mitotic cells in H&E stained sections and by immunohistochemical analysis using an antibody against the proliferation marker Ki67 on histological sections of long bones removed from control and experimental animals. The Number of mitotic figures in tumor cells was counted in 10 randomly selected fields of vision under high magnification (400X). Total mitotic index was established as a percentage of total tumor cells by blinded evaluation to the origin of the slides (82). Positive immunohistochemical reactions were quantified and represented as Ki67 index.

Statistical analysis

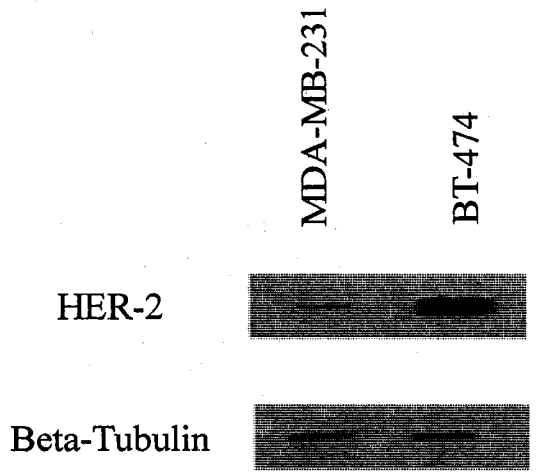
All results are expressed as mean ± SEM. Statistical significance of the difference in numbers of osteolytic metastasis and tumor volume between control and Herceptin treated groups were analyzed by Mann-Whitney test for non-parametric samples and tumor progression was analyzed by Kaplan-Meier.

Results

Effect of Herceptin on human breast cancer cell growth in vitro

Human breast cancer cell lines MDA-MB-231 and BT-474 were characterized for the levels of expression of HER-2. Western blot analysis showed marked overexpression of HER-2 in BT-474 breast cancer cells (Figure 2). Results are representative of the mean ± SEM of two separate experiments. Significant change in HER-2 production is represented by asterisk ($p < 0.05$).

In order to evaluate the effect of Herceptin on cell growth, human breast cancers expressing low (MDA-MB-231) and high (BT-474) levels of HER-2 were treated with different doses (1.25-20.0 μg/ml) daily of Herceptin for 5 days. Herceptin had no significant effect on MDA-MB-231 cell growth (Figure 3A). In contrast Herceptin treatment resulted in a dose dependent inhibition of BT-474 cell growth (Figure 3B). Results are representative of the mean ± SEM of three separate experiments. Significant difference in cell growth is represented by asterisk ($p < 0.05$).



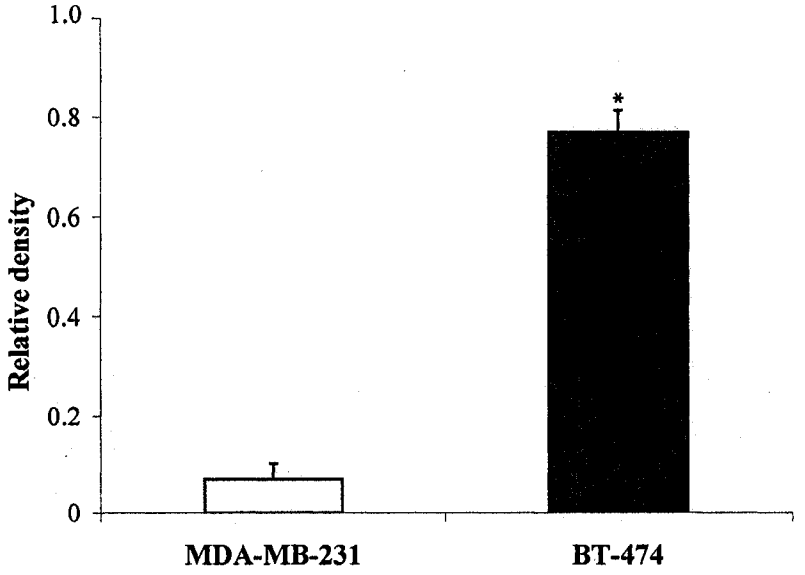
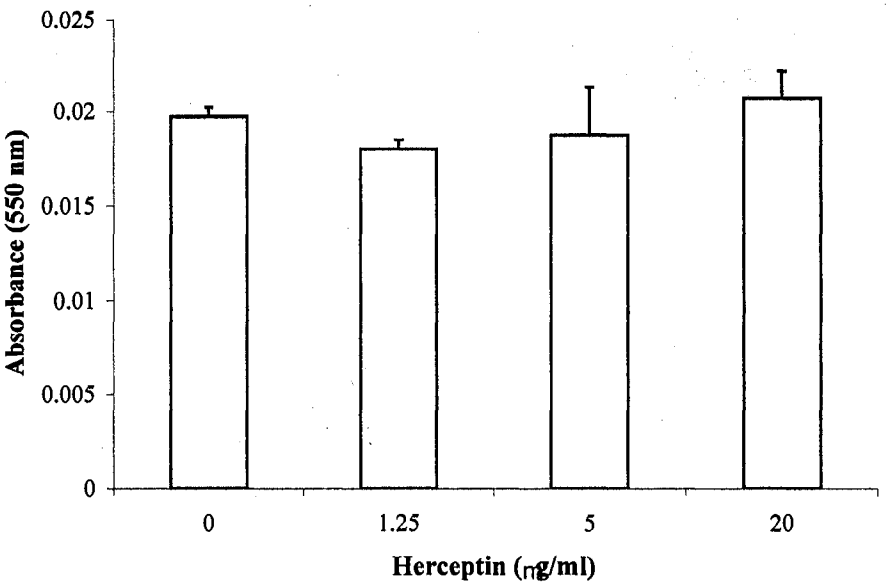


Figure 2. Expression of HER-2 protein in human breast cancer cells

A.



B.

