Leader

Biological indices in the assessment of breast cancer

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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 deaths² 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 form-

ation, 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. Bonnadonna⁶ 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,

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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 exons, 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 progesterone 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 progesterone 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 tran-

scription 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-erbB-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-erbB-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 prognostic³⁶; 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 G₀.⁴² 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% ($p < 0.05$). At a cut off of 16% positivity, only 50% survival was seen in the high expressers ($p < 0.01$).

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 ($p = 0.05$, $r = 0.3$) 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 G₁ and peaking during S phase, and declining during G₂ 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 cells⁵³ 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-1⁵⁷ 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 Richards⁶² 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 *erbB*-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-erbB*-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-erbB-2 oncogene

The *c-erbB-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-erbB-2* was a human gene discovered by its homology to the retroviral gene *v-erbB*, and HER-2 was isolated by screening a human genomic DNA library for homology with *v-erbB*. When the DNA sequences were determined subsequently, *c-erbB-2*, HER-2, and *neu* were found to represent the same gene.

The *c-erbB-2* DNA is located on human chromosome 17q21 and codes for *c-erbB-2* mRNA (4.6 kb), which translates *c-erbB-2* to protein (p185). This protein is a normal component of cytoplasmic membranes and the *c-erbB-2* oncogene is homologous with, but not identical to, *c-erbB-1*, which is located on chromosome 7 and codes for the epidermal growth factor receptor.⁷⁸⁻⁸² The *c-erbB-2* protein is a cell membrane protein with extracellular, transmembrane and intracellular tyrosine kinase activity. It is expressed in all epithelial cells.

c-erbB-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-erbB-2* gene amplification with overexpression.⁸³⁻⁸⁶ In one study of invasive breast cancers,⁸⁵ 27% of tumours showed *c-erbB-2* amplification and overexpression, and 63% had no amplification and low levels of *c-erbB-2* gene product; however, 10% of tumours displayed overexpression without detectable gene amplification. *c-erbB-2* has been shown to be amplified in about 20–30% of invasive breast carcinomas in various studies.

c-erbB-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-erbB-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-erbB-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 carcinoma¹⁰⁷⁻¹¹¹; in contrast, in situ lobular carcinomas seldom overexpress *c-erbB-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-erbB-2* in the initiation and progression of breast cancer. Slamon *et al*¹¹⁸ first reported the association between *c-erbB-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-erbB-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-erbB-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-erbB-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-erbB-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-erbB-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-erbB-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-erbB-2* may also serve as a predictor of response to adjuvant treatment. Some studies have shown a correlation between *c-erbB-2* overexpression and poor response to chemotherapy,^{116 131} and in one study there was a dose-response effect in patients with *c-erbB-2* overexpression but not in patients with no or minimal *c-erbB-2* expression.¹⁵⁰ Thus *c-erbB-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-erbB-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 anti-tumour effects mediated by monoclonal antibodies on cultured cells expressing high levels of *c-erbB-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 antiprogesterins 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 xenografts.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-erbB-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 functioning 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 cancer²³⁵ and familial breast and ovarian cancer²³⁶ 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 tumorigenicity 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 associates²⁸¹ 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 mm²) 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 METALLOPROTEASES

The matrix metalloproteases 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 metalloproteases related to cancer invasion and metastasis include type IV collagenase or matrix metalloprotease-2 (MMP-2), matrix metalloprotease-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 multivariate 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 multivariate 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 cells³¹¹ 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, *erbB-2* may predict for improved response to doxorubicin, rather than CMF (cyclophosphamide-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 relevant for prognosis. The need for proper validation of these markers has been emphasised by McGuire³ 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.

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