Leader

Biological indices in the assessment of breast cancer

A S-Y Leong, A K C Lee

Breast cancer is the most common malignant tumour among women in Australia and the United States, afflicting one in 13 and one in eight women respectively during their lifetime. It is a major public health problem worldwide with an estimated 600 000 new cases per year.[1] While most occur among women in the so called developed countries, where breast cancer accounts for nearly 25% of all new cancers, developing countries are not spared, with nearly 250 000 cases per year, representing 14% of the total cancer incidence. In the United States over a 30 year period from 1950, it was estimated that breast cancer accounted for nearly 1 000 000 deaths[2] and the annual mortality in the United Kingdom is approximately 15 000. Despite our ability to diagnose the disease at an earlier stage of its evolution and the refinements in treatment, both of which have collectively resulted in improvements in five year survivals, overall breast cancer mortality rates have not improved significantly in the last 25 years, with about one third of patients dying of their disease.

In the last decade there has been a tremendous increase in our knowledge of the biology of normal and abnormal breast development but despite this the treatments available to women with breast cancer are mainly empirical and in general have not been refined by the impact of our new knowledge of breast cancer biology. Biological indices that are clinically relevant should provide information about aetiology, allow assessment for future risk to the patient or her family, or directly influence treatment and be relevant to prognosis. An ideal biomarker should reflect all these three areas. While many of the biological factors which have been described appear to have prognostic value when studied retrospectively in a univariate analysis, such information may be of limited use until we identify the factors that are independently relevant to prognosis in multivariate analysis.[3]

The relative importance of the various prognostic factors also varies with time after the initial diagnosis; a prognostic factor that is highly significant for outcome in the first year after diagnosis may have little relevance after five years. For example, Lipponen *et al*,[4] in a long term study of a cohort of 464 patients with breast cancer, found that tumour diameter, axillary lymph node status, glandular formation, and proportion of intraduct growth were of prognostic value up to five years; mitotic index was significant in the first two years, but histological grade and morphometric nuclear factors had short term value only.

Current treatments for breast cancer are strongly influenced by the patient's choice, her age and menopausal status, tumour size, grade, axillary lymph node status, and the expression of oestrogen and progesterone receptor proteins in the tumours.[5] Aside from the histological variables such as tumour size, histological type and tumour grade, tumour stage—particularly with respect to lymph node status—is one of the most important prognostic variables in breast cancer. Bonnadonna[6] showed the 10 year disease-free survival to be approximately 80%, 40%, 35%, and 15% for the respective lymph node groups of 0 involved nodes, 1–3 positive nodes, 4–10 positive nodes, and more than 10 positive nodes. The NSABP experience provides data that are essentially similar to this Italian experience, the 10 year follow up statistics being 80%, 53%, 36%, and 13% for the nodal groups of 0, 1–3, 4–12, and more than 13 involved nodes.[7]

The number of treatments, which include conservative surgery, local-regional radiotherapy, and different chemotherapy regimens with or without hormone treatment, has increased considerably, combining to highlight the growing importance of prognostic factors in the management of patients with breast cancer. Breast cancer is not a single disease, with every patient receiving the same treatment. The wide choice of treatments and the participation of patients in treatment decisions makes it all the more necessary to be able to match subsets of patients with breast cancers through their clinical and biological features with the most appropriate treatment.

Besides oestrogen and progesterone receptor status, other possible prognostic indices in breast cancer have been investigated. These include the overexpression of oncogenes, the expression of mutant tumour suppressor genes, growth factors and receptors, and the assessment of tumour proliferation indices. In addition, the interaction of breast cancer with the microenvironment, particularly tumour angiogenesis, the expression of proteases including cathepsin D, and matrix metalloproteases, tumour adhesion molecules,

Division of Tissue Pathology, Institute of Medical and Veterinary Science and Department of Pathology, University of Adelaide, Adelaide, South Australia
A S-Y Leong

Department of Pathology, Lahey Clinic Medical Center and Harvard Medical School, Burlington, Massachusetts, USA
A K C Lee

Correspondence to:
Professor Anthony S-Y Leong,
Division of Tissue Pathology, Institute of Medical and Veterinary Science,
PO Box 14, Rundle Mall, Adelaide,
South Australia 5000.

Accepted for publication
6 April 1995

plasminogen activators, and tumour associated leucocytes are areas of current investigation.

Oestrogen receptor (OR) and progesterone receptor (PR)

Nearly 100 years ago, it was observed that oophorectomy was associated with clinical remission in two of three premenopausal women with metastatic breast cancer. Subsequently, adrenalectomy and hypophysectomy were found to be effective means of hormonal ablation in postmenopausal women with advanced breast cancer. Despite the usefulness of hormonal manipulation in some women, only approximately 30% of unselected women with metastatic breast cancer responded to the treatment. There was therefore a need to distinguish those women whose breast cancers were hormone dependent from those whose tumours were hormone independent.

Employing cytosol based ligand binding assays, it has been shown that approximately 50–60% of women with OR-rich breast cancers responded to hormone treatment, while less than 10% with OR-poor breast cancers showed similar response. The relevance of oestrogen receptor status and hormonal treatment in node negative tumours, however, is less clear. Most of the inaccuracy in predicting hormone responsiveness was due to unresponsive OR positive tumours. This could be the result of heterogeneity in oestrogen receptor expression in tumour cells, which has been shown to exist; however, it could also result from the presence of an oestrogen receptor that is present but is abnormal and fails to have a biological action. In selected target tissues, oestrogens have been found to stimulate not only mitogenesis but also the synthesis for specific proteins. One of these oestrogen induced proteins is the progesterone receptor. Since progesterone receptor is an oestrogen inducible protein, its expression indicates an intact oestrogen receptor pathway and may identify tumours that are hormonally responsive to oestrogen, thereby improving the overall predictive value of steroid hormone receptor assays. The value of oestrogen and progesterone receptor assays in predicting response to hormonal treatment in advanced breast cancer patients has been well supported both by studies employing cytosol based ligand binding methods and by immunohistochemical assays. Those women whose cancers express both oestrogen and progesterone receptors show the greatest likelihood of responding to endocrine treatment. Using conventional biochemical assays the response rate is about 77% for OR+ PR+ tumours, 46% for OR− PR+, 27% for OR+ PR−, and 11% for OR− PR− tumours.[8] In some series, however, the prognostic advantage of receptor positivity was lost after 4–5 years of follow up.[9–11] Nonetheless, it is clinically recognised that a small proportion of patients whose tumours are receptor negative will show a positive response to hormone treatment, and as many as one third of those with receptor positive tumours may fail to respond to such treatment. On the basis of comparative immunohistochemical studies, it is believed that some of these discrepancies are caused by errors of the biochemical method, dilutional effect of abundant stroma, or inadequate tumour sampling.[12] Until recently, histological methods of oestrogen and progesterone receptor assays were unreliable and largely unused. The introduction of monoclonal antibodies which recognise epitopes of these receptor molecules has led to reliable histological assays, initially in frozen tissue sections, but more recently, with the advent of second generation antibodies coupled with refined methods of immunostaining, immunohistochemical assays for these receptors can be performed on routinely fixed, paraffin embedded material.[13]

MOLECULAR BIOLOGY OF THE OESTROGEN RECEPTOR

The human oestrogen receptor is a member of a family of nuclear receptors for small hydrophobic ligands such as the steroid hormones, thyroid hormone, vitamin D, and retinoic acid. Genes of the steroid and thyroid hormone receptor family have common structural and functional domains. Each receptor has a ligand binding domain, a hinge region, a DNA binding domain, and a variable or regulatory domain. The oestrogen receptor gene is located on the long arm of chromosome 6 (band q24-27) and the progesterone receptor gene on chromosome 11. The oestrogen receptor gene of eight exons and intervening introns spans at least 140 kilobases.[14 15] The four domains are coded for as follows: amino terminal hypervariable region predominantly coded for by exon 1; DNA binding domain by exons 2 and 3; hinge region by exon 4, and the large hydrophobic hormone binding domain by five different axons, namely exons 4, 5, 6, 7, and part of exon 8.

Mutagenesis studies have shown that certain amino acids in the steroid binding domains of oestrogen and progesteron receptors are critical for hormone recognition. Alteration of some amino acids abolishes steroid binding altogether or may result in reduced affinity for the steroid. Binding of the ligand to the receptor is thought to result in an allosteric alteration that allows the receptor hormone complex to bind to its DNA response element in the promoter region of a target gene. In the absence of hormone binding, the domain appears to be inhibitory in function, preventing transcriptional activation. There seem to be other functions of the hormone binding domain, including binding of heat shock protein 90 and a role in receptor dimer formation. In between the hormone binding and DNA binding domains are amino acids which have been referred to as the hinge region, thought to have an important role in establishing the allosteric association of the hormone binding and the regulatory domains, inhibiting transcription activity by the latter domain in the absence of ligand. The sequences contained in the hinge region are critical in directing the oestrogen and progesterone receptor proteins to the nucleus after they are synthesised in the cytoplasm.[16 17]

A second signal is also present in the hormone binding domain that specifies nuclear localisation of receptor proteins in the presence of hormone.

The DNA binding domain in both oestrogen receptors and progesterone receptors has many basic amino acids, nine of which are cysteine residues, some being repeating units folded into a "fingered" structure coordinated by a zinc ion, known as the "zinc finger". The amino terminal zinc finger is responsible for recognition of the specific DNA hormone response element and, with the hormone binding domain, is thought to be involved in protein-protein interactions leading to receptor dimerisation.[18 19]

It thus appears that a wide variety of responses is elicited in various tissues by oestrogens, with many genes being activated; however, few of them have been identified. Oestrogen and progesterone receptors appear to enhance transcriptional activity of selected target genes. The actual mechanism is not known but probably involves interactions between receptors and other transcription factors with the promoter regions of the respective genes. Hormone induction or transcription seems to involve receptor mediated establishment of a transcription complex at the promoter, a function which may be mediated by the hypervariable or regulatory domain of oestrogen and progesterone receptors.

In the current model of oestrogen receptor action, oestradiol diffuses into the cell and binds to the receptor, leading to its dimerisation and tight binding to its specific DNA target, the oestrogen response element in target genes. Although initial assays employing hypotonic lysis of cells led to the extraction of oestrogen receptor in the cytosolic fraction, immunohistochemical stains using monoclonal antibodies to the receptor have shown that it is predominantly located in the nucleus even in the absence of hormone. However, hormone binding does alter the interaction of oestrogen receptor with the nucleus in an unknown fashion, as evidenced by an altered ability to extract the receptor biochemically.

Like several members of the nuclear receptor family, oestrogen receptor is thought to be associated with a complex of heat shock proteins (hsp) including hsp90. The binding may be through the ligand binding domain, and its interaction remains controversial. One suggestion is that hsp90 acts to keep the unliganded receptor in an inactive state, while another suggestion is that the heat shock complex may function as a protein "chaperone", aiding in the correct folding of the oestrogen receptor. Recent immunohistochemical studies have suggested that the association of unliganded progesterone receptor with hsp90 may be an artefact generated through the extraction of progesteron receptor. The exact role of hsp90 in oestrogen receptor function thus remains to be elucidated.

Following the binding of specific DNA sequences to the oestrogen response element in target genes, there is stimulation of general transcriptional factors to increase the transcription of target genes. Some of these genes directly or indirectly lead to the establishment of both autocrine and paracrine growth stimulatory loops. The activation of the transcription of target genes occurs by an unknown mechanism and only a few genes have so far been shown to be the direct targets of oestrogen receptor action. These include progesterone receptor and pS2, the latter a gene of unknown function. In addition, there is evidence that both growth factors such as transforming growth factor-α and insulin-like growth factor-1, and growth factor receptors such as EGF receptor and *c-erb*B-2 can be upregulated in breast cancer cells following treatment with oestradiol.[20] Thus oestrogen and progesterone receptors function as transcriptional activating proteins in breast cancer and the expression of these receptors in tumour tissue is correlated with patient prognosis and response to endocrine treatment. Although the mechanism of establishment of both autocrine and paracrine growth regulatory circuits is not entirely known, further understanding of the molecular biology of these receptor proteins will provide new insights into the mechanisms of tumour cell regulation and their control.

Patients with PR positive tumours have shown a longer disease-free interval and longer overall survival.[21-24] Some studies report a trend of improved prognosis in PR positive, node negative cases.[21 25] Oestrogen and progesterone receptor values are most useful for prognosis in premenopausal women.[26] The measurement of both oestrogen receptor and progesterone receptor together also has prognostic significance: the incidence of breast cancers which are OR+ PR+ is 50%, OR+ PR− 20%, OR− PR+ 5%, and OR− PR− 25%; their five year disease-free intervals are 73%, 75%, 68%, and 64%; and the overall five year survival is 91%, 93%, 88%, and 77% respectively.[21 26-29] It appears, therefore, that oestrogen receptor and progesterone receptor are both prognostic indicators but neither is a strong predictor.

The hormone receptor content of breast cancers shows a significant association with the degree of tumour differentiation and histological subtype.[28 30 31] Tumours overexpressing *c-erb*B-2 oncoprotein, a poor prognostic indicator, are more likely to be OR- and PR-,[32 33] and receptor-poor tumours are also more likely to show higher proliferation indices by thymidine labelling, flow cytometry, or immunostaining of cell proliferation antigens.[31 34 35]

Tumour proliferation indices

The rate of growth of tumours is determined by the differences in the rates of tumour cell proliferation and tumour cell death by necrosis and apoptosis. Tumours with high rates of cell proliferation might be expected to have a worse prognosis; however, these tumours may also be more sensitive to treatment because most anticancer drugs are more active against rapidly proliferating cells. The distribution of cellular

DNA content can be estimated by flow cytometry and by static cytometry or densitometry. A recent study investigating the significance of DNA ploidy and S phase fraction in a large cohort of 747 breast cancer patients found that neither variable was independently prognostic[36]; other studies have reported a higher response rate to combination chemotherapy in aneuploid tumours and the probability of tumour response to be positively correlated with tumour S phase fraction.[37 38]

Tritiated thymidine labelling index (TLI) is generally considered the gold standard for measuring cell cycle kinetics and has been shown to be an independent marker of prognosis in invasive breast carcinoma, particularly in lymph node negative patients.[29 39–41] The requirements for fresh tissue and autoradiography have hampered the use of TLI on a routine basis. Monoclonal antibodies to the thymidine analogue 5-bromodeoxyuridine have been developed that allow determination of labelling index without the need for autoradiography. Double labelling experiments using tritiated thymidine and 5-bromodeoxyuridine have shown that essentially all cells labelled with one precursor are also labelled with the other. Labelling indices correlate with other histopathological indices of unfavourable prognosis.