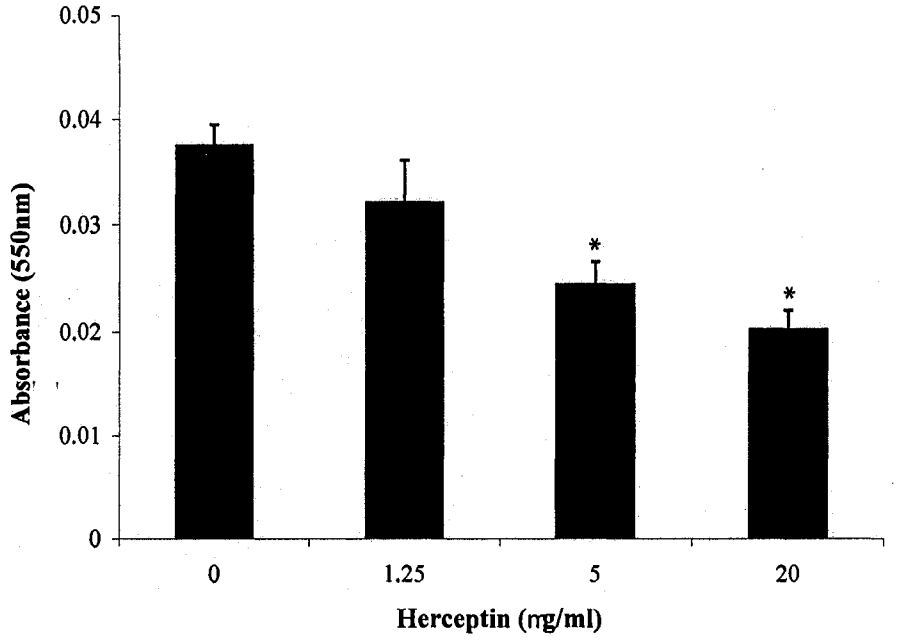


Figure 3. Effect of Herceptin on human breast cancer cell proliferation

Effect of Herceptin on the development of skeletal metastasis

The effect of Herceptin administration on the development of skeletal metastasis was evaluated in the xenograft model of human breast cancer using HER-2 overexpressing BT-474 cells as described in Figure 1, protocol A.

Distinct skeletal lesions were seen in control group of animals receiving non-specific IgG at week 10 post-tumor cell inoculation. In contrast, experimental animals receiving Herceptin either failed to develop any skeletal lesions or exhibited lesions of significantly smaller size compared to control group as assessed by radiography using X-ray analysis. Representative hind limb radiographs of a control and an experimental animal showed appearance of distinct skeletal lesions in the proximal tibia in the control animal, whereas minimal or no distinct skeletal lesions were seen in Herceptin-treated animal at week 10 post-tumor cell inoculation (Figure 4A). Area of skeletal lesions in all of the control and Herceptin treated animals was scored at week 10 as described in "Materials and methods". Treatment with Herceptin resulted in a significant decrease in the total lesion area as compared to the animals receiving control IgG (Figure 4B). Results are representative of the mean ± SEM of at least 12 animals in each group. Significant difference in lesion area from control animals is shown by asterisk ($p < 0.05$).

In order to effectively determine the metastatic tumor burden in the bone, histological analysis of tibia removed from control and Herceptin-treated animals was performed as described in "Materials and methods". The presence of histologically detectable tumor cells was seen in both control and experimental animals; however there was a significant decrease in the lesion area in mice treated with Herceptin. Tumor cells (T) filled marrow cavity, which was associated with the loss of trabecular bone (Figure 5A). Determination

of tumor volume/tissue volume ratio as described in "Materials and Methods" showed a significant decrease in tumor burden in experimental group of animals treated with Herceptin (Figure 5B). Results are representative of the mean ± SEM of at least 12 animals in each group. Significant changes in tumor volume/ tissue volume ratio (TuV/TV) as compared to control animals is shown by asterisk ($p<0.05$).

These results showed that Herceptin is highly effective at preventing the development of BT-474 breast cancer bone metastasis as observed by both radiological and histological analysis.

