Prognostic factors in primary breast cancer

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Prognostic Factors in Primary Breast Cancer

PROEFSCHRIFT

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R L H Jansen

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Promotores

Prof dr HFP Hillen
Prof dr J-W Arends

Co-promotor

Dr HC Schouten

Beoordelingscommissie

Prof dr MF von Meyenfeldt (voorzitter)
Prof dr GH Blijham (Universiteit Utrecht)
Prof dr ir PA van den Brandt
Dr JGM Klijn (Erasmus Universiteit Rotterdam)
Prof dr FCS Ramaekers

Aan: Carla, Mark, Lenno en Rodi

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Chapter IA

Prognostic and predictive factors in breast cancer

RLH Jansen, HFP Hillen, HC Schouten

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Introduction

Breast cancer is the most frequent cancer type in Dutch women and accounts for 22% of all cancer deaths¹. To date the axillary lymph node status is the most important prognostic factor for patients with breast cancer. In general, the 10-year survival of node-negative patients is about 70% whereas the 10-year survival in node-positive patients is only about 30%², whereby the prognosis is directly related to the number of involved axillary nodes². Traditionally the axillary lymph node status has guided the need for adjuvant therapy. However, in terms of the absolute effect on mortality after 10 years less than 10% of the patients benefit from adjuvant treatment^{3,4}. There is a need to prognosticate which patients with node-positive disease will not benefit from standard adjuvant treatment or will be cured without it. In node-negative patients we need more information on which patients should be treated with adjuvant therapy to improve survival. Therefore, there is an urgent need for prognostic factors derived from the primary tumour to make better patient selection. So far, numerous prognostic factors have been investigated. To be useful in clinical practice a prognostic factor should have a good prognostic value, should be cheap and easy to perform. Here we shall briefly review the most important prognostic and predictive factors. A prognostic factor is a factor associated with disease-free or overall survival in the absence of systemic adjuvant therapy. A predictive factor is defined as a factor associated with response to a particular therapy.

Primary treatment

Treatment of the primary tumour with either mastectomy, lumpectomy with radiation therapy or lumpectomy without radiation therapy does not influence overall survival^{5,6}. Lumpectomy without radiation therapy, however, greatly increases the risk of local recurrence^{5,6}. Although the axillary lymph node status is the most important prognostic factor, the influence of the routine axillary nodal dissection itself on overall survival has especially recently become a matter for discussion^{7,8}. Such an influence is at best very limited, whereby it has to be kept in mind that an axillary lymph node dissection can cause substantial morbidity.

Remarkably, a recent study demonstrated that the surgeon *per se* can be a prognostic factor: care by a specialist surgeon resulted in a 8% improvement in 10-year survival compared with a non-specialist surgeon⁹.

Patient characteristics

Studies on the influence of age on the clinical outcome of breast cancer have been controversial. However, most studies have found a worse clinical outcome in younger patients¹⁰⁻¹⁵. Despite a high correlation of younger age with poor prognostic factors like S-phase fraction and p53 abnormalities in two of these studies age younger than 35 years was in multivariate analyses still a poor prognostic factor^{14,15}. Based on these studies and a more recently published study of 3722 patients¹⁶ it seems reasonable to conclude that women younger than 35-40 years have a worse prognosis.

In breast cancer black women have a higher mortality rate than white women^{17,18}. This is because black women have tumours that are more advanced at diagnosis but also because of different tumour biology, and confounding comorbid conditions and socioeconomic factors¹⁸.

Histopathological factors

Looking at histopathological features of the primary tumour itself, ductal carcinoma is by far the most common **histological type** of invasive breast cancer and is diagnosed in approximately