It has been a tradition in histopathology to use mitotic activity as an indicator of cell cycle kinetics, but mitosis counting requires standardisation and involves some degree of subjectivity. More importantly, the number of mitotic figures in any tissue sample has been shown to be influenced by the interval between removal of the biopsy and fixation of the tumour sample. With delays in fixation, completion of mitosis occurs, hence reducing the number of mitotic figures. Techniques are now available that allow immunohistochemical assessment of cell proliferation by the detection and quantitation of cellular proteins that are unique to proliferating cells. Several proliferation dependent antigens have been identified and these include Ki-67 and proliferating cell nuclear antigen (PCNA). Ki-67 is a non-histone nuclear protein found in all phases of the cell cycle except G_0.[42] The development of antibodies which label the protein in frozen sections allowed immunohistochemical evaluation of cycling cells in tissue sections from the percentage of nuclei staining for Ki-67, indicating the proliferating fraction. A high proportion of tumour cells staining for Ki-67 correlates with poor tumour differentiation,[30 43] inversely correlates with oestrogen receptor status,[30 44] correlates with thymidine labelling index,[45] and indicates a worse prognosis.[46]

A recent study of 54 invasive ductal and nine invasive lobular carcinomas with a median follow up of 37 months found progressively worse disease-free and overall survival with increased Ki-67 levels.[47] At cut off values of 12% of nuclear staining for Ki-67, survival of low expressers was 95%, whereas that of high expressers approached 60% ($p < 0{\cdot}05$). At a cut off of 16% positivity, only 50% survival was seen in the high expressers ($p < 0{\cdot}01$). The parameter was independent of age, nodal status, or hormone status. Worsened survival at higher cut off values of proliferative index and independence from several other factors have been shown with flow cytometric studies of proliferation. A significant, albeit imperfect, correlation ($p = 0{\cdot}05$, $r = 0{\cdot}3$) between Ki-67 and flow cytometric estimation of proliferation has been found.[48]

Proliferating cell nuclear antigen (PCNA, cyclin) is a 36 kDa non-histone nuclear protein that functions as an accessory protein to DNA polymerase delta. The level of PCNA has been shown to correlate with cellular proliferation and specifically with DNA synthesis, rising during late G_1 and peaking during S phase, and declining during G_2 and M phases. PCNA measurements in a variety of tumours have been shown to correlate with mitotic activity and tumour grade and parallel flow cytometric determination of the S phase.[49] One problem of immunostaining of PCNA lies in the susceptibility of the antigen to formalin fixation. The ability to immunostain the antigen is progressively diminished with increasing durations of fixation so that interpretation of PCNA values should be done with caution.[50] There are also variations between clones with marked differences in sensitivity between two common commercial clones, PC10 and 19A2, the former producing much stronger staining intensity as well as labelling a significantly larger number of tumour cells.[50]

There may be a demonstrable correlation between PC10 and 19A2 counts with Ki-67, although values with the former are much higher,[51] perhaps because of the long half life of PCNA, so that non-cycling cells may show stainable antigen.

Several other markers may reflect measurement of tumour proliferation. AgNOR (argyrophilic nucleolar organiser regions) counts correlate with DNA ploidy and cell proliferation.[31 52] P120 is a cell cycle associated nucleolar antigen which is detected in rapidly dividing cells[53] and P120 immunostaining in breast carcinoma was correlated with patient survival, nodal status, and mitotic index,[54] with multivariate analysis showing that the worst prognosis was found in patient with positive axillary lymph nodes and P120 positive tumours, 73% of these patient dying of the disease. Furthermore, node negative patients could be divided into two groups based on P120 expression. Ninety percent of patients with P120 negative tumours were alive at five years compared to only 67% in those with P120 positive tumours.[54] Topoisomerase II and histone III mRNA are unambiguously associated with cycling cells,[55 56] probes to the latter now being commercially available.

Recently, two monoclonal antibodies, MIB-1[57] and Ki-S5,[58] directed against a formalin resistant epitope of the Ki-67 antigen, have been developed and a polyclonal antibody to Ki-67 is available which allows the detection of the Ki-67 antigen in formalin fixed, paraffin embedded sections. These markers show good correlation with Ki-67 counts obtained in frozen sections.[51]

PLOIDY AND S PHASE FRACTION

The distribution of cellular DNA content can be estimated by flow cytometry, based on the measurement of fluorescence from dyes that bind in a stoichiometric manner to DNA. DNA content reflects the number of chromosomes, and ploidy can be determined by comparing the DNA distribution of tumour cells with that of normal diploid cells. The distribution of DNA can also be analysed to estimate the proportion of cells that are undergoing DNA synthesis, but this calculation may be difficult in some aneuploid tumours. Diploid tumours may have only a slightly better prognosis than aneuploid tumours in axillary node negative breast cancer although multivariate analyses do not always support the ability of DNA ploidy to provide independent prognostic information.[59-61] In a review of 20 studies investigating DNA ploidy and 12 studies investigating S phase fraction, O'Reilly and Richards[62] found that in general aneuploidy was associated with worse prognosis, particularly with high S phase fraction. There is a clear association between high S phase fraction and increased risk of recurrence and mortality for axillary node negative and positive breast cancer patients. S phase fraction is highly correlated with tumour grade and may not attain independent significance in multivariate analyses; however, it is less subjective than histological grading. A major problem which has been identified is a lack of standardisation of preparative techniques and histogram evaluation methods, making it difficult to compare data among various centres. Interinstitutional studies using identical specimens have shown poor agreement among laboratories with regard to the measurement of S phase fraction. The proportion of tumour cells comprising the cell suspension can vary greatly and dilution with benign tissue elements can significantly alter the measurement of the proliferative fraction, especially in diploid tumours. Few studies have compared response to treatment for groups of patients separated on the basis of S phase fraction or ploidy. Brifford *et al*[37] reported a higher response rate to combination chemotherapy in a small sample of 25 aneuploid tumours and Remvikos *et al*[38] found the probability of tumour response to be positively correlated with S phase. In contrast, no effect of ploidy or S phase fraction on relapse-free survival was shown in patients who received adjuvant therapy for early breast cancer.[63] Overall, there appears to be evidence for increased recurrence and death in patients with higher S phase fractions, and a weaker association with aneuploidy, although the methodological differences and arbitrary cut off values employed to generate discriminant groups make it difficult to apply such results for general use.

Growth factors and receptors

It has long been known that breast tumour cells cultured in vitro require exogenous serum derived factors for optimal growth. Protein fractions responsible for such activity have been found with variable frequency in primary human breast tumour samples and include the epidermal growth factor receptor (EGF-R), the *erb*B-2 oncoprotein, insulin and insulin-like growth factor receptors (I-R, IGF-I-R, IGF-II-R), transforming growth factor β receptors, fibroblast growth factor receptors (FGF-R), and the somatostatin receptor (SR). Only the more important of these will be discussed.

Cell surface growth factor receptors constitute a family of homologous transmembrane proteins. Receptor binding by a specific extracellular ligand induces an activated state associated with membrane dimerisation of receptor, autophosphorylation, and activation of its cytoplasmic tyrosine kinase activity. A complex and poorly understood sequence of "second messenger" events usually includes the liberation of inositol polyphosphatases and diacylglycerol, increase in intracellular calcium, altered cytoskeleton, and the transcription induction of genes such as *c-myc*, *c-fos*, and *c-jun*.[64,65] The end result of ligand stimulation can be quite variable and depends on the receptor being stimulated, the cell type, and the environment in which this occurs. The cellular response can range from proliferation to differentiation, and it may be associated with increased motility, production of specific proteins, or growth inhibition. The presence or overexpression of certain receptors in primary breast tumours has been found to correlate with prognosis and response to systemic therapy. These correlations are mostly based on archived tumour specimens and retrospective analysis of corresponding clinical data. The conclusions are often too preliminary to alter clinical practice, but nevertheless support the role of growth factors in the development and progression of human breast cancer and indicate their potential as a new method of treatment.

EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor (EGF) is a mediator of cell proliferation and is one of the growth factors necessary for the maintenance of normal breast epithelium. Its action is mediated by its receptor (EGFR), which is a transmembrane glycoprotein with an extracellular ligand binding domain and an intracellular tyrosine kinase domain, structurally closely related to *c-erb*B-2. The EGFR binds not only EGF, but also transforming growth factor α and other factors. Transfection and overexpression of human EGFR in immortalised human fibroblasts can lead to EGF dependent malignant transformation. Furthermore, transgenic mice bearing a constitutively expressed EGF-α gene develop hyperplasia in breast alveoli and terminal ducts, followed by the stochastic appearance of breast adenomas and adenocarcinomas after pregnancy.[66] About 35-45% of primary human breast cancers overexpress EGF-α and EGFR,[67,68] and numerous clinical studies have shown that EGFR overexpression is associated with other markers of increased tumour aggressiveness and a worse clinical outcome,[69-72] although this remains controversial.[73,74] Inexplicably, the increased

EGFR expression is usually due to increased levels of EGFR mRNA and the gene is only amplified infrequently.[75-77] In conclusion, EGFR may have useful prognostic value, but further studies on larger series with longer follow up and with multivariate analysis are necessary to define its clinical usefulness.

*c-erb*B-2 oncogene

The *c-erb*B-2 oncogene was discovered in the 1980s by three different avenues of investigation. The *neu* oncogene was detected as a mutated transforming gene in neuroblastomas experimentally induced in fetal rats. The *c-erb*B-2 was a human gene discovered by its homology to the retroviral gene *v-erb*B, and HER-2 was isolated by screening a human genomic DNA library for homology with *v-erb*B. When the DNA sequences were determined subsequently, *c-erb*B-2, HER-2, and *neu* were found to represent the same gene.

The *c-erb*B-2 DNA is located on human chromosome 17q21 and codes for *c-erb*B-2 mRNA (4·6 kb), which translates *c-erb*B-2 to protein (p185). This protein is a normal component of cytoplasmic membranes and the *c-erb*B-2 oncogene is homologous with, but not identical to, *c-erb*B-1, which is located on chromosome 7 and codes for the epidermal growth factor receptor.[78-82] The *c-erb*B-2 protein is a cell membrane protein with extracellular, transmembrane and intracellular tyrosine kinase activity. It is expressed in all epithelial cells.

*c-erb*B-2 gene alterations have been reported in diverse human neoplasms, and almost exclusively involve amplification of the gene. Gene amplification involves the repeated duplication of a particular gene sequence, resulting in multiple gene copies within each cell. This results in overexpression of the gene product, as reflected in the levels of mRNA and gene oncoprotein. There is in general good correlation of *c-erb*B-2 gene amplification with overexpression.[83-86] In one study of invasive breast cancers,[85] 27% of tumours showed *c-erb*B-2 amplification and overexpression, and 63% had no amplification and low levels of *c-erb*B-2 gene product; however, 10% of tumours displayed overexpression without detectable gene amplification. *c-erb*B-2 has been shown to be amplified in about 20–30% of invasive breast carcinomas in various studies.

*c-erb*B-2 may be evaluated by a variety of techniques. Gene amplification can be detected by Southern blot,[84 85] slot blot,[85] quantitative polymerase chain reaction (PCR),[87 88] in situ hybridisation,[89] and fluorescent in situ hybridisation (FISH)[90]; RNA overexpression can be detected by northern blot, reverse transcriptase polymerase chain reaction (RT-PCR), and in situ hybridisation,[84 85 88] while protein overexpression is conveniently detected with immunohistochemical techniques or by western blot and flow cytometry.[84 91 92 93]

Various studies have correlated *c-erb*B-2 amplification or overexpression with other prognostic variables in breast cancer patients. Although these studies give insufficient or no information on survival outcome to evaluate the prognostic implications, almost all of them showed a strong relationship with various established adverse factors.[94-103] These include large tumour size, unfavourable histological type, poor histological grade, high mitotic rate, high proliferative activity, positive nodal status, presence of haematogenous metastasis, and aneuploidy.

*c-erb*B-2 overexpression is more common in invasive ductal and medullary carcinomas than in lobular, colloid, and papillary carcinomas.[104-106] In intraductal carcinomas, it is almost exclusively seen in large cell, high nuclear grade, oestrogen receptor negative, comedo type intraductal carcinoma[107-111]; in contrast, in situ lobular carcinomas seldom overexpress *c-erb*B-2.[104 106 112 113] Overexpression is more common in intraductal than in invasive carcinomas,[111 114] more common in invasive tumours associated with intraductal component than in those without,[78 115 116] and there is usually concordance between the invasive and intraductal components of an individual tumour.[109 117] These observations have important implications regarding the role of *c-erb*B-2 in the initiation and progression of breast cancer. Slamon *et al*[118] first reported the association between *c-erb*B-2 gene amplification and adverse prognosis in breast cancer patients. The large number of ensuing studies have produced conflicting results.[85 92 110 119-128] However, the majority of larger studies appear to show a correlation with disease recurrence and survival, especially in node positive patients. The conflicting observations may have resulted from differences in methodology and reagents, nature of tissue studied, patient characteristics, and sample size.

The importance of an adequate cohort size is underscored by a report on node negative tumours,[129] which combined the data from three previous immunohistochemical studies on archival material from three different institutions using the same antibody to *c-erb*B-2.[130-132] Only one of the three studies was initially able to show a correlation with prognosis.[132] Reanalysis of the combined data from a total of 483 patients showed that *c-erb*B-2 was a significant independent prognostic variable for the entire population, and for node negative and node positive patients separately. However, the prognostic impact of *c-erb*B-2 in node negative patients remains unsettled. The results of a large number of studies performed on node negative patients are about evenly divided.[85 92 98 100 116 122 124 129 131-149] It should be noted that in some of the negative studies, *c-erb*B-2 overexpression showed prognostic significance in the node positive tumours in the same series. Again, an adequate cohort size is critical in node negative patients because of the low frequency of *c-erb*B-2 alteration and low relapse rate in this subset of patients (20–30% at 10 years). Another confounding factor is the great degree of variability in immunohistochemistry, as elegantly illustrated by Press *et al*.[91]

The conflicting data regarding the prognostic value of *c-erb*B-2, despite universal observation of a strong correlation with various adverse

prognostic factors, suggest that it may not be a powerful predictor by itself, and in any individual patient it should be employed as part of a multivariate approach to guiding treatment and determining prognosis.

Aside from its role as a potential prognostic factor, overexpression of *c-erb*B-2 may also serve as a predictor of response to adjuvant treatment. Some studies have shown a correlation between *c-erb*B-2 overexpression and poor response to chemotherapy,[116 131] and in one study there was a dose-response effect in patients with *c-erb*B-2 overexpression but not in patients with no or minimal *c-erb*B-2 expression.[150] Thus *c-erb*B-2 may be useful in predicting a poor response to chemotherapy and identifying patients who are most likely to benefit from high dose regimens.