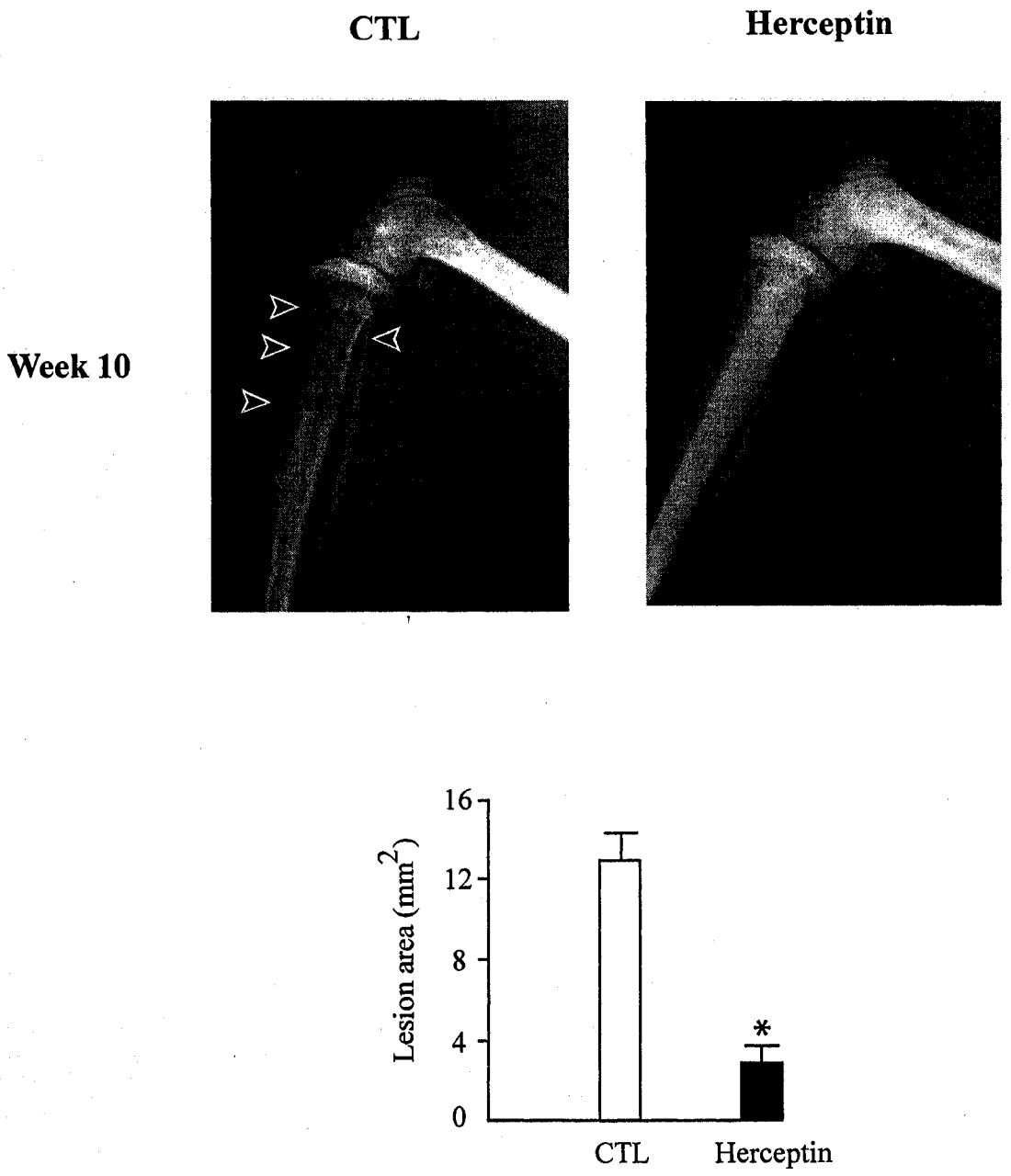
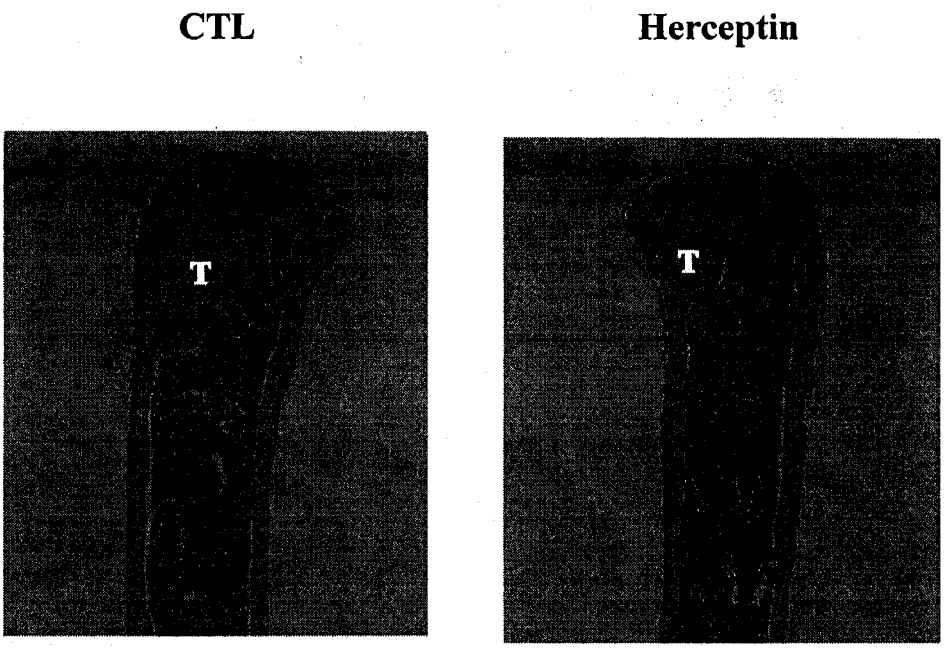


Figure 4. Radiographic analysis of skeletal metastasis in BT-474 tumor bearing animals

A:



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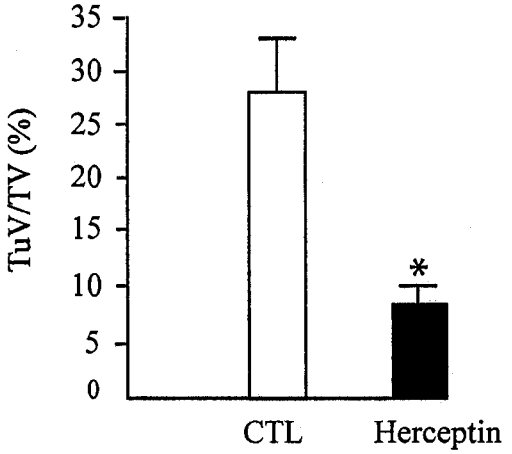


gure 5. Bone histology and histomorphometric analysis of skeletal metastasis in BT-474 tumor bearing animals

Effect of Herceptin on established skeletal metastasis

To evaluate the effectiveness of Herceptin on the progression of established skeletal metastasis in BT-474 tumor bearing animals, we used protocol B as outlined in Figure 1. All animals exhibiting skeletal metastasis at week 5 were selected, randomized and divided into two groups. For the next 5 weeks these animals were treated with either 1 mg/kg twice weekly non-specific IgG or Herceptin. Effect of this treatment on the progression of skeletal metastasis was evaluated at weekly intervals by X-ray analysis.

At week 10 post tumor cell inoculation and after 5 weeks of treatment, all control animals receiving non-specific IgG showed continued progression of their skeletal metastasis. In contrast, experimental animals receiving Herceptin exhibited either a decrease or complete arrest in the progression of their skeletal metastasis. Representative radiographs of long bones at week 5 and 10 following treatment with control IgG or Herceptin is shown in figure 6 (panel A). Area of skeletal lesions in control and Herceptin treated animals at week 5 and 10 was scored as described in "Materials and methods". While the total lesion area at the start of these studies was similar by radiographic analysis in control and experimental groups of animals, treatment with Herceptin for 5 weeks resulted in preventing any further increase in lesion area (Figure 6B). Results are representative of the mean ± SEM of 12 animals in each group which were subjected individually to radiographic analysis. Significant changes in lesion area from control at each time point are shown by asterisk ($p<0.05$).