Because *c-erb*B-2 protein has an extracellular domain and tends to be expressed in more aggressive tumours, it is a potential target for immunotherapy. This is supported by antitumour effects mediated by monoclonal antibodies on cultured cells expressing high levels of *c-erb*B-2 proteins and on nude mice bearing breast carcinoma xenografts.[151 152]

pS2

pS2 is a 6660 Da protein which shows some similarity to growth factors, although its function remains unknown. The protein may reflect a functioning steroid dependent stimulatory pathway; thus pS2, like EGFR, has been found to be more predictive than oestrogen receptor measurement for the likelihood of tumour response to endocrine treatment. Low concentrations of the protein have been associated with a poor prognosis.[153 154] The five year recurrence-free survival and overall survival were 85% and 95% respectively for OR+/PR+/pS2+ tumours, but only 50% and 54% for patients with OR+/PR+/pS2− tumours.[153] In another study of 72 advanced breast cancer cases, 76% of pS2+ cases had stable disease, complete remission, or partial remission as compared with 37% of the pS2− cases; the authors proposed that pS2, a marker of an intact oestrogen pathway, may help differentiate the 35–50% of OR+ breast cancer patients who do not respond clinically to hormone treatment, and the rare OR− patients who do.[155]

OTHER GROWTH FACTORS

Various of other growth factors may have a role in the stimulation of breast cancer. The fibroblast growth factor (FGF) family is composed of seven mitogenic peptides found in many normal and malignant human tissues. They are surface molecules that require binding to heparin to induce mitogenesis, and some function as embryonic morphogens, mitogens, and mediators of angiogenesis. Breast cancer cells produce FGF-4 and other proteins with FGF-like activity and they proliferate in response to FGF although the frequency of expression of FGFs and their receptors in primary human breast cancer is not yet established.[156 157]

The platelet derived growth factor receptor (PDGFR) and its ligand, PDGF, is a potent mitogen for cells of mesenchymal origin, being a tyrosine kinase growth factor receptor. It is known to be expressed on some human breast cancer cell lines that are mitogenically stimulated by PDGF and, in a small series of patients, significantly raised plasma PDGF was found in patients with advanced metastatic breast cancer compared to those with early stage disease.[158–160]

The insulin and insulin-like growth factor receptors belong to a subclass of tyrosine kinase receptors and appear to regulate normal cellular growth and metabolism, including that of mammary epithelial cells. There is increasing evidence that dysregulation of pathways using these receptors may contribute to breast carcinogenesis. Overexpression of functional insulin receptor in primary breast tumours as compared with normal breast tissue and fibroadenomas has been reported, correlating with other indices of increased aggressiveness such as size and histological grading.[161] The modulating role that oestrogen and progesterone have on insulin mediated growth in vitro suggest that antioestrogens and antiprogestins may be able to down modulate the insulin receptor signalling system.

The insulin-like growth factors (IGF-I) and (IGF-II), or somatomedins, are homologous to insulin, but they have distinct receptor binding and biological effects. Most primary breast tumours express receptors for IGF-I and IGF-II and show in vitro growth stimulation by IGF-I more than by IGF-II. The findings that plasma IGF-I is higher in patients with breast cancer compared with controls supports its role as an in vivo mitogen for breast cancers.[162] An antibody to the IGF-I receptor inhibits the growth of breast cancer cells in vitro and in nude mouse tumour xenographs.[163]

Other growth factors and receptors such as somatostatin, somatostatin receptor, and transforming growth factor β have an established role in cell growth but little is known of their in vivo effects on breast cancer.

Tumour suppressor genes

Tumour suppressor genes or antioncogenes normally regulate cell growth and differentiation and play an important role in the deterrence of tumorigenesis in normal cells.[164 165] Genetic alteration of tumour suppressor genes leads to loss of tumour suppressor function, thereby contributing to the development of cancer. Mutations may occur in both alleles, as in familial retinoblastoma; or more commonly, a mutation in one allele (loss of heterozygosity or reduction to homozygosity) is followed by loss of the second allele (double knockout). Both alleles have to be inactivated for tumour suppressor function to be lost and these mutations may occur in the germline or somatic cells. Germline mutations may arise spontaneously in the gametes or persist in a family as a dominant trait.

p53 TUMOUR SUPPRESSOR GENE

Abnormalities of p53 are probably the most common genetic abnormalities in human can-

cer.[166–168] The gene is located in the short arm of chromosome 17 and plays an important role in regulating the cell cycle, programmed cell death or apoptosis, DNA synthesis, cellular proliferation, and coordinates a complex system of response to DNA damage, hence the designation "guardian of the genome".[166 168 169] The gene protein binds to specific DNA sequences and acts as a transcription factor that positively or negatively regulates the expression of specific genes. The effect of p53 suppressor gene on gene transcription can be influenced by modifications in the gene itself, by post-transcriptional modifications such as phosphorylation and changes in physical conformation, or by interaction with other cellular proteins, such as *mdm*-2 gene protein,[170–173] and oncoviral proteins, such as adenovirus E1B protein and human papilloma virus E6 protein.[174] Nuclear exclusion and sequestration in the cytoplasm also affect the normal function of p53.[175]

Mutations of the p53 gene result in genomic instability that leads to development of cancers in diverse organs.[167 176] The most common alteration is missense mutation; other alterations include non-sense mutation, splicing mutation, and frame shift mutation. The mutations are clustered in the evolutionarily conserved regions of the p53 gene, namely exons 5 to 8, between codons 120 and 290 out of the 393 amino acid residues, with hot spots in codons at residues 175, 248, 249, 273, and 281.[177] In breast cancer, no tumour specific "hot spots" have been reported,[177] and about 25% of mutations are detected in codons 175, 194, 273, and 280.[178]

The wild type p53 protein is a nuclear phosphoprotein with a very short half life and it is expressed at low levels in normal cells. As a result, it is virtually undetectable in normal cells by immunohistochemical methods. p53 gene alterations result in various mutant p53 proteins which have in common a change in conformation and greater stability. The resulting longer half life permits their accumulation and demonstration by immunohistochemistry.[179] The mutant proteins not only lose tumour suppressor function, but can act as dominant oncogenes.[174]

It has been estimated that p53 gene mutations and protein accumulation occur in 14–58%[176 178 180–186] of invasive breast cancers. These mutations may be characterised by molecular biological techniques including direct sequencing, single strand conformation polymorphism, and polymerase chain reaction.[187–191] Alternatively, since missense mutation results in a mutant protein and accounts for the majority of p53 mutations,[177] immunohistochemistry is a satisfactory way of detecting this mutation.[189 192–194] However, it does not identify less common mutations such as deletion, non-sense, splice site, and frame shift mutations.

The majority of publications to date, most of which have used immunohistochemical staining, indicate that p53 gene mutation is an independent prognostic variable in invasive breast carcinomas.[184 195–208] In studies that did not include the clinical outcome, and even in those that failed to show a significant prognostic value, p53 gene mutation correlated with traditional adverse prognostic variables, such as nodal status, tumour size, histological grade, mitotic rate, oestrogen receptor/progesterone receptor status, ploidy, high S phase index, and *neu* oncoprotein overexpression.[180 195 209–220] However, several studies did not substantiate a significant role for p53 mutations in predicting survival in breast cancer.[211 216 221 222]

p53 protein expression is seen most often with invasive ductal and medullary carcinomas, and is uncommon in invasive lobular carcinoma and other less aggressive histological types.[205 223–225] p53 protein accumulation is also observed in in situ ductal carcinoma.[184 186 216 220 226] Concordant expression is often seen between the in situ and invasive components,[186] and between the primary tumour and metastases.[226 227] In intraductal carcinomas, p53 accumulation is seen mostly in the large cell, high nuclear grade, ER-negative, comedo type.[185 186 216 220] Interestingly, *c-erb*B-2 overexpression is also seen in this subtype of intraductal carcinoma. However, current data seem to indicate that there is no correlation between these two markers in intraductal carcinoma and that they may be expressed in different cell populations.[186 216]

Cytoplasmic p53 protein staining has been observed in a minority of breast and colon carcinomas[175 185 186 228] which may not be accompanied by a corresponding gene mutation.[175 185] The significance of such cytoplasmic staining in the absence of gene mutation is not clear, but it may represent one alternative mechanism to point mutation whereby the tumour suppressor function is compromised.

The Li-Fraumeni syndrome is a rare autosomal dominant familial syndrome in which the kindreds develop multiple cancers at a young age.[229–231] The cancers include sarcomas and adrenal cortical carcinomas during infancy, osteosarcomas in adolescence, acute leukaemia and brain tumours throughout childhood and young adulthood, premenopausal breast carcinomas, and possibly gonadal germ cell tumours. The genetic basis of this syndrome consists of the loss or inactivation of one of the alleles of p53 in the germ line, with the consequent inheritance of a single junctioning allele in all somatic cells. This leads to genomic instability which may in turn generate further and multiple genetic alterations, thereby predisposing the patient to cancer development.[232–234] Treatment of patients with this underlying genetic abnormalities by radiotherapy and chemotherapy may increase the risk of a second cancer.[233]

There is also firm evidence linking early onset familial breast cancer[235] and familial breast and ovarian cancer[236] to the putative BRCA-1 gene on chromosome 17q21.

Transfection experiments reintroducing wild-type p53 gene in human breast cancer cell lines often results in the inhibition of tumorogenicity and the growth of cancer cells.[237 238] This may have potential application for gene

therapy, based on renewal of p53 function.

One study showed that p53 dependent apoptosis modulated the cytotoxic effects of chemotherapy and radiotherapy; cells lacking wild-type p53 were resistant to these treatments while cells expressing wild-type p53 responded and underwent cell death by apoptosis.[239] These observations raise the prospect that p53 mutations may provide a genetic basis for drug resistance, and have important treatment implications if verified by clinical studies.

Circulating serum antibodies to mutant p53 protein have been detected in about 20% of breast cancer patients,[240] and these may have potential use as a tumour marker for diagnosis and for monitoring treatment and follow up of breast cancer patients. Immunotherapeutic strategies may also be developed on the basis of cytotoxic response to mutant p53 protein epitope in tumour cells.[177]

RETINOBLASTOMA SUSCEPTIBILITY SUPPRESSOR GENE

The retinoblastoma susceptibility gene was the first tumour suppressor gene to be identified. Retinoblastoma gene alterations have been reported in 22% of human breast carcinoma cell lines and in 19-28% of invasive cancers.[241-243] *Rb* gene alterations occur more frequently in advanced breast cancers and the loss of protein expression is often heterogeneous within a tumour. Furthermore, allele loss may not be correlated with loss of gene protein expression.[241] These observations suggest that *Rb* gene alteration may not be an initiating event in breast carcinogenesis but is an event associated with the disease progression.[241 242 244 245] To date, no convincing association of *Rb* gene deletion in breast cancer and prognosis has been convincingly demonstrated.[241 246-248]

THE BRCA-1 GENE AND FAMILIAL EARLY ONSET BREAST CANCER

There is now firm evidence linking a putative BRCA-1 gene, located on chromosome 17q12-21, to familial early onset breast cancer[249] and familial breast and ovarian cancers.[250] The genetic basis of how this gene confers susceptibility to early onset breast cancer and the clinical relevance remain to be elucidated.[251-260]

nm23 Anti-metastasis gene

The nm23 gene family was originally identified in the murine melanoma cell line and nm23-H1 was found to be transcribed at a 10-fold higher rate in cells of lower metastatic potential. Somatic allelic deletion of the nm23-H1 gene, carried on chromosome 17q, has been reported in human breast, renal, colorectal, and lung carcinoma. There is accumulating evidence that reduced levels of the nm23 gene product in a tumour lead to increased metastatic capacity of the tumour cells. Transfection of the nm23 gene into melanoma K1735 cells results in a reduction of metastatic tumour formation.

Several clinical studies evaluating nm23 gene expression in invasive breast carcinoma have shown that reduced expression was associated with positive lymph node status, high histological grade, and poor prognosis.[261-266] There are few studies on in situ carcinoma of the breast. In one study, loss of nm23 expression was observed in comedo type intraductal carcinoma, but not in non-comedo type.[265] Another study of carcinoma in situ reported a heterogeneous staining pattern. In pure non-invasive carcinomas, lobular and comedo ductal types of carcinoma in situ expressed more nm23 than non-comedo ductal carcinoma in situ; however, no difference was observed in the different subtypes of carcinoma in situ with concomitant invasive cancer. A greater expression of nm23 was seen in comedo ductal carcinoma in situ without concomitant invasion than comedo ductal carcinoma in situ with invasion.[267]

The observed association of reduced expression to poor prognosis is not universal. One group studied invasive breast carcinoma and other solid tumours with enzymatic assays of nucleoside diphosphate (NDP) kinase and with immunohistochemistry and western blot with antibodies to NDP kinase. These workers reported an increased NDP expression in malignant tumours compared to benign tissue, and observed no relationship between NDP kinase activity and nodal status, size, proliferative activity, hormone receptor expression, and histoprognostic index.[268 269] Another study of lung carcinomas also reported an increased expression nm23-H1 and H2 mRNA in more advanced and poorly differentiated tumours, and no prognostic correlation was observed.[270]

Tumour-host interaction factors

ANGIOGENESIS AND MICROVESSEL DENSITY

The growth and spread of neoplasms depend on their interactions with host tissue, including the vasculature, stroma, and immune system. Experimental data provide strong evidence that angiogenesis plays an important role in neoplastic progression, facilitating expansion of the primary neoplasm and increasing its proliferation rate.[271-273] In vitro studies indicate that angiogenesis is the limiting factor in tumour growth and metastasis, with inhibitors of angiogenesis limiting tumour growth.[274-276] Inhibition of vascular endothelial growth factor induced angiogenesis suppressed tumour growth and a human carcinoma cell line implanted in mice showed a twofold increase in tumour size and increased tumour vasculature when exposed to basic fibroblast growth factor (bFGF), a powerful angiogenesis promoting factor. The tumour growth was significantly retarded with administration of neutralising antisera against bFGF and since this cell line lacked bFGF receptors and did not respond to bFGF in vitro, these effects were presumably mediated by the bFGF receptors on endothelial cells, leading to angiogenesis which in turn promoted tumour growth.[277]

Angiogenesis may also play an important role in the complicated process of metastasis, during which tumour cells must gain access to the vasculature, disseminated to distant organs,

escape from the vasculature, and establish metastatic deposits in the target organs. Cancer cells rarely invade the vasculature in the absence of neovascularisation.[278] Angiogenesis may thus facilitate metastasis by presenting a larger vascular area as the target for invading cancer cells, and this process is enhanced by the fragmented, leaky nature of the new vessels.[279] Furthermore, endothelial cells of the new vessels secrete degradative enzymes such as collagenases and plasminogen activators which promote the motility of the vessels and facilitate their interaction with and invasion by tumour cells.[280]

Weidner and associates[281] performed a quantitation of intratumoral microvascular density in breast carcinomas as a measure of angiogenetic activity. By selecting the most vascular areas of a breast carcinoma (hot spots) and counting the number of microvessels in a microscopic field at 200 × magnification, using factor VIII related antigen immunostaining to accentuate the vessels, they indicated that the mean microvascular density was significantly higher in primary breast carcinomas from patients who already had developed metastases compared with those patients without metastases (microvascular density 101 versus 45 per 200 × field). They subsequently confirmed a significant relationship of microvascular density to disease-free and overall survival in both node negative and node positive patients, and showed that in node negative patients it was the most significant prognostic variable by multivariate analysis.[282] Bosari *et al*[283] confirmed that the microvascular density was significantly greater in node positive patients than in node negative patients; it was an important prognostic variable in predicting disease-free survival and overall survival and an independent variable by multivariate analysis.

Employing immunohistochemical staining for CD31, a platelet-endothelial cell adhesion molecule, to highlight the new vessels, Horak *et al*[284] showed that microvascular density was the most significant prognostic variable in predicting survival. Other studies have since confirmed the correlation of microvascular density with metastases in breast carcinomas.[285–290] While there have been reports which failed to find an association between microvascular density and prognosis in breast carcinoma, the discordance was most probably due to methodological differences. The optimal counting field area appears to be a 200 × microscopic field (or 0·74 mm^2) and the use of smaller counting areas results in greater field to field variability of vessel count; the exclusion of positively stained cells without lumen may account for the lack of correlation of microvascular density with prognosis.

CATHEPSIN D

Cancer invasion and metastasis is a complex process, and in breast cancer the tumour has to penetrate the basement membrane of the mammary duct, invade and spread through the stroma, gain access into and disseminate through blood and lymphatic vessels, find its way out of the vascular system, and implant and proliferate in distant sites. The elaboration of various proteolytic enzymes to degrade basement membrane and matrix components aids this process. Such proteolytic enzymes include urokinase-type plasminogen activator, matrix metalloproteases, and cathepsins. The expression of these enzymes and their inhibitors, such as plasminogen activator inhibitors and tissue inhibitors of metalloproteases, may have important biological significance and prognostic value.

Cathepsin D may be a potential prognostic variable in breast cancer. It is an oestrogen inducible and constitutively produced lysosomal enzyme, which functions at an acidic pH and is widely distributed in tissues. The enzyme is capable of digesting extracellular matrix and also acts as a growth factor.[291 292] It may therefore play a role in determining tumour invasiveness and proliferative activity and it is tempting to speculate that tumours which elaborate a high content of cathepsin D may have a more aggressive behaviour because of these properties. The results, however, have been discordant on the relation of raised cathepsin D levels to other indices of prognosis in breast cancer such as nodal status, tumour size and histological grade. However, most studies indicate that cathepsin D levels are independent of oestrogen receptor and progesterone receptor status.[293–296]

MATRIX MATELLOPROTEASES

The matrix matelloproteases comprise a family of genes that share structural and functional similarities, the most important of which is the ability to degrade one or more of the molecules that make up the extracellular matrix. The major matrix matelloproteases related to cancer invasion and metastasis include type IV collagenase or matrix matelloprotease-2 (MMP-2), matrix matelloprotease-9 (MMP-9), stromelysin-1 (MMP-3), and stromelysin-2 (MMP-10). The enzyme collagenase type IV (MMP-2) degrades type IV collagen, the major structural protein component of basement membranes. MMP-2 can also degrade elastase and gelatin which form the major structural collagens of the interstitial matrix. In a study of 55 breast samples, 20 of 23 cases of intraduct carcinoma showed positive staining for MMP-2.[297] Thirty six of 40 cases of invasive carcinoma showed positive staining, and metastatic cells in lymph nodes were positive in 10 of 12 cases. Immunohistochemical evaluation of the protein indicated an increasing intensity and percentage of positive staining with increasing stage/depth of invasion in cases of breast, colonic, gastric, and hepatocellular carcinoma.[298 299] In another study of 187 cases of node negative breast cancer, MMP-2 and laminin receptor levels were related in 52% of the cases, although MMP-2 did not appear related to size, proliferation, histological grade, or hormonal status. Neither disease-free survival nor overall survival at six years were significantly associated with tumour levels of MMP-2, but high levels of MMP-2 were related

to increased local-regional recurrence rather than distant metastases.[300] In situ hybridisation studies have shown that MMP-2 is synthesised in the stromal fibroblasts surrounding invading nests of tumour,[301] although immunohistochemical staining for the protein was observed in only some and not all fibroblasts,[297] suggesting that the stromal cells may contribute proteolytic activity which enhances the ability of tumour cells to invade.

PLASMINOGEN ACTIVATORS

Urokinase plasminogen activator (uPA) is secreted as a pro-enzyme which binds to specific, localisable receptors on the cell surface where it is cleaved to an active form. Tumour cells transfected with the uPA sense gene showed an increased ability to metastasise, whereas in cells transfected with antisense to prepro-uPA, a significant decrease in metastasis was found.[302] When evaluated in 166 breast cancer patients, uPA was found to be an independent prognostic marker.[303] The relative risk of disease recurrence in patients with high as opposed to low levels of uPA was 4:1. The relative risk of death was 8:76 and in a cohort of 115 patients, uPA was found to distinguish high and low risk patients with either node positive or node negative tumours.[304] uPA may be useful as a predictor of early relapse, as multivariant analysis of hormone status, nodal status, and vascular invasion showed the relative risk of disease-free survival for uPA was 21·1 at 12·5 months, falling to 3·4 at 25 months.[304] uPA and PAI-1 (plasminogen activator inhibitor) predict poor prognosis. When 113 of 115 patients were analysed, PAI-1 was higher in tumour than in benign tissues. PAI-1 correlated with uPA in the breast cancers, but despite this, in multivariant analysis both PAI-1 and uPA were found to be independent prognostic factors. Furthermore, those patients with high levels of both proteins carried the maximum risk.[305] It has been suggested that PAI-1 has a role in protecting angiogenesis, thus causing it to act in a manner that enhances tumour growth. In contrast, tissue plasminogen activator (tPA), although sharing many features of uPA, is oestrogen induced in breast cancer and therefore serves as a marker of an intact oestrogen receptor pathway, similar to pS2. Accordingly, high levels of the protein have been shown to be a good prognostic indicator.[303]

ADHESION MOLECULES

Cellular adhesions consist of two basic types: focal adhesions, and hemi-desmosomes or larger adhesions. Coupled with the action of proteolytic enzymes, adhesions become a dynamic process by which migration occurs. Specific interactions with blood protein fragments create cellular emboli and the interaction of proteases and adhesion proteins; a co-operative, regulated process of signal transduction through receptors for adhesion glycoprotein stimulates protease gene expression. Important molecules for cell adhesion include the non-collagenous glycoproteins (fibronectin, vitronectin, thrombospondin, laminin, von Willebrand's factor, entactin, and tenascin), the collagens, glycosaminoglycans, proteoglycans, cell adhesion receptors (laminin receptor, cadherins, selectins, CD44, and cell adhesion molecules), and the family of integrins.[306] Loss of integrin α-2-β-1 is associated with poorly differentiated breast carcinoma, and decreased α-6-β-1 and increased α-v-β-3 are also associated with poorly differentiated breast carcinoma.[307] Some breast cancer cells have a laminin receptor which may play a role in the attachment and invasion of primary tumours and metastases. Of 187 breast cancer cases, the presence of laminin receptor did not correlate with survival, but correlated with an increased risk of local-regional recurrence.[300] In another study of 235 breast cancers, patients with tumours expressing laminin receptor had 40% less risk of recurrence, but this did not approach statistical significance.[308]

Drug resistance

One of the most perplexing problems in chemotherapy is the resistance of certain tumours to all chemotherapeutic regimens, while other tumours start off being chemosensitive to a particular agent but show resistance to treatment over time and with tumour progression. Tumour cells which become resistant to one drug often show cross resistance to a wide variety of other structurally unrelated drugs. For example, a cell resistant to doxorubicin can show cross resistance to diverse drugs to which it had never been exposed, including *VINCA* alkaloids and mitomycin C, but not to other drugs such as alkylating agents. This phenomenon of multidrug resistance (mdr) is associated with overexpression of a 170 kDa protein known as the P glycoprotein, the product of the *mdr-1* gene. P glycoprotein is a transmembrane protein which has been termed a "drug efflux pump", although its precise function has not been clarified. The protein can be detected with immunohistochemical stains and amplification or increased expression of *mdr* genes that encode the glycoprotein can be assessed by standard genetic techniques. When expressed, the transmembrane protein is an active transporter that removes cellular toxins and drugs, and the presence of amplified *mdr* genes or of P glycoprotein correlates well with resistance to these agents. There is low expression of P glycoprotein in primary breast cancer, but increased expression is seen in tumours treated with anthracyclines. This effect is probably due to selection of drug resistant cells during treatment and is likely to be clinically irrelevant. The expression or amplification of *mdr* genes in association with the natural progression of tumours in patients not undergoing chemotherapy has also recently been described.[309]

The highly conserved heat shock proteins (hsp) have been reported to be increased in breast cancer cell lines and clinical cases. In a series of 200 node negative tumours analysed for all three heat shock proteins, a significantly

shorter relapse-free survival was found in relation to increasingly raised concentrations of heat shock proteins. The five year actuarial recurrence increased from 24% to 80% as the number of high level heat shock proteins increased from 0 to 4 (McGuire WL, Tandon A, Allred DC, unpublished data). In another study, analysis of 385 node negative breast cancer specimens showed that high levels of hsp27 and low levels of oestrogen were independent significant prognosticators of shortened disease-free survival.[310] Heat shock therapy induces doxorubicin resistance in MDA-MB-234 cells[311] and transfection of hsp27 cDNA into cell lines increases hsp27 expression, producing a twofold increase in growth rate of colonies overall and a fourfold increase in soft agar colonies. The transfection produces a 2·5-fold increase in resistance to doxorubicin compared to controls.

Conclusions

Breast cancer is a complex disease which has posed many perplexing clinical problems. Despite improvements in diagnosis and treatment, approximately one third of patients with breast cancer continue to die from their disease, a situation which has not changed very much in the last 50 years. One third of node negative breast cancer patients develop recurrence and metastases from their disease, making it necessary to attempt to identify this group of patients who require adjuvant chemotherapy. It is also becoming increasingly apparent that breast cancer is not a biologically homogeneous disease, and multiple alterations from normal mammary cells are required to achieve a transformed phenotype. Furthermore, as there may be several possible alterations that will produce the transformations leading to the malignant state, the identification of specific sets of common alterations within a given cancer may provide necessary information as to its uniqueness and how it may best be treated.

Currently, traditional morphological indices such as nodal status, tumour size, histological type, and tumour grade remain unsurpassed as prognostic indicators in breast cancer, but there is strong evidence that these indices are inadequate to predict the behaviour and response to treatment of a significant percentage of such tumours. While oestrogen receptor and progesterone receptor are proven indices for predicting response to hormonal therapy, several newer biological markers of breast cancer may also provide specific treatment information. For example, *erb*B-2 may predict for improved response to doxorubicin, rather than CMF (cyclophospamide-methotrexate-5-fluorouracil); hsp27 may predict for failure of doxorubicin and pS2; or epidermal growth factor receptor may provide supplementary information predicting response to hormonal therapy. The cell proliferation markers and oncoproteins associated with cell cycle regulation have shown strong promise of providing important prognostic information, and studies to date indicate that these markers are independent of one another. The advent of immunohistochemical methods to measure most of these markers make retrospective as well as prospective studies possible. The area of tumour-stromal interaction provides numerous potentially important markers of cancer prognosis and they include growth factors, cathepsin D, nm23, and angiogenesis, all of which have been shown in small cohorts to have prognostic importance. Even if biological markers such as these cannot be applied to develop new treatments, their identification and quantitation may be relevance for prognosis. The need for proper validation of these markers has been emphasised by McGuire[3] and the influence of time after diagnosis on the relative importance of various prognostic factors has been demonstrated by Lipponen *et al.*[4] For example, tumour diameter, axillary lymph node status, tubule formation, and a fraction of intraductal growth were shown to be of prognostic value up to five years, mitotic index was of prognostic significance in the first two years, but histological grade and morphometric nuclear factors had short term value only. Such time dependent variation may help to explain discrepant results from different studies investigating the value of possible prognostic indicators.

1 Parkin DM, Läärä E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers in 1980. *Int J Cancer* 1988;41:4–97.
2 Riggan WB, van Bruggon JA, Acquavella JF. *US cancer mortality rates and trends, 1950–1979.* Washington, DC: US Government Printing Office, 1993.
3 McGuire WL. Breast cancer prognostic factors: evaluation guidelines. *J Natl Cancer Inst* 1990;**83**:154–5.
4 Lipponen P, Aaltomaa S, Eskelinen M. The changing importance of prognostic factors in breast cancer during long term follow-up. *Int J Cancer* 1992;**51**:698–702.
5 Glick JH, Gelber RD, Goldhirsch A, Seen H-J. Meeting highlights: adjuvant therapy for primary breast cancer. *J Natl Cancer Inst* 1992;**19**:1479–85.
6 Bonnadonna G. Conceptual and practical advances in the management of breast cancer. *J Clin Oncol* 1989;7: 1380–7.
7 Fisher ER, Sass R, Fisher B. Pathologic findings from the NSABP. Discriminants for 10-year treatment failure. *Cancer* 1984;**53**:712–20.
8 Osborne CK, Yochmowitz MG, Knight WA, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 1980;**46**:2884–8.
9 Hahnel R, Woodings T, Vivian AB. Prognostic value of oestrogen receptors in primary beast cancer. *Cancer* 1979; **44**:671–5.
10 Raemaekers JMM, Beex LVAM, Koenders AJM. Disease-free interval and oestrogen receptor activity in tumour tissue of patients with primary breast cancer: analysis after long-term follow-up. *Breast Cancer Res Treat* 1985; **6**:123–30.
11 Spyratos F, Hacene K, Tubiana-Hulin M. Prognostic value of oestrogen and progesterone receptors in primary infiltrating ductal breast cancer. A sequential multivariate analysis of 1262 patients. *Eur J Cancer Clin Oncol* 1989; **25**:1233–40.
12 Battifora H. *Estrogen receptor and progestogen receptor in breast cancer prognosis: the role of immunohistochemistry.* Arthur Purdie-Stout Society of Surgical Pathologists Handout, March 13, 1994.
13 Leong AS-Y, Milios J. Comparison of antibodies to estrogen and progesterone receptors and the influence of microwave-antigen retrieval. *Appl Immunohistochem* 1993; **1**:282–8.
14 Law ML, Kaof FT, Wei Q. The progesterone receptor gene maps to human chromosome band 11q13, the site of mammary oncogene *int-2*. *Proc Natl Acad Sci USA* 1987;**84**:2877–81.
15 Ponglikitmongkol M, Green S, Chambon P. Genomic organisation of the human estrogen receptor gene. *EMBO J* 1988;**7**:3385–8.
16 Picard D, Kumar V, Chambon P, Yamamoto KR. Signal transduction by steroid hormones: nuclear localisation is differentially regulated in estrogen and glucocorticoid receptors. *Cell Regul* 1990;**1**:291–9.
17 Fuller PJ. The steroid receptor super-family: mechanisms of diversity. *FASEB J* 1991;5:3092–9.
18 Hard T, Kellenbach E, Boelens R. Solution structure of glucocorticoid receptor DNA-binding domain. *Science* 1990;**249**:157–60.
19 Schwabe JWR, Newhause D, Rhodes D. Solution structure of the DNA-binding domain of oestrogen receptor. *Nature* 1990;**348**:458–61.