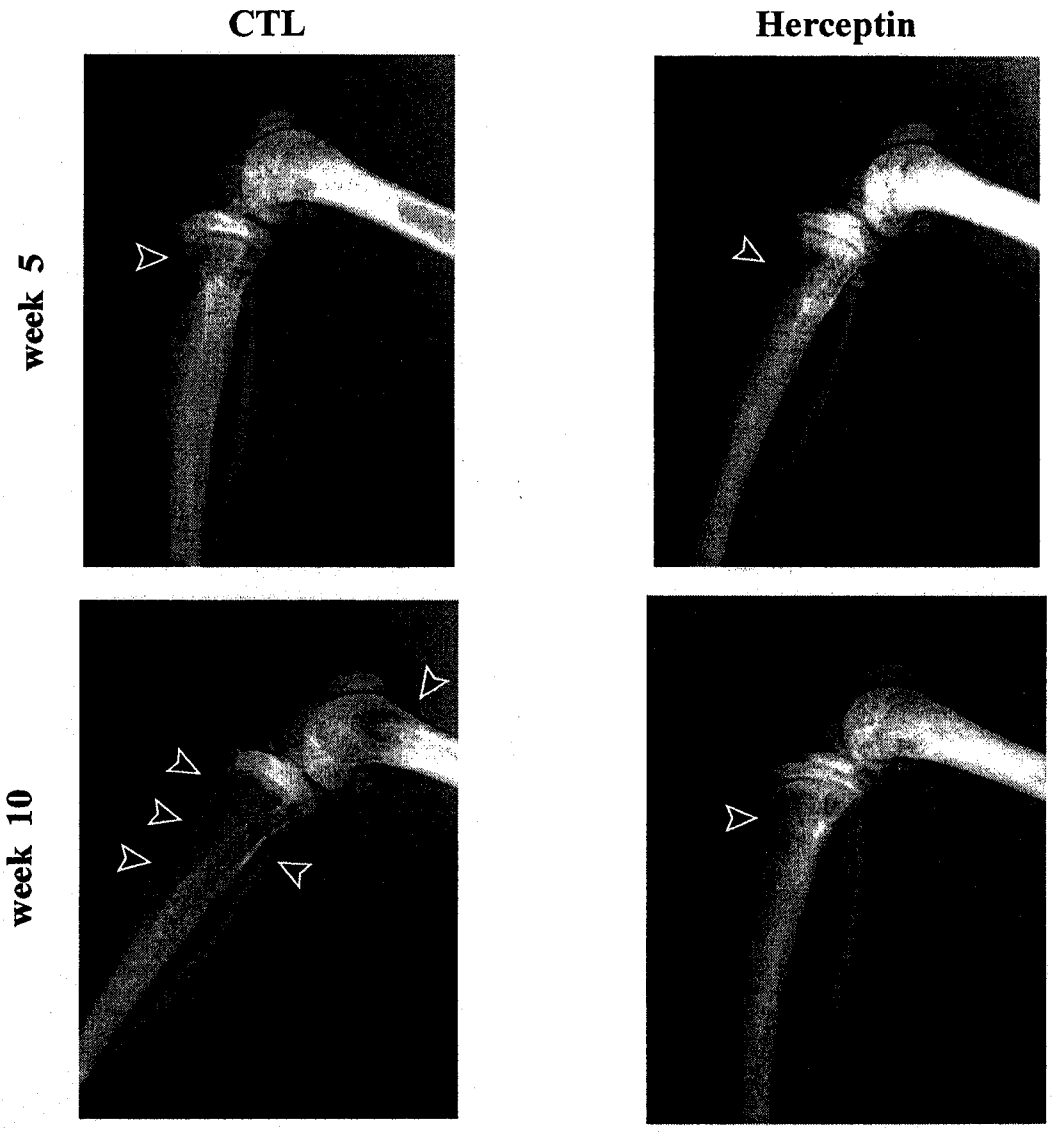
Histological analysis of tibia removed from control and Herceptin-treated animals was performed as described in "Materials and methods". Bone histology and histomorphometric analysis of long bones of control animals showed the presence of

extensive skeletal metastasis where tumor cells (T) filled the bone marrow space and caused the destruction of cortical and trabecular bone (Figure 7A). In contrast, treatment with Herceptin resulted in significantly lower tumor volume/tissue volume ratios (Figure 7B). Results are representative of the mean ± SEM of at least 12 animals in each group. Significant changes in tumor volume/ tissue volume ratio (TuV/TV) as compared to control animals is shown by asterisk ($p<0.05$).

These results showed that Herceptin is also effective in impeding the progression of established BT-474 breast cancer bone metastasis as observed by both radiological and histological analysis.

Figure 6. Radiographic analysis of established skeletal metastasis in BT-474 tumor bearing animals

A:



B:

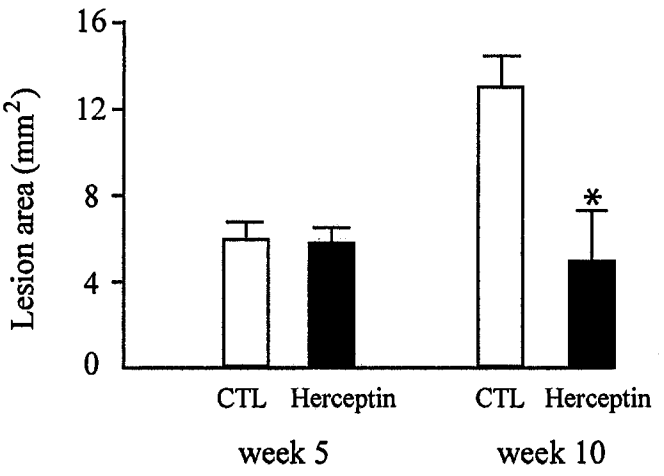
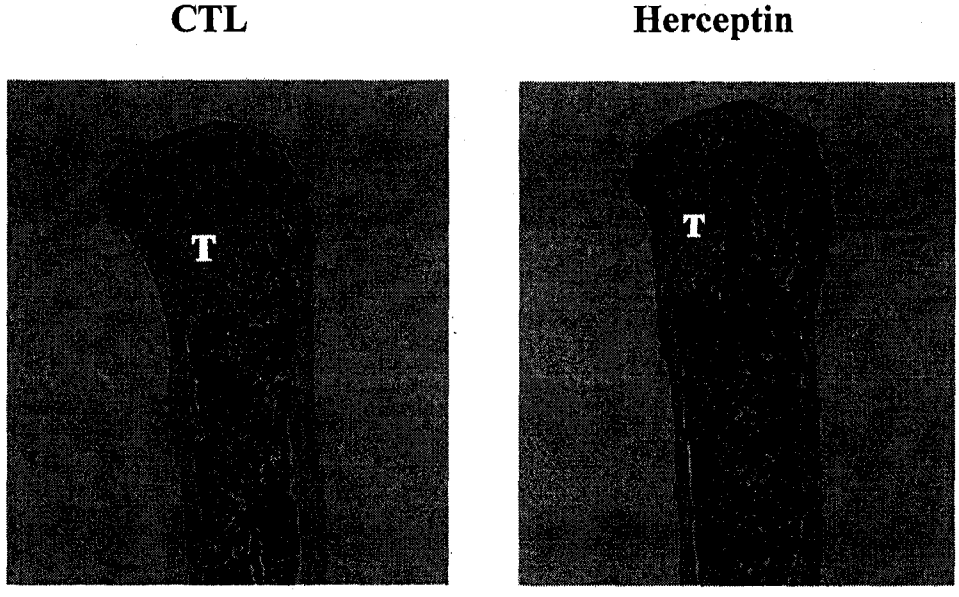
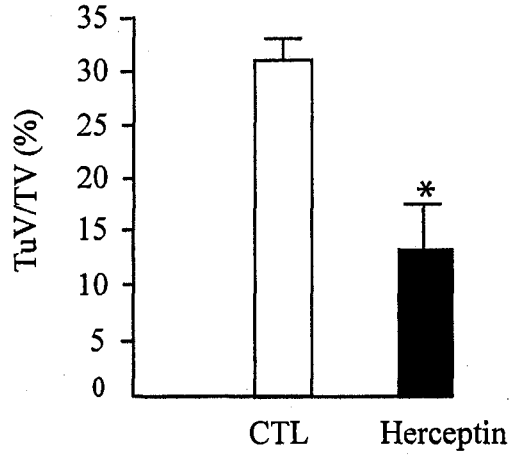


Figure 7. Bone histology and histomorphometric analysis of established skeletal metastasis in BT-474 tumor bearing animals

A:



B:



Effect of Herceptin on non-HER-2 overexpressing experimental skeletal metastasis and on normal bone.

In order to establish the specificity of Herceptin in blocking the progression of experimental skeletal metastasis via the HER-2 signaling pathway, we also utilized MDA-MB-231 human breast cancer cell line which does not overexpress HER-2. Following the inoculation of MDA-MB-231, animals were treated with either control non-specific human IgG or Herceptin as described in protocol A.

X-ray examination showed the presence of experimental skeletal metastasis at week 5 in both control and Herceptin treated groups which continued to show a progressive increase in lesion area and number up to week 10 post tumor cell inoculation. Representative radiographs of long bones of control and experimental animals at week 5 and 10 post-tumor cell inoculation is shown in figure 8 (panel A). Analysis of radiographs from both groups of animals did not show any statistically significant change in lesion area following treatment with Herceptin in these non-HER-2 overexpressing cells (Figure 8B). Results are representative of 12 animals in each group which were individually subjected to radiographic analysis.

To rule out the possibility of immune response that can be attributed to the effects on tumor metastasis following treatment with Herceptin, we evaluated the effect of Herceptin on non-tumor bearing animals. Normal non-tumor bearing animals were injected with non-specific human IgG or Herceptin for five weeks as described in protocol A. Treatment with either control IgG or Herceptin failed to show any change in bone remodeling as assessed by radiological and bone histomorphometric analysis (data not shown).

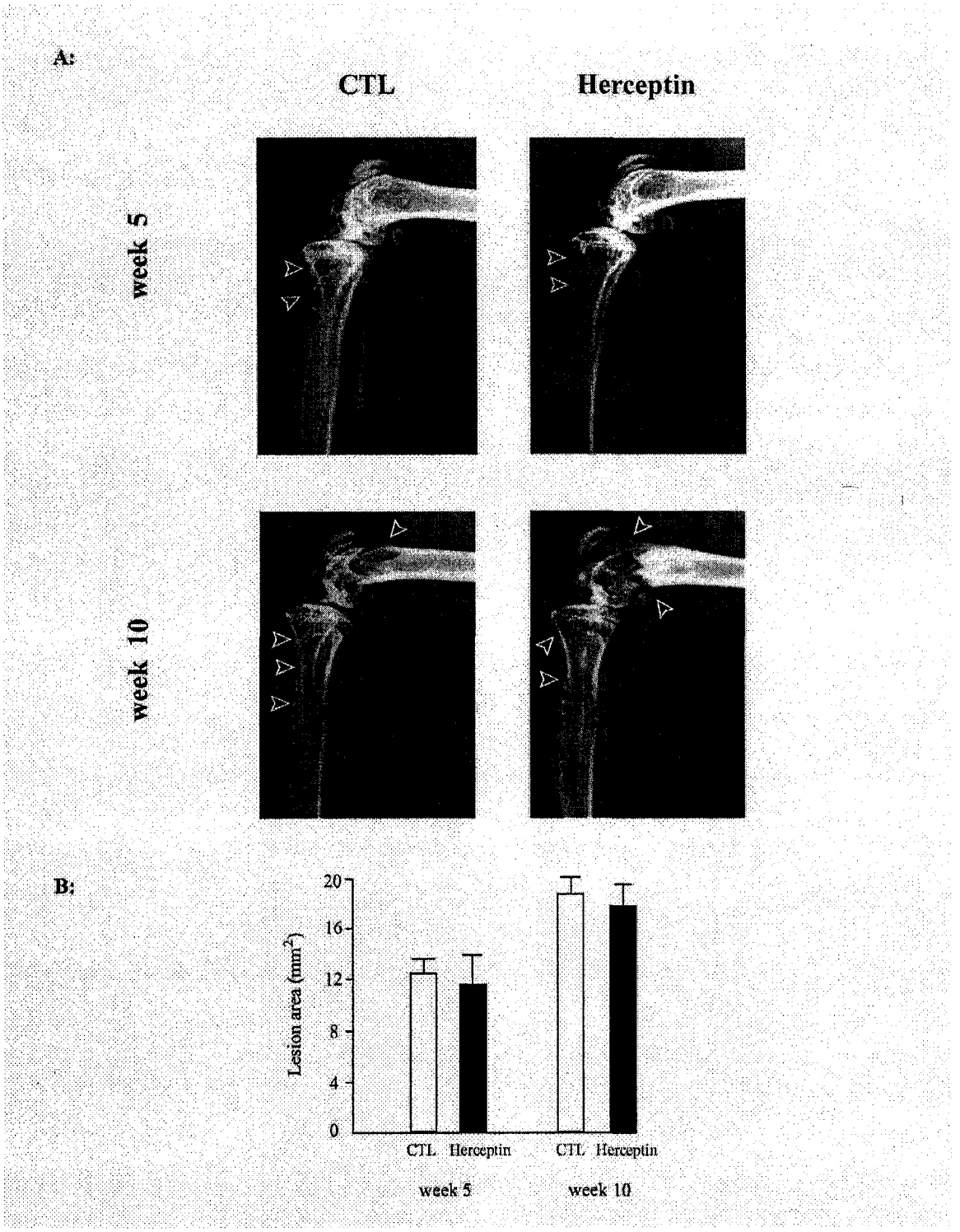


Figure 8. Radiographic analysis of skeletal metastasis in MDA-MB-231 tumor bearing animals

Effect of Herceptin on BT-474 tumor cell proliferation

Since HER-2 activation promotes tumor cell proliferation, we evaluated the effect of Herceptin on bone metastatic BT-474 breast cancer cell growth *in vivo*. Mitotic index and expression of the proliferation marker Ki67 were determined in sections from animals in protocol A and B as described in "Materials and methods".

Mitotic index and Ki67 expression was significantly reduced in both studies when Herceptin treatment was initiated either from day 0 (Figure 9A) or at week 5 when skeletal metastasis were established (Figure 9B). However these effects were more significant in Herceptin treated group of animals from protocol A when treatment was initiated at the time of tumor cell inoculation. Results are representative of the mean ± SEM of six animals in each group from two separate experiments. Significant difference from control is shown by asterisks ($p<0.05$).

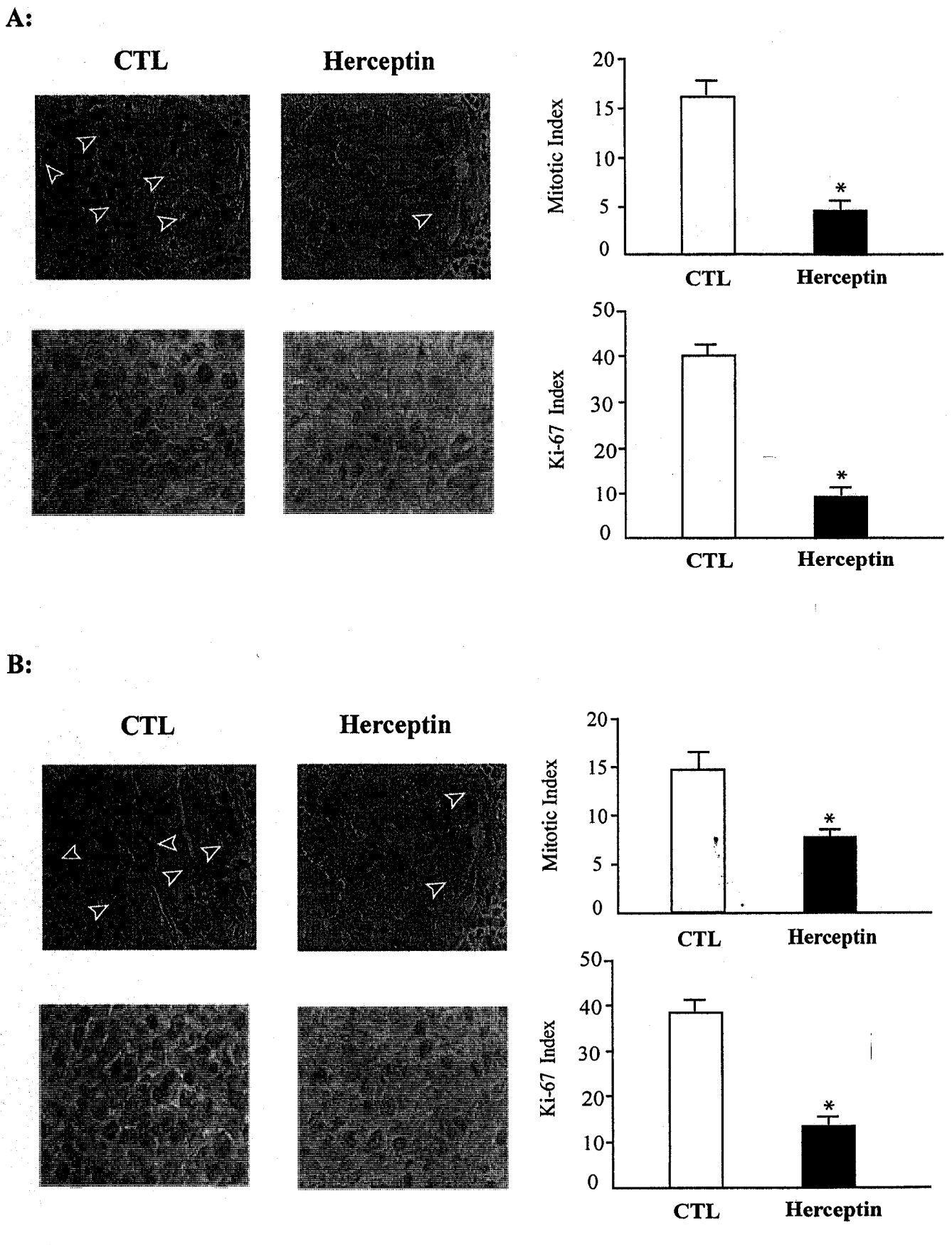


Figure 9. Effect of Herceptin on mitotic index and Ki67 expression of bone metastatic BT-474 cells.

Effect of Herceptin on HER-2 mediated signaling pathway

In order to define the molecular mechanism of Herceptin mediated effect on blocking the development of skeletal metastasis, histological sections of control and experimental animals treated with Herceptin were analyzed by immunohistochemistry by determining the levels of production of HER-2, phosphorylated (P) P-HER-2, MAPK and P-MAPK. Positive immunohistochemical reactions were quantified and represented as total density as described in "Materials and methods".

These studies showed no significant change in the levels of production of HER-2, P-HER-2 or MAPK between control and experimental animals when Herceptin treatment was initiated at day 0 which resulted in a significant decrease in skeletal metastasis as seen by radiological and histological analysis. However, due to the ability of Herceptin to block phosphorylation of MAPK (P-MAPK) histological sections of experimental animals receiving Herceptin exhibited a significantly lower level of production of P-MAPK (Figure 10). Results are representative of mean ± SEM of at least three sections for six animals in each group. Representative photomicrographs are shown (200X).

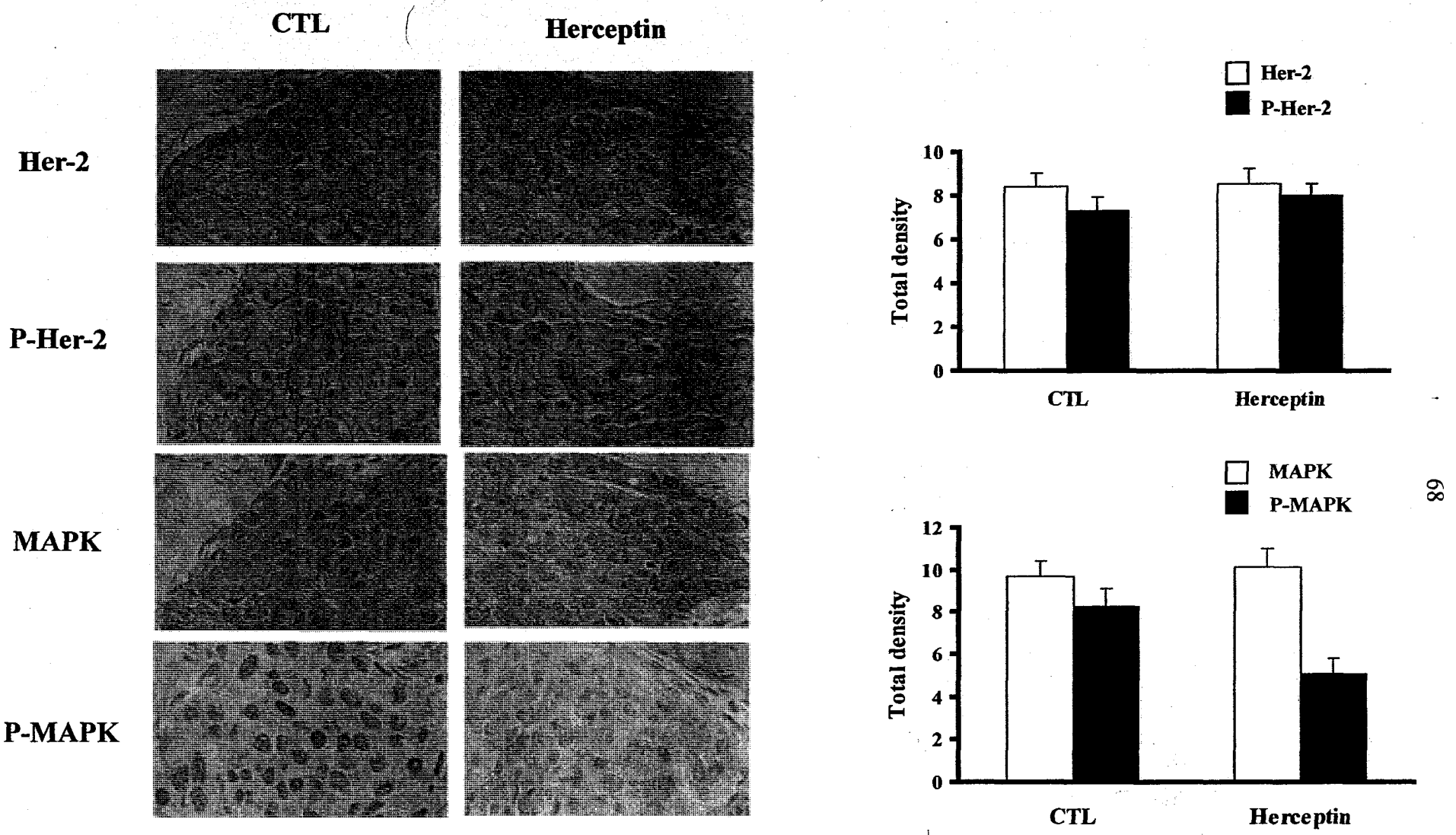


Figure 10. Effect of Herceptin on HER-2 mediated signaling pathway

Conclusion and Discussion

Despite the advances in the last two decades in elucidating breast cancer biology, understanding the complex multi-step process of metastatic progression of this common malignancy to the skeleton and its control continues to be an elusive goal.

In the present study, we directly evaluated the efficacy of Herceptin in abrogating the development and progression of established skeletal metastases using a xenograft model of breast cancer. The dose of Herceptin used in the current study has previously been shown to reduce tumor growth *in vivo* (77). The *in vivo* model used in the current study is well characterized and has been extensively used (79). In this model implantation of estradiol pellets has been shown to provide a supportive role for breast cancer cell growth (76). However, due to the well described role of estradiol in bone remodeling and its interaction with HER-2 and in order to rule out the unlikely but possible interference of short term estradiol administration in a bone metastasis model, the estradiol pellets were implanted uniformly in all experiments. We have used estrogen receptor (ER) positive human breast cancer cells BT-474 since they are known to endogenously produce high levels of HER-2. Furthermore, the anti-tumor effects of Herceptin have been demonstrated in HER-2 overexpressing cell lines regardless of their ER status (77). To evaluate the effectiveness of Herceptin, we used two experimental protocols as outlined in Figure 1. In protocol A where Herceptin treatment was initiated at the time of tumor cell inoculation, 5 weeks of treatment with Herceptin resulted in a significant decrease in the number of animals developing skeletal metastases and a reduction in skeletal lesion area as compared to vehicle treated control groups. Although treatment was stopped after 5 weeks, these anti-tumor effects of Herceptin were maintained until

week 10 post tumor cell inoculation as compared to animals receiving non-specific IgG. However this response was less robust in protocol B when a similar therapeutic approach was used to block the progression of established skeletal metastases. To effectively assess the therapeutic response, we applied several criteria including radiological analysis, bone histology and evidence of any side effects of this treatment. Since Herceptin does not have efficacy in HER-2 negative patients, we did not see any statistically significant change in the incidence or degree of skeletal metastases in animals inoculated with non-HER-2 overexpressing MDA-MB-231 cells following Herceptin treatment (83). These results are in agreement with previous reports where Herceptin was effective in reducing the growth of HER-2 overexpressing BT-474 and not MDA-MB-231 cells *in vivo* (75,77). Furthermore, previous studies have shown that HER-2 overexpressing variant of MDA-MB-231 cells obtained by gene transfection, unlike the wild type MDA-MB-231 cells, show decrease in tumor growth in response to Herceptin treatment (86). These findings in addition to failure of Herceptin to hinder the development and progression of osteolytic bone metastasis in MDA-MB-231 bearing animals in our *in vivo* model suggests that in this model Herceptin has a general anti-proliferative effect on cells overexpressig HER-2 rather than being a specific treatment for bone metastasis. By treating non-tumor bearing animals with either control non-specific IgG or Herceptin under similar experimental conditions, we have also ruled out the non-specific effect of Herceptin on bone via an immune response.

From a mechanistic point Herceptin is known to inhibit tumor growth via several potential mechanisms including antibody dependent cell-mediated cytotoxicity (ADCC), downregulation of HER-2, cell cycle arrest at G1 phase, induction of p27 preventing

HER-2 cleavage, and blocking tumor associated angiogenesis (72). In our studies, Herceptin exhibited a marked change in tumor cell mitotic index and downregulation of HER signaling pathway. The ability of Herceptin to exhibit more significant anti-mitotic effects as seen in protocol A and maintenance of these effects during these studies can be attributed to the early initiation of treatment and the ability of Herceptin to inhibit the MAPK signal transduction pathway through inhibiting its phosphorylation. This can consequently result in less expression of a number of proto-oncogenes such as *c-fos* and *c-myc* which has been implemented in growth and progression of cancer (67, 87, 88). In agreement with previous studies Herceptin was selectively able to decrease P-MAPK without changing MAPK, HER-2 or P-HER in BT-474 cells (84).

These effects of Herceptin on skeletal metastases are of particular interest since a potential limitation in developing effective therapeutic regiment for skeletal metastases could be the low bioavailability of these agents in the skeleton.

This study clearly demonstrates the ability of this targeted therapeutic approach in limiting the development of skeletal metastases and underscores the need to identify and initiate such therapy at the earliest time point in patients who are HER-2 positive. Furthermore, these results warrant the use of such an approach even in late stages of disease where any reduction in skeletal tumor burden is likely to have a direct impact on tumor associated morbidity by reducing bone metastases associated pain and fractures. Combining immunotherapy with chemotherapeutic agents like anthracyclines and taxanes has shown benefit in reducing tumor burden however the effect of these approaches on blocking skeletal metastases needs to be investigated (77). In order to improve targeted delivery of these agents additional studies are warranted using cytotoxic conjugates alone

or encapsulated into liposomes which have already shown their effectiveness to improve delivery and bioavailability (85). An area of caution in using such an approach remains the expression of HER-2 in normal tissue where it may not only promote an immune response but can also localize these cytotoxic conjugates in normal cells. However, due to significantly higher level of expression of HER-2 in tumor cells, this approach may be effective by decreasing the availability of these agents to normal tissues and avoiding drug resistance.

In conclusion results from this study have demonstrated the benefit of Herceptin in blocking breast cancer associated skeletal metastases in HER-2 positive tumors. Since Herceptin is currently indicated in hormone refractory metastatic breast cancer, results presented here provide the rationale to initiate this treatment at an early time point. This can be done at the time of establishment of HER-2 positive status of tumor cells and will lead to prevention of developing skeletal metastases which occur in the later stages of the disease. In summary, use of such immunotherapeutic regimens alone and in combination with chemotherapeutic and anti-angiogenic agents can prove to be of benefit in decreasing breast cancer associated morbidity and mortality.

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Chapter 4: Appendix