20 Tora L, White J, Brou C. The human estrogen receptor has two independent non-acidic transcriptional activation functions. *Cell* 1989;**59**:477–87.
21 Fisher B, Redmon DC, Fisher ER. Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: findings from national surgical adjuvant breast and bowel project protocol B-06. *J Clin Oncol* 1988;**6**:1076–87.
22 Chevallier B, Heintzman F, Mosseri V. Prognostic value of estrogen and progesterone receptors in operable breast cancer. *Cancer* 1988;**62**:2517–24.
23 Brocklehurst D, Wilde CE, Finbow JAH. Relative importance of estrogen and progesterone receptor assays as prognostic indicators in primary breast cancer: a short-term study. *Clin Chem* 1989;**35**:238–40.
24 Sigurdsson H, Baldetorp B, Borg A. Indicators of prognosis in node negative breast cancer. *N Engl J Med* 1990;**322**: 1045–53.
25 Mason BH, Holdaway KM, Mullins progesterone receptor. Progesterone and estrogen receptors as prognostic variables in breast cancer. *Cancer Res* 1983;**43**:2985–90.
26 Thorpe SM, Rose C, Rasmussen BB. Prognostic value of steroid hormone receptors: multivariate analysis of systemically untreated patients with node negative primary breast cancer. *Cancer Res* 1987;**47**:6125–33.
27 McCarty KS, Barton TK, Fetter BF. Correlation of estrogen and progesterone receptors with histologic differentiation in mammary carcinoma. *Cancer* 1980;**46**: 2851–58.
28 Mohammed RH, Lakatua DJ, Haus E, Yasmineh WJ. Estrogen and progesterone receptors in human breast cancer: correlation with histologic subtype and degree of differentiation. *Cancer* 1986;**58**:1076–81.
29 Courdi A, Heri M, Dahan E. Factors affecting relapse in node negative breast cancer: a multivariate analysis including the labelling index. *Eur J Cancer Clin Oncol* 1989;**25**:351–6.
30 Raymond WA, Leong AS-Y. The relationship between growth fractions and oestrogen receptors in human breast carcinoma, as determined by immunohistochemical staining. *J Pathol* 1989;**158**:203–11.
31 Raymond WA, Leong AS-Y. Silver stained nucleolar organiser regions in human breast carcinoma related to tumour growth fraction, as determined by Ki-67 immunostaining. *Hum Pathol* 1989;**20**:741–6.
32 Heintz NH, Leslie KO, Rogers LA, Howard PL. Amplification of the *c-erb*B-2 oncogene and prognosis of breast adenocarcinoma. *Arch Pathol Lab Med* 1990;**114**: 160–63.
33 Marx D, Schauer A, Reiche C. *c-erb*B-2 expression and correlation to other biological parameters of breast cancer. *J Cancer Res Clin Oncol* 1990;**116**:15–20.
34 Silvestrini R, Diadone MG, Di Fronzo G. Relationship between proliferative activity and estrogen receptors in breast cancer. *Cancer* 1979;**44**:665–70.
35 Klute TE, Muss HB, Anderson D. Relationship of steroid receptor, cell kinetics and clinical status in patients with breast cancer. *Cancer Res* 1981;**41**:3524–9.
36 Stanton PD, Cooke TG, Oakes SJ. Lack of prognostic significance of DNA ploidy and S- phase fraction in breast cancer. *Br J Cancer* 1992;**66**:925–9.
37 Brifford M, Spyratos F, Tubiana-Hulin M. Sequential cytopunctures during pre-operative chemotherapy for primary breast carcinoma. Cytomorphic changes, initial tumour ploidy and tumour regression. *Cancer* 1989;**63**: 631–7.
38 Remvikos Y, Beuzeboc P, Zajdela A. Correlation of pretreatment proliferative activity of breast cancer with the response to cytotoxic chemotherapy. *J Natl Cancer Inst* 1989;**81**:1383–7.
39 Meyer JS, Province M. Proliferative index of breast carcinoma by thymidine labelling: prognostic power independent of stage, oestrogen and progesterone receptors. *Breast Cancer Res Treat* 1988;**12**:191–204.
40 Silvestrini R, Diadone MG, Di Fronzo G. Prognostic implication of labelling index versus estrogen receptors and tumour size in node negative breast cancer. *Breast Cancer Res Treat* 1986;7:161–9.
41 Tubiana M, Pejovic MH, Koscielny S. Growth rate, kinetics of tumour cell proliferation and long-term outcome in human breast cancer. *Int J Cancer* 1989;**44**:17–22.
42 Gerdes J, Lemke H, Baisch H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984;**133**: 1710–5.
43 Leong AS-Y, Raymond WA. Prognostic parameters in breast carcinoma. *Pathology* 1989;**21**:169–75.
44 Veronese SM, Gambacorta M. Detection of Ki-67 proliferation rate in breast cancer. Correlation with clinical and pathologic features. *Am J Clin Pathol* 1991;**95**:30–4.
45 Kamel OW, Franklin WA, Ringus JC. Thymidine labelling index and Ki-67 growth fraction in lesions of the breast. *Am J Pathol* 1990;**134**:107–13.
46 Sahih AA, Ro J, Ro JY. Ki-67 immunostaining in node negative stage I/II breast carcinoma. Significant correlation with prognosis. *Cancer* 1991;**68**:549–7.
47 Wintzer HO, Zipfel I, Schulte-Monting J. Ki-67 immunostaining in breast tumours and its relationship to prognosis. *Cancer* 1991;**67**:421–8.
48 Viehl P, Chevillard S, Mosseri V. Ki-67 index and S-phase fraction in human breast carcinomas. Comparison and correlations with prognostic factors. *Am J Clin Pathol* 1990;**94**:681–6.
49 Garcia RL, Coltrera MD, Gown AM. Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. *Am J Pathol* 1989;**134**: 733–8.
50 Leong AS-Y, Milios J, Tang SK. Is immunolocalisation of proliferating nuclear antigen (PCNA) in paraffin sections a valid index of cell proliferation? *Appl Immunohistochem* 1993;**1**:127–35.
51 Leong AS-Y, Vinyuvat S, Suthipintawong C, Milios J. A comparative study of cell proliferation markers in breast carcinomas. *J Clin Pathol: Mol Pathol* 1995;**48**:M83–7.
52 Mourad WA, Erkman-Balis B, Livingston S. Argyrophilic nucleolar organiser regions in breast carcinoma: correlation with DNA flow cytometry, histopathology, and lymph node status. *Cancer* 1992;**69**:1739–44.
53 Freeman JW, Busch RK, Gyorkey F. Identification and characterisation of a human proliferation-associated nucleolar antigen with a molecular weight of 120 000 expressed in early g_1 phase. *Cancer Res* 1988;**48**:1244–51.
54 Freeman JW, McGrath P, Bondada V. Prognostic significance of proliferation-associated nucleolar antigen P120 in human breast carcinomas. *Cancer Res* 1991;**51**: 1973–8.
55 Heck MMS, Ernshaw WC. Topoisomerase II: a specific marker for cell proliferation. *J Cell Biol* 1986;**103**:2569–81.
56 Chou MY, Chang AL, McBride J. A rapid method to determine proliferation patterns of normal and malignant tissues by H3 mRNA in situ hybridisation. *Am J Pathol* 1990;**136**:729–33.
57 Cattoretti G, Becker MHG, Key G. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB-1 and MIB-3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* 1992; **168**:357–63.
58 Kreipe H, Wacker H-H, Heidebrecht HJ. Determination of the growth fraction in non-Hodgkin's lymphomas by monoclonal antibody Ki-S5 directed against a formalin-resistant epitope of the Ki-67 antigen. *Am J Pathol* 1993; **142**:1689–94.
59 Clark GM, Dressler LG, Owens MA, Pounds G, Oldaker T, McGuire WL. Prediction of relapse or survival in patients with node negative breast cancer by DNA flow cytometry. *N Engl J Med* 1989;**320**:627–33.
60 O'Reilly SM, Camplejogn RS, Barnes DM. DNA index, S-phase fraction, histological grade and prognosis in breast cancer. *Br J Cancer* 1990;**61**:671–4.
61 McGuire WL, Clark GM. Prognostic factors and treatment decision in axillary node negative breast cancer. *N Engl J Med* 1992;**326**:1756–61.
62 O'Reilly SM, Richards MA. Is DNA flow cytometry a useful investigation in breast cancer? *Eur J Cancer* 1992; **28**:504–7.
63 Hedley DW, Rugg CA, Gelber RD. Association of DNA index and S-phase fraction with prognosis of node positive early breast cancer. *Cancer Res* 1987;**47**:4729–35.
64 Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell* 1990;**61**:203–12.
65 Majerus PW, Ross TS, Cunningham TW. Recent insights into phosphatidylinositol signalling. *Cell* 1990;**63**:459–61.
66 Matsui Y, Halter SA, Holt JT. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. *Cell* 1990;**61**:1147–52.
67 Rios MA, Macios A, Perez R, Lage A, Skorg L. Receptors for epidermal growth factor and estrogen as predictors of relapse in patients with mammary carcinoma. *Anticancer Res* 1988;**8**:173–6.
68 Sainsbury JRC, Farndon IR, Needham GK, Malcolm A, Harris AL. Epidermal growth factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet* 1987;i:1398–402.
69 Klijn JG, Berns PM, Schmitz PI, Foekens JA. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr Rev* 1992;**13**:3–17.
70 Koenders PG, Beex LV, Kienhuis CB, Kloppenborg PW, Benraad TJ. Epidermal growth factor receptor and prognosis in human breast cancer: a prospective study. *Breast Cancer Res Treat* 1993;**25**:21–7.
71 Lewis S, Locker A, Todd JH, Bell JA, Nicholson R, Elston CW. Expression of epidermal growth factor receptor in breast carcinoma. *J Clin Pathol* 1990;**43**:385–9.
72 Nicholson RI, McClelland RA, Finlay P, Eaton CL, Gullick WJ. Relationship between EGF-R, c-erbB-2 protein expression and Ki-67 immunostaining in breast cancer and hormone sensitivity. *Eur J Cancer* 1993;**29**:1018–23.
73 Foekens JA, van Putten WI, Portengen J, Rodenburg CJ, Reubi JC. Prognostic value of p53 protein and receptors for epidermal growth factor (EGR-R), insulin-like growth factor-1 (IGF-1-R) and somatostatin (SS-R) in patients with breast and ovarian cancer. *J Steroid Biochem Mol Biol* 1990;**37**:815–21.
74 Spyratos F, Delarue JC, Andrieu R, Champeme MH, Hacene K. Epidermal growth factor receptors and prognosis in primary breast cancer. *Breast Cancer Res Treat* 1990;**17**:83–9.
75 Harris AL, Nicholson S. Epidermal growth factor receptors in human breast cancer. *Cancer Treat Res* 1988;**40**:93–118.
76 Ro J, North SM, Gullick GE, Hortobagyi GN, Gutterman JU, Blick MC. Amplified and over-expressed epidermal growth factor receptor gene in uncultured primary human breast carcinoma. *Cancer Res* 1988;**48**:161–4.
77 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987;**235**:177–82.

78 Horiguchi J, Iino Y, Takei H, Yakoe T, Ishida T. Immunohistochemical study on the expression of c-*erb*B-2 oncoprotein in breast cancer. *Oncology* 1994;**51**:47–51.
79 Lupu R, Colomer R, Kannan B, Lippman ME. Characterization of a growth factor that binds exclusively to the *erb*B-2 receptor and induces cellular responses. *Proc Natl Acad Sci USA* 1992;**89**:2287–91.
80 Peles E, Bacus SS, Koski RA. Yarden J. Isolation of the *neu*/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* 1992;**69**:205–16.
81 Sarkar FH, Smith MR, Hoover R, Princler G, Crissman JD. c-*erb*B-2 promoter-specific DNA-binding protein isolated from human breast cancer tissues displays mitogenic activity. *J Biol Chem* 1994;**269**:12285–9.
82 Wen D, Peles E, Cupples R, Yarden J. *Neu* differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* 1992;**69**:559–72.
83 Gramlich TL, Cohen C, Fritsch C, DeRose PB, Gansler T. Evaluation of c-*erb*B-2 amplification in breast carcinoma by differential polymerase chain reaction. *Am J Clin Pathol* 1994;**101**:493–9.
84 Naber SP, Tsutsumi Y, Yin S, Zolnay SA, Mobtaker H. Strategies for the analysis of oncogene overexpression: studies of the *neu* oncogene in breast carcinoma. *Am J Clin Pathol* 1990;**94**:125–36.
85 Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science* 1989;**44**:707–12.
86 Venter DJ, Kumar S, Tuzi NL, Gullick WJ. Overexpression of the c-*erb* B-2 oncoprotein in human breast carcinomas: Immunohistological assessment correlates with gene amplification. *Lancet* 1987;ii:69–72.
87 Sestini R, Orlando C, Zentilin L, Gelmini S, Pinzani P. Measuring c-*erb*B-2 oncogene amplification in fresh and paraffin-embedded tumors by competitive polymerase chain reaction. *Clin Chem* 1994;**40**:630–6.
88 Loda M. Polymerase chain reaction-based methods for the detection of mutations in oncogenes and tumor suppressor genes. *Hum Pathol* 1994;**25**:564–71.
89 Smith KL, Robbins PD, Dawkins HJS, Papadimitriou JM, Redmond SL. C-*erb*B-2 amplification in breast cancer: detection in formalin-fixed, paraffin-embedded tissue by in situ hybridization. *Hum Pathol* 1994;**25**:413–8.
90 Kallioniemi O-P, Kallioniemi A, Kurisu W, Thor A, Chen L-C. *ERBB2* amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 1992;**89**:5321–5.
91 Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/*neu* antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res* 1994;**54**:2771–7.
92 Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2/neu oncogene protein and prognosis in breast cancer. *J Clin Oncol* 1989;7:1120–8.
93 Li BD, Harlow SP, Budnick RM, Sheedy DL, Stewart CC. Detection of HER-2/neu oncogene amplification in flow cytometry-sorted breast ductal cells by competitive polymerase chain reaction. *Cancer* 1994;**73**:2771–8.
94 Adenine J, Gaudray P, Simon M-P, Simony-Lafontaine J, Jeanteur P, Theillet C. Proto-oncogene amplification and human breast tumor phenotype. *Oncogene* 1989;**4**:1389–95.
95 Bacus S, Bacus J, Slamon DJ, Press MF. HER-2/*neu* oncogene expression and DNA ploidy analysis in breast cancer. *Arch Pathol Lab Med* 1990;**114**:164–9.
96 Bacus SS, Ruby SG, Weinberg DS, Chin D, Ortiz R, Bacus JW. HER-2/*neu* oncogene expression and proliferation in breast cancers. *Am J Pathol* 1990;**137**:103–11.
97 Barbareschi M, Leonardi E, Mauri FA, Serio G, Palma PD. p53 and c-*erb*B-2 protein expression in breast carcinomas. An immunohistochemical study including correlations with receptor status, proliferation markers, and clinical stage in human cancer. *Am J Clin Pathol* 1992;**98**:408–18.
98 Berns EM, Klijn JG, van Staveren IL, Portengen H, Noordegraaf E, Foekens JA. Prevalence of amplification of the oncogenes c-*myc*, HER2/*neu*, and *int*-2 in one thousand human breast tumors: correlation with steroid receptors. *Eur J Cancer* 1992;**28**:697–700.
99 Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD. Correlation of c-*erb*B-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res* 1988;**48**:1238–43.
100 Borg A, Baldetorp B, Ferno M, Killander D, Olsson H, Sigurdsson H. *erb* B2 amplification in breast cancer with a high rate of proliferation. *Oncogene* 1991;**6**:137–43.
101 Ciocca DR, Fujimura FK, Tandon AK, Clark GM, Mark C. Correlation of HER-2/*neu* amplification with expression and with other prognostic factors in 1103 breast cancers. *J Natl Cancer Inst* 1992;**84**:1279–82.
102 Cline MJ, Battifora H, Yokota J. Proto-oncogene abnormalities in human breast cancer: Correlations with anatomic features and clinical course. *J Clin Oncol* 1987; 5:999–1006.
103 dePotter CR. Beghin C, Marak AP, Vandekerokmove D, Roels HJ. The *neu* oncogene protein as a predictive factor for haematogenous metastases in breast cancer patients. *Int J Cancer* 1990;**45**:55–8.
104 Porter PL, Garcia R, Moe R, Corwin DJ, Gown AM. c-*erb*B-2 oncogene protein in in situ and invasive lobular breast neoplasia. *Cancer* 1991;**68**:331–4.
105 Soomro S, Shousha S, Taylor P, Shepard JM, Feldmann M. c-*erb*B-2 expression in different histological types of invasive breast carcinoma. *J Clin Pathol* 1991;**44**:11–14.
106 Somerville JE, Clarke LA, Biggart JD. c-*erb*B-2 overexpression and histological type of in situ and invasive breast carcinoma. *J Clin Pathol* 1992;**45**:16–20.
107 Kobayashi S, Iwase H, Itoh Y, Fukuoka H, Yamashita H. Estrogen receptor, c-*erb*-2 and nm23/NDP kinase expression in the intraductal and invasive components of human breast cancers. *Jpn J Cancer Res* 1992;**83**:859–65.
108 Liu E, Thor A, He M, Barcos M, Ljung BM, Benz C. The HER2(c-*erb*B-2) oncogene is frequently amplified in in situ carcinomas of the breast. *Oncogene* 1992;7: 1027–32.
109 Lodato RF, Maguire HC, Greene MI, Weiner DB, Livolsi VA. Immunohistochemical evaluation of c-*erb*B-2 oncogene expression on ductal carcinoma in situ ductal hyperplasia of the breast. *Mol Pathol* 1990;**3**:449–54.
110 Maguire HC, Hellman ME, Greene MI, Yeh I. Expression of c-*erb*B-2 in in situ and in adjacent invasive ductal adenocarcinomas of the female breast. *Pathobiology* 1992; **60**:117–21.
111 Van de Vijver MJ, Peterse JL, Mooi WJ, Wisman P, Lomans J. *Neu*-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. *N Engl J Med* 1988;**319**:1239–45.
112 Ramachandra S, Machin L, Ashley S, Monaghan P, Gusterson BA. Immunohistochemical distribution of c-*erb*B-2 in in situ breast carcinoma—a detailed morphological analysis. *J Pathol* 1990;**161**:7–14.
113 Wilbur DC, Barrows GH. Estrogen and progesterone receptor and c-*erb*B-2 oncoprotein analysis in pure in situ breast carcinoma: an immunohistochemical study. *Mod Pathol* 1993;**6**:114–20.
114 Barnes DM, Bartkova J, Camplejohn RS, Gullick WJ, Smith PJ, Millis RR. Overexpression of the c-*erb* B-2 oncoprotein: why does this occur more frequently in ductal carcinoma in situ than in invasive mammary carcinoma and is this of prognostic significance? *Eur J Cancer* 1992;**28**:644–8.
115 Allred DC, Clark GM, Molina R, Tandon AK, Schnitt S. Overexpression of HER-2/*neu* and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. *Hum Pathol* 1992;**23**: 974–9.
116 Allred DC, Clark G, Tandon AK, Molina R, Tormey DC. HER-2/neu in node negative breast cancer: Prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J Clin Oncol* 1992;**10**:599–605.
117 Iglehart JD, Kraus MH, Langton BC, Huper G, Kerns BJ, Marks JR. Increased *erb*B-2 gene copies and expression in multiple stages of breast cancer. *Cancer Res* 1990;**50**: 6701–7.
118 Slamon DJ, Clark GM, Wong SG. Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987;**235**:177–82.
119 Osaki A, Toi M, Yamada H, Kawami H, Kuroi K, Toge T. Prognostic significance of co-expression of c-*erb*B-2 oncoprotein and epidermal growth factor receptor in breast cancer patients. *Am J Surg* 1992;**164**:323–6.
120 Tervahauta A, Eskelinen M, Syrjanen S, Lipponen P, Pajarinen P, Syrjanen K. Immunohistochemical demonstration of c-*erb*B-2 oncoprotein expression in female breast cancer and its prognostic significance. *Anticancer Res* 1991;**11**:1677–81.
121 Tetu B, Brisson J. Prognostic significance of HER-2/*neu* oncoprotein expression in node positive breast cancer. *Cancer* 1994;**73**:2359–65.
122 Thor AD, Schwartz LH, Koerner FC, Edgerton SM, Skates SJ. Analysis of c-*erb* B-2 expression in breast carcinomas with clinical follow-up. *Cancer Res* 1989;**49**: 7147–52.
123 Toikkanen S, Helin H, Isola J, Joensuu H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30 year follow-up. *J Clin Oncol* 1992;**10**:1044–8.
124 Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S. Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: *hst-l/int-2* and *c-erbB-2/ear-1*. *Cancer Res* 1989;**9**:3104–8.
125 Varley JM, Swallow JW, Breammar WJ. Alterations to either c-*erb*B-2 or c-*myc* proto-oncogenes in breast carcinomas correlate with poor short term prognosis. *Oncogene* 1987; **1**:423–30.
126 Walker RA, Gullick WJ, Varley JM. An evaluation of immunoreactivity for c-*erb* B-2 protein as a marker of poor short-term prognosis in breast cancer. *Br J Cancer* 1989;**60**:426–9.
127 Yamashita H, Kobayashi S, Iwase H, Itoh Y, Kuzushima T. Analysis of oncogenes and tumor suppressor genes in human breast cancer. *Jpn J Cancer Res* 1993;**84**:871–8.
128 Zhou DJ, Ahuja H, Cline MJ. Proto-oncogene abnormalities in human breast cancer: c-*erb*B-2 amplification does not correlate with recurrence of disease. *Oncogene* 1989;**4**:105–8.
129 Gullick WJ, Love SB, Wright C. c-*erb*B-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br J Cancer* 1991; **63**:434–8.
130 Barnes DM, Lammie GA, Millis RR, Gullick WL, Allen DS, Altman DG. An immunohistochemical evaluation of c-*erb*B-2 expression in human breast carcinoma. *Br J Cancer* 1988;**58**:448–52.
131 Gusterson BA, Gelber RD, Goldhirsch A. The In-

ternational (Ludwig) Breast Cancer Study Group. Prognostic importance of c-*erb*B-2 expression in breast cancer. *J Clin Oncol* 1992;**10**:1049–56.
132 Wright C, Angus B, Nicholson S, Sainsbury RC, Cairns J. Expression of c-*erb*B-2 oncoprotein: a prognostic indicator in human breast cancer. *Cancer Res* 1989;**49**: 2087–90.
133 Bianchi S, Paglierani M, Zampi G, Cardona G, Cataliotti L. Prognostic significance of c-*erb*B-2 expression in node negative breast cancer. *Br J Cancer* 1993;**67**:625–9.
134 Borg A, Tandon AK, Sigurdsson H, Clark GM, Ferno Met. HER-2/*neu* amplification predicts poor survival in node positive breast cancer. *Cancer Res* 1990;**50**:4332–7.
135 Clark GM, McGuire WL. Follow-up study of HER-2/*neu* amplification in primary breast cancer. *Cancer Res* 1991; **51**:944–8.
136 Gasparini G, Gullick WJ, Maluta S, Palma PD, Caffo O. c-*erb*B-3 and c-*erb*B-2 protein expression in node negative breast carcinoma—an immunocytochemical study. *Eur J Cancer* 1994;**30**:16–22.
137 Kallioniemi O-P, Holli K, Visakorpi T, Koivula T, Helin HH, Isola JJ. Association of c-*erb*B-2 oncogene overexpression with high rate of cell proliferation, increased risk for visceral metastasis and poor long-term survival in breast cancer. *Int J Cancer* 1991;**49**:650–5.
138 Lovekin C, Ellis IO, Locker A. c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. *Br J Cancer* 1991;**63**:439–43.
139 Marks JR, Humphrey PA, Wu K, Berry D, Bandarenko N. Overexpression of p53 and HER-2/*neu* proteins as prognostic makers in early stage breast cancer. *Ann Surg* 1994;**219**:332–41.
140 O'Reilly SM, Barnes DM, Camplejohn RS, Bartkova J, Gregory WM, Richards MA. The relationship between *c-erb* B-2 expression, S-phase fraction and prognosis in breast cancer. *Br J Cancer* 1991;**63**:444–6.
141 Ottestad L, Andersen TI, Nesland JM, Skrede M, Tveit KM. Amplification of c-*erb*B-2, *int*-2 and c-*myc* genes in node negative breast carcinoma. Relationship to prognosis. *Acta Oncol* 1993;**32**:289–94.
142 Paik S, Hazan R, Fisher ER, Sass RE, Fisher B, Redmond C. Pathologic findings from the national surgical adjuvant breast and bowel project: prognostic significance of *erb* B-2 protein over-expression in primary breast cancer. *J Clin Oncol* 1990;**8**:103–12.
143 Paterson MC, Dietrich KD, Danyluk J, Paterson AHG. Correlation between c-erb B-2 amplification and risk of early relapse in node negative breast cancer. *Cancer Res* 1991;**51**:556–67.
144 Press MF, Pike MC, Chazin VR, Hung G, Udove JA. HER-2/*neu* expression in node negative breast cancers: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res* 1993;**53**:4960–70.
145 Richner J, Gerber HA, Locher GW, Goldhirsch A, Gelber RD. c-*erb* B-2 protein expression in node negative breast cancer. *Ann Oncol* 1990;**1**:263–8.
146 Rilke F, Colnaghi MI, Cascinelli N, Andreola S, Baldini MT. Prognostic significance of HER-2/*neu* expression in breast cancer and its relationship to other prognostic factors. *Int J Cancer* 1991;**49**:44–9.
147 Ro J, El-Naggar A, Ro JY, Blick M, Fraschini F. c-*erb*B-2 amplification in node negative breast cancer. *Cancer Res* 1989;**49**:6941–4.
148 Schimmelpenning H, Eriksson ET, Falkmer UG, Rutqvist LE, Johansson H. Prognostic significance of immunohistochemical c-*erb*B-2 proto-oncogene expression and nuclear DNA content in human breast cancer. *Eur J Surg Oncol* 1992;**18**:530–7.
149 Winstanley J, Cooke T, Murray GD, Platt-Higgins A, George WD. The long term prognostic significance of c-*erb*B-2 in primary breast cancer. *Br J Cancer* 1991;**63**: 447–50.
150 Muss HB, Thor AD, Berry DA, Kute T, Liu ET. c-*erb*B-2 expression and response to adjuvant therapy in women with node positive early breast cancer. *N Engl J Med* 1994;**330**:1260–6.
151 Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* 1985;**41**:695–706.
152 Drebin JA, Link VC, Greene MI. Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects *in vivo*. *Oncogene* 1988;**2**:387–94.
153 Foekens JA, Riol M-C, Seguin P. Prediction of relapse and survival in breast cancer patients by pS2 protein status. *Cancer Res* 1990;**50**:3832–7.
154 Predine J, Spyratos F, Prud'homme JF. Enzyme-linked immunosorbent assay of pS2 in breast cancers, benign tumours, and normal breast tissues: correlation with prognosis and adjuvant hormone therapy. *Cancer* 1992;**69**: 2116–23.
155 Schwartz LH, Koerner FC, Edgerton SM. pS2 expression and response to hormonal therapy in patients with advanced breast cancer. *Cancer Res* 1991;**51**:624–8.
156 Theillet C, LeRoy X, De Lapeyriere O. Amplification of FGF-related genes in human tumours. Possible involvement of *HST* in breast carcinomas. *Oncogene* 1989; **4**:915–21.
157 Tripathy D, Benz CC. Activated oncogenes and putative tumour suppressor genes involved in human breast cancers. In: Benz CC, Liu E, eds. *Oncogenes and tumour suppressor genes in human malignancies*. Norwell, MA: Kluwer Academic Publishers, 1993:15–28.
158 Bronsert DA, Pantazis P, Antoniadis HN. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci USA* 1987;**84**: 5763–70.
159 Kasid A, Lippman ME. Oestrogen and oncogene mediated growth regulation of human breast cancer cells. *J Steroid Biochem* 1987;**27**:465–71.
160 Ariad S, Seymour L, Bezwoda WR. Platelet-derived growth factor (PDGF) in plasma of breast cancer patients: correlation with stage and rate of progression. *Breast Cancer Res Treat* 1991;**20**:11–16.
161 Papa V, Pezzino V, Constandino A. Elevated insulin receptor content in human breast cancer. *J Clin Invest* 1990; **86**:1503–9.
162 Peyrad J-P, Bonneterre J, Vennin PH. Insulin-like growth factor 1 receptors (IGF-1-R) and IGF-1 in human breast tumours. *J Steroid Biochem Mol Biol* 1990;**37**:823–30.
163 MacAulay VM. Insulin-like growth factors and cancer. *Br J Cancer* 1992;**65**:311–7.
164 Friend SH, Dryja TP, Weinberg RA. Oncogenes and tumor-suppressing genes. *N Engl J Med* 1988;**318**:618–22.
165 Knudson AG. Antioncogenes and human cancer. *Proc Natl Acad Sci USA* 1993;**90**:10914–21.
166 Lane DP. p53, guardian of the genome. *Nature* 1992;**358**: 15–26.
167 Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991;**351**:453–6.
168 Vogelstein B, Kinzler KW. p53 function and dysfunction. *Cell* 1992;**70**:523–6.
169 Levine AJ. The p53 tumor suppressor gene and product. *Cancer Surv* 1992;**12**:59–79.
170 Finlay CA. The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol Cell Biol* 1993;**13**:301–6.
171 Hupp TR, Meek DW, Midgley CA, Lane DP. Regulation of the specific DNA binding function of P53. *Cell* 1992; **71**:875–86.
172 Momand J, Zambeti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992;**9**:1237–45.
173 Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumor suppressor p53. *Nature* 1993; **363**:857–60.
174 Lane DP, Benchimol B. p53: oncogene or anti-oncogene? *Genes Dev* 1990;**4**:1–8.
175 Moll UM, Riou G, Levine AJ. Two distinct mechanisms alter p53 in breast cancer; mutation and nuclear exclusion. *Proc Natl Acad Sci USA* 1992;**89**:7262–6.
176 Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;**53**:49–53.
177 Harris CC, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 1993;**329**:1318–27.
178 Coles C, Condie A, Chetty U, Steel CM, Evans HJ, Prosser J. p53 mutations in breast cancer. *Cancer Res* 1992;**52**: 5291–8.
179 Gannon JV, Greaves R, Iggo R, Lane DP. Activating mutations in p53 produce a common conformational effect: A monoclonal antibody specific for the mutant form. *EMBO J* 1990;**9**:1595–6022.
180 Cattoretti G, Rilke F, Andreola S, S'Amato, Delia D. p53 expression in breast cancer. *Int J Cancer* 1988;**41**:178–83.
181 Davidoff AM, Humphrey PA, Iglehart JD, Marks JR. Genetic basis for p53 overexpression in human breast cancer. *Proc Natl Acad Sci USA* 1991;**88**:5006–10.
182 Harris AL. p53 expression in human breast cancer. *Adv Cancer Res* 1992;**59**:69–88.
183 Soini Y, Paakko P, Nuorva K, Kamel D, Lane DP, Vahakangas K. Comparative analysis of p53 protein immunoreactivity in prostatic, lung and breast carcinoma. *Virchows Arch (A Pathol Anat Histopathol)* 1992;**421**:223–38.
184 Thor AD, Moore DH II, Edgerton SM, Kawasaki ES, Reihsaus E. Accumulation of p53 tumor suppressor gene protein: An independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992;**84**:845–55.
185 Varley JM, Brammar WJ, Lane DP. Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. *Oncogene* 1991;**6**:413–21.
186 Walker RA, Dearing SJ, Lane DP, Varley JM. Expression of p53 protein in infiltrating and in-situ breast carcinoma. *J Pathol* 1991;**165**:203–11.
187 Borresen AL, Hovig E, Smith-Sorensen B. Constant denaturant gel electrophoresis as a rapid screening technique for p53 mutations. *Proc Natl Acad Sci USA* 1991;**88**: 8405–9.
188 Deng G, Chen LC, Schott DR, Thor A, Bhargava V. Loss of heterozygosity and p53 gene mutations in breast cancer. *Cancer Res* 1994;**54**:499–95.
189 Dunn JM, Hastrich DJ, Newcomb P, Webb JC, Maitland NJ. Correlation between p53 mutations and antibody staining in breast carcinoma. *Br J Surg* 1993;**80**:1410–12.
190 Marchetti A, Buttitta F, Pellegrini S, Diella F, Campani D. p53 mutations and histologic type of invasive breast carcinoma. A polymerase chain reaction-single-strand conformation polymorphism and immunohistochemical analysis. *Ann NY Acad Sci* 1993;**698**:114–9.
191 Loda M. Polymerase chain reaction-based methods for the detection of mutations in oncogenes and tumor suppressor genes. *Hum Pathol* 1994;**25**:564–71.
192 Bartek J, Bartkova J, Vojtesek B, Staskoa Z, Rejthar A. Patterns of expression of the p53 tumor suppressor in human breast tissues and tumours in situ and *in vitro*. *Int J Cancer* 1990;**46**:839–44.

193 Kerns BJM, Jordan PA, Moore MBH. p53 overexpression in formalin-fixed, paraffin-embedded tissue detected by immunohistochemistry. *J Histochem Cytochem* 1992;**40**: 1047–51.
194 Vojtesek B, Fisher CJ, Barnes DM, Lane DP. Comparison between p53 staining in tissue sections and p53 protein levels measured by and ELISA technique. *Br J Cancer* 1993;**67**:1254–8.
195 Allred DC, Clark GM, Elledge R. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node negative breast cancer. *J Natl Cancer Inst* 1993;**85**:200–6.
196 Anderson TI, Holm R, Nesland JM, Heimdal KR, Ottestad L, Borresen AL. Prognostic significance of TP53 in breast carcinoma. *Br J Cancer* 1993;**68**:540–8.
197 Barnes DM, Dublin EA, Fisher CJ, Levison DA, Millis RR. Immunohistochemical detection of p53 protein in mammary carcinoma: an important new independent indicator of prognosis? *Hum Pathol* 1992;**24**:469–76.
198 Elledge RM, Fuqua SA, Clark GM, Pujol P, Allred DC, McGuire WL. Prognostic significance of p53 gene alterations in node negative breast cancer. *Breast Cancer Res Treat* 1993;**26**:225–35.
199 Gasparini G, Weidner N, Bevilacqua P, Maluta S, Dalla Palma P. Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node negative breast carcinoma. *J Clin Oncol* 1994;**12**:456–66.
200 Isola J, Visakorpi T, Holli K, Kallioniemi OP. Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node negative breast cancer patients. *J Natl Cancer Inst* 1992;**4**: 1109–14.
201 Iwaya K, Tsuda H, Hiraide H. Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. *Jpn J Cancer Res* 1991;**2**:835–40.
202 Lipponen P, Ji H, Aaltomaa S, Syrjanen S, Syrjanen K. p53 protein expression in breast cancer as related to histopathological characteristics and prognosis. *Int J Cancer* 1993;**55**:51–6.
203 Lipponen P, Aaltomaa S, Syrjanen S, Syrjanen K. c-*erb*B-2 oncogene related to p53 expression, cell proliferation and prognosis in breast cancer. *Anticancer Res* 1993;**13**: 1147–52.
204 Marks JR, Humphrey PA, WU K, Berry D, Barenko N. Overexpression of p53 and HER-2/*neu* proteins as prognostic markers in early stage breast cancer. *Ann Surg* 1994;**219**:332–41.
205 Poller DN, Hutchings CE, Galea M, Bell JA, Nicholson RA. p53 protein expression in human breast carcinoma: relationship to expression of epidermal growth factor receptor, *c-erb*B-2 protein overexpression, and estrogen receptor. *Br J Cancer* 1992;**66**:583–8.
206 Silvestrini R, Benini E, Daidone MG. p53 as an independent prognostic marker in lymph node negative breast cancer patients. *J Natl Cancer Inst* 1993;**85**:965–70.
207 Thorlacius S, Borreson AL, Eyfyord JE. Somatic p53 mutation in human breast carcinomas in an Icelandic population: A prognostic factor. *Cancer Res* 1993;**53**: 1637–41.
208 Visscher DW, Sarkar FH, Wykes S, Kothari K, Macoska J. Clinicopathologic significance of p53 immunostaining in adenocarcinoma of the breast. *Arch Pathol Lab Med* 1993;**117**:973–6.
209 Barbareschi M, Leonardi E, Mauri FA, Serio G, Palma PD. p53 and c-*erb*B-2 protein expression in breast carcinomas. An immunohistochemical study including correlations with receptor status, proliferation markers, and clinical stage in human cancer. *Am J Clin Pathol* 1992; **98**:408–18.
210 Bhargava V, Thor A, Deng G, Ljung B-M, Moore DH. The association of p53 immunoreactivity with tumor proliferation and other prognostic markers in breast cancer. *Mod Pathol* 1994;**7**:361–8.
211 Bosari S, Lee AK, Viale G, Heatey GJ, Coggi G. Abnormal p53 immunoreactivity and prognosis in node negative breast carcinomas with long-term follow-up. *Virchows Arch (A)* 1992;**421**:291–5.
212 Davidoff AM, Herndon JE, Glover NS, Kerns BJ, Pence JC. Relation between p53 overexpression and established prognostic factors in breast cancer. *Surgery* 1991;**110**: 259–64.
213 Domagala W, Harezga B, Szadowska A, Markiewski M, Weber K. Nuclear p53 protein accumulates preferentially in medullary and high-grade ductal but rarely in lobular breast carcinomas. *Am J Pathol* 1993;**142**:669–74.
214 Marzars R, Spinardi L, Bencheikh M, Simony-Lafontaine J, Jeanteur P, Theillet C. p53 mutations occur in aggressive breast cancer. *Cancer Res* 1992;**52**:3918–23.
215 Merlo GR, Bernardi A, Diella F, Venesio T, Cappa AP. In primary human breast carcinoma mutations in exons 5 and 6 of the p53 gene are associated with a high s-phase index. *Int J Cancer* 1993;**54**:531–5.
216 Poller DN, Roberts EC, Bell JA, Elston CW, Blamey RW. p53 protein expression in mammary ductal carcinoma in situ: relationship to immunohistochemical expression of estrogen receptor and c-*erb*B-2 protein. *Hum Pathol* 1993; **24**:463–8.
217 Riou G, Le MG, Travaagli JP, Levine AJ, Moll UM. Poor prognosis of p53 gene mutation and nuclear overexpression of p53 protein in inflammatory breast carcinoma. *J Natl Cancer Inst* 1993;**85**:1765–7.
218 Thompson AM, Anderson TJ, Condie A, Prosser J, Chetty U. p53 allele losses, mutations and expression in breast cancer and their relationship to clinico-pathological parameters. *Int J Cancer* 1992;**50**:528–32.
219 Trudel M, Mulligan L, Cavenee W, Margolese R, Cote J, Gariepy G. Retinoblastoma and p53 gene product expression in breast carcinoma: immunohistochemical analysis and clinicopathologic correlation. *Hum Pathol* 1992;**23**:1388–94.
220 Tsuda H, Iwaya K, Fukutomi T, Hirohashi S. p53 mutations and c-erbB-2 amplification in intraductal and invasive breast carcinomas of high histologic grade. *Jpn J Cancer Res* 1993;**84**:394–401.
221 Caleffi M, Teague MW, Jensen RA, Vnencak-Jones CL, Dupont WE, Parl FF. p53 gene mutations and steroid receptor status in breast cancer. Clinicopathologic correlations and prognostic assessment. *Cancer* 1994;**73**: 2147–56.
222 Hanzal E, Gitsch G, Kohlberger P, Dadak C, Miechowiecka N, Breitenecker G. Immunohistochemical detection of mutant p53-suppressor gene product in patients with breast cancer; influence on metastasis-free survival. *Anticancer Res* 1992;**12**:2325–9.
223 Marchetti A, Buttitta F, Pellegrini S, Campani D, Diella F. P53 mutations and histological type of invasive breast carcinoma. *Cancer Res* 1993;**53**:4665–9.
224 Martinazzi M, Crivelli F, Zampatti C, Martinazzi S. Relationship between p53 expression and other prognostic factors in human breast carcinoma. An immunohistochemical study. *Am J Clin Pathol* 1993;**100**: 213–7.
225 Martinazzi M, Crivelli F, Zampatti C, Pisani P. Low, intermediate and high grade breast carcinomas as determined by histotyping, immunohistochemical prognosticators and histological grading. *Pathologica* 1992; **84**:33–47.
226 Davidoff AM, Kerns BJ, Iglehart JD, Marks JR. Maintenance of p53 alterations throughout breast cancer progression. *Cancer Res* 1991;**51**:2605–10.
227 Bartkova J, Bartek J, Vojtesek B, Lukas J, Rejthar A. Immunochemical analysis of the p53 oncoprotein in matched primary and metastatic human tumors. *Eur J Cancer* 1993;**29**:881–6.
228 Bosari S, Viale G, Bossi P, Maggioni M, Coggi G, Lee AK. Cytoplasmic p53 protein accumulation: an independent prognostic indicator in colorectal adenocarcinomas. *J Natl Cancer Inst* 1994;**86**:681–7.
229 Li FP, Fraumeni JF. Soft-tissue sarcomas, breast cancer and other neoplasms: a familial syndrome? *Ann Intern Med* 1969;**71**:747–52.
230 Malkin D, Li FP, Strong LC. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 1990;**250**:1233–8.
231 Srivastava S, Zou ZQ, Pirollo K, Blattner WA, Chang EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* 1990; **348**:747–9.
232 Jolly KW, Malkin D, Douglass EC, Brown TF, Sinclair AE, Look AT. Splice-site mutation of the p53 gene in a family with hereditary breast-ovarian cancer. *Oncogene* 1994;**9**:97–102.
233 Malkin D, Jolly KW, Barbier N. Germline mutations of the p53 tumor-suppressor gene in children and young adults with second malignant neoplasms. *N Engl J Med* 1992;**326**:1309–15.
234 Togochida J, Yamaguchi T, Dayton SH. Prevalence and spectrum of germline mutations of the p53 gene among patients with sarcoma. *N Engl J Med* 1992;**326**:1301–8.
235 Hall JM, Lee MK, Newman B. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990;**250**:1684–9.
236 Narod SA, Feunteun J, Lynch HT. Familial breast-ovarian cancer locus on chromosome 17q21-q23. *Lancet* 1991; **338**:82–3.
237 Casey G, Lo-Hsueh MM, Lopez ME, Vogelstein B, Stanbridge EJ. Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. *Oncogene* 1991;**6**:1791–7.
238 Negrini M, Sabbioni S, Haldar S, Posati L, Castagnoli A. Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. *Cancer Res* 1994; **54**:1818–24.
239 Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993;**74**:957–67.
240 Crawford LV, Pim DC, Bulbrok RD. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. *Int J Cancer* 1982;**30**:403–8.
241 Borg A, Zhang Q-X, Alm P, Olson H, Sellberg G. The retinoblastoma gene in breast cancer; Allele loss is not correlated with loss of gene protein expression. *Cancer Res* 1992;**52**:2991–4.
242 Fung YK, T'Ang A. The role of the retinoblastoma gene in breast cancer development. *Cancer Treat Res* 1992;**61**: 59–68.
243 Lee EYHP, To H, Shew JY, Bookstein R, Scully P. Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* 1988;**241**:218–21.
244 Bowcock AM, Hall JM, Hebert JM, Kong MC. Exclusion of the retinoblastoma gene and chromosome 13q as the site of a primary lesion for human breast cancer. *Am J Hum Genet* 1990;**46**:12–17.
245 Wang NP, To H, Lee W-H, Lee Y-HP. Tumor suppressor activity of Rb and p53 in human breast carcinoma cells. *Oncogene* 1993;**8**:279–88.
246 Sawan A, Randall B, Angus B, Wright C, Henry JA. Retinoblastoma and p53 gene expression related to re-

lapse and survival in human breast cancer: an immunohistochemical study. *J Pathol* 1992;**168**:23–8.
247 Varley JM, Aromour J, Swallow JE, Jeffreys AJ, Ponder BAJ. The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. *Oncogene* 1989;**4**:725–9.
248 Walker RA, Varley JM. The molecular pathology of human breast cancer. *Cancer Surv* 1993;**16**:31–57.
249 Hall JM, Lee MK, Newman B. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990;**250**:1684–9.
250 Narod SA, Feunteun J, Lynch HT. Familial breast-ovarian cancer locus on chromosome 17q21-q23. *Lancet* 1991; **338**:82–3.
251 Abel KJ, Boehnke M, Prahalad M, Ho P, Flejter WL. A radiation hybrid map of the BRCA-1 region of chromosome 17q12-21. *Genomics* 1993;**17**:632–41.
252 Anderson LA, Friedan L, Osborne-Lawrence S, Lynch E, Weissenbach J. High-density genetic map of the BRCA1 region of chromosome 17q12-21. *Genomics* 1993;**17**:618–23.
253 Bowcock AM, Anderson LA, Friedman LS, Black DM, Osborne-Lawrence S. THRA1 and D17S183 flank an interval of <4 cM for the breast-ovarian cancer gene (BRCA1) on chromosome 17q21. *Am J Hum Genet* 1993; **52**:718–22.
254 Casey G, Plummer S, Hoeltge G, Scanlon D, Fasching C, Stanbridge EJ. Functional evidence for a breast cancer growth suppressor gene on chromosome 17. *Hum Mol Genet* 1993;**2**:1921–7.
255 Devilee P, Cornelis RS, Bootsma A, Bardoel A, van Vliet M. Linkage to markers for the chromosome region 17q12-21 in 13 Dutch breast cancer kindreds. *Am J Hum Genet* 1993;**52**:730–5.
256 Flejter Wl, Barcroft CL, Guo SW, Lynch ED, Boehnke M. Multicolor FISH mapping with Alu-PCR-amplified YAC clone DNA determines the order of markers in the BRCA-1 region on chromosome 17q12-21. *Genomics* 1993;**17**:624–31.
257 Hall JM, Friedman L, Guenther C, Lee MK, Weber JL. Closing in on a breast cancer gene on chromosome 17q. *Am J Hum Genet* 1992;**50**:1235–42.
258 Lalle P, De Latour M, Rio P, Bignon YJ. Detection of allelic losses on 17q12-21 chromosomal region in benign lesions and malignant tumors occurring in a familial context. *Oncogene* 1994;**9**:437–42.
259 Smith SA, Easton DF, Ford D, Peto J, Anderson K. Genetic heterogeneity and localization of a familial breast-ovarian cancer gene on chromosome 17q12-21. *Am J Hum Genet* 1993;**52**:767–76.
260 Teare MD, Santibanez-Koref MF, Wallace SA, White GR, Evans DG. A linkage study in seven breast cancer families. *Am J Hum Genet* 1993;**52**:786–8.
261 Bevilacqua G, Sobel ME, Liotta LA, Steeg PS. Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. *Cancer Res* 1989;**49**:5185–90.
262 Cohn K, Wang F, DeSoto-LaPaix F, Solomon WB, Patterson LG. Association of nm23-H1 allelic deletions with distant metastases in colorectal carcinoma. *Lancet* 1991;**338**:722–4.
263 Hennessy C, Henry JA, May FE. Expression of the anti-metastatic gene nm23 in human breast cancer; an association with good prognosis. *J Natl Cancer Inst* 1991; **83**:281–5.
264 Hirayama R, Sawai S, Takagi Y, Mishima Y, Kimura N. Positive relationship between expression of anti-metastatic factor (nm23 gene product or nucleoside diphosphate kinase) and good prognosis in human breast cancer. *J Natl Cancer Inst* 1991;**83**:1249–50.
265 Royds JA, Stephenson TJ, Rees RC, Shorthouse AJ, Silcocks PB. Nm23 protein expression in ductal in situ and invasive human breast carcinoma. *J Natl Cancer Inst* 1993; **85**:727–31.
266 Tokunaga Y, Urano T, Furukawa K, Kondo H, Kanematsu T, Shiku H. Reduced expression of nm23-H1, but not of nm23-H2, is concordant with the frequency of lymph-node metastasis of human breast cancer. *Int J Cancer* 1993;**55**:66–71.
267 Simpson JF, O'Malley F, DuPont WD, Page DL. Heterogeneous expression of nm23 gene product in non-invasive breast carcinoma. *Cancer* 1994;**73**:2352–8.
268 Lacombe M-L, Sastre-Garau X, Lascu I, Vonica A, Wallet V, Thiery JP, *et al.* Overexpression of nucleoside diphosphate kinase (Nm23) in solid tumors. *Eur J Cancer* 1991;**27**:1302–7.
269 Sastre-Garau X, Lacombe ML, Jouve M, Veron M, Magdelenat H. Nucleoside diphosphate kinase/nm23 expression in breast cancer: lack of correlation with lymph-node metastasis. *Int J Cancer* 1992;**50**:533–8.
270 Engel M, Theisinger B, Seib T, Seitz G, Huwer H. High levels of nm23-H1 and nm23-H2 messenger RNA in human squamous cell lung carcinoma are associated with poor differentiation and advanced tumor stages. *Int J Cancer* 1993;**55**:375–9.
271 Folkman J. Angiogenesis and breast cancer. *J Clin Oncol* 1994;**12**:441–3.
272 Folkman J, Hochberg M, Knighton D. Self-regulation of growth in three dimensions: the role of surface area limitations. In: Clarkson B, Baserga R, eds. *Control of animal cell proliferation*. Cold Spring Harbor, New York: Cold Spring Harbor Press, 1974:833–42.
273 Hayes DF. Angiogenesis and breast cancer. *Hematol Oncol Clin North Am* 1994;**8**:51–71.
274 Folkman J. Angiogenesis and its inhibitors. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Important advances in oncology*. Philadelphia: JB Lippincott Co, 1985:42–62.
275 Ingber D, Fujita T, Kishimoto S, Katsuichi S, Kanamaru T, Brem H, *et al.* Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. *Nature* 1990;**348**:555–7.
276 Yamaoka M, Yamamoto T, Masaki T. Inhibition of tumor growth and metastasis of rodent tumors by the angiogenesis inhibitor O-(chloroacetyl-carbamoyl) fumagillol (TNP-470; AGM-1470). *Cancer Res* 1993;**53**: 426–7.
277 Hori A, Sasada R, Matsutani E. Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Res* 1991;**51**:6180–4.
278 Liotta LA, Kleinerman J, Saidel G. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Res* 1976;**36**:889–94.
279 Nagy JA, Brown LF, Senger DR, Lanir N, Van de Water L, Dvorak AM, *et al.* Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochem Biophys Acta* 1989;**948**:305–26.
280 Moscatelli D, Gross J, Rifkin D. Angiogenic factors stimulate plasminogen activator and collagenase production by capillary endothelial cells [abstr]. *J Cell Biol* 1981;**91**: 201a.
281 Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991;**324**:1–8.
282 Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, *et al.* Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;**84**:1875–87.
283 Bosari S, Lee AKC, DeLellis RA. Microvessel quantitation and prognosis in invasive breast carcinoma. *Hum Pathol* 1992;**23**:755–61.
284 Horak E, Leek R, Klenk N. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* 1992;**340**:1120–4.
285 Fox SB, Leek RD, Smith K, Hollyer J, Greenall M, Harris AL. Tumor angiogenesis in node negative breast carcinomas— relationship to epidermal growth factor receptor, estrogen receptor, and survival. *Breast Cancer Res Treat* (in press).
286 Gasparini G, Weidner N, Bevilacqua P, Pozza F, Maluta S, Palma PD, *et al.* Microvessel density (a measure of tumor angiogenesis): a new prognostic factor in node negative invasive breast carcinoma. *J Clin Oncol* 1994; **12**:456–66.
287 Sahin AA, Sneige N, Ordonez GN. Tumor angiogenesis detected by *Ulex europaeus* agglutinin 1 lectin (UEA 1) and factor VIII immunostaining in node negative breast carcinoma (NNBC) treated by mastectomy: prediction of tumor recurrence [abstr]. *Mod Pathol* 1993;**6**:19a.
288 Sneige N, Singletary E, Sahin A. Multiparameter analysis of potential prognostic factors in node negative breast cancer patients [abstr]. *Mod Pathol* 1992;**5**:18a.
289 Toi M, Kashitani J, Tominaga T. Tumor angiogenesis is an independent prognostic indicator in primary breast carcinoma. *Int J Cancer* 1993;**55**:371–4.
290 Visscher DW, Smilanetz S, Drozdowicz S, Wykes SM. Prognostic significance of image morphometric microvessel enumeration in breast carcinoma. *Anal Quant Cytol Histol* 1993;**15**:88–92.
291 Montcourrier P Mangeat PH, Salazar G, Morissset M, Sahuquet A, Pochefort H. Cathepsin D in breast cancer cells can digest extracellular matrix in large acidic vesicles. *Cancer Res* 1990;**50**:6045–54.
292 Vignon G, Capony F, Chambon M, Freiss G, Garcia M, Rochefort H. Autocrine growth stimulation of the MCF-7 breast cancer cells by the estrogen-regulated 52K protein. *Endocrinology* 1986;**118**:1537–45.
293 Charpin C, Devictor B, Bonnier P. Cathepsin D immunohistochemical assays in breast carcinomas: Image analysis and correlation to prognostic factors. *J Pathol* 1993;**170**:463–70.
294 Kandalaft PL, Chang KL, Ahn CH, Traweek ST, Mehta P, Battifora H. Prognostic significance of immunohistochemical analysis of cathepsin D in low-stage breast cancer. *Cancer* 1993;**71**:2756–63.
295 Marsigliante S, Biscozzo L, Greco S, Leo G, Storelli C. Relation of cathepsin D level to the estrogen receptor in human breast cancer. *Int J Clin Lab Res* 1992;**22**:52–7.
296 Sacks NP, Smith K, Norman AP, Greenall M, LeJeune S, Harris AL. Cathepsin D levels in primary breast cancers: relationship with epidermal growth factor receptor, oestrogen receptor and axillary nodal status. *Eur J Cancer* 1993;**29**:426–8.
297 Monteagudo C, Merino MJ, San-Juan J. Immunohistochemical distribution of type IV collagenase in normal, benign and malignant breast tissue. *Am J Pathol* 1990;**136**:585–92.
298 D'Errico A, Garbisa S, Liotta LA. Augmentation of type IV collagenase, laminin receptor and Ki-67 proliferation antigen associated with human colon, gastric, and breast carcinoma progression. *Mod Pathol* 1991;**4**:239–46.
299 Grigioni WF, Garbisa S, D'Errico A. Evaluation of hepatocellular carcinoma aggressiveness by a panel of extracellular matrix antigens. *Am J Pathol* 1991;**138**:647–54.
300 Daidone MG, Silvestrini R, D Errico A. Laminin receptors, collagenase IV and prognosis in node negative breast cancers. *Int J Cancer* 1991;**48**:529–32.
301 Pyke C, Ralfkiaer E, Huhtala P. Localisation of messenger

RNA for Mr72000 and 92000 type IV collagenases in human skin cancers by in situ hybridisation. *Cancer Res* 1992;**52**:1336–41.
302 Yu H, Schultz RM. Relationship between secreted urokinase plasminogen activator activity and metastatic potential in murine B16 cells transfected with human urokinase sense and anti-sense genes. *Cancer Res* 1990; **50**:7623–33.
303 Duffy MJ, O'Grady P, Devaney D. Tissue-type plasminogen activator, a new prognostic marker in breast cancer. *Cancer Res* 1989;**49**:6008–14.
304 Schmitt M, Janicke F, Graeff H. Tumour-associated fibrinolysis: the prognostic relevance of plasminogen activators uPA and pPA in human breast cancer. *Blood Coagul Fibrinolysis* 1990;**1**:695–702.
305 Janicke F, Schmitt M, Graef H. Clinical relevance of the urokinase-type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer. *Semin Thromb Hemost* 1991;**17**:303–12.
306 Faassen AE, Drake SL, Iida J. Mechanisms of normal cell adhesion to the extracellular matrix and alterations associated with tumour invasion and metastasis. In: Weinstein RS, Graham AR, eds. *Advances in pathology and laboratory medicine*, vol 5. St Louis: Mosby Year Book, 1992:229–50.
307 Pignatelli M, Cardillo MR, Hanby A. Integrins and their accessory adhesion molecules in mammary carcinomas: loss of polarisation in poorly differentiated tumours. *Hum Pathol* 1992;**23**:1159–66.
308 Marques LA, Franco ELF, Tortoni H. Independent prognostic value of laminin receptor expression in breast cancer survival. *Cancer Res* 1990;**50**:1479–83.
309 Lonn U, Lonn S, Nylen U, Stenkvist B. Appearance and detection of multiple copies of *mdr-1* gene in clinical samples of mammary carcinoma. *Int J Cancer* 1992;**51**: 682–6.
310 Chamness GC. Eestrogen-inducible heat shock protein hsp27 predicts recurrence in node negative breast cancer. *Proc Am Assoc Cancer Res* 1989;**30**:252–61.
311 Ciocca DR, Fuqua SA, Lock-Lim S. Response of human breast cancer cells to heat shock and chemotherapeutic drugs. *Cancer Res* 1992;**52**:3648–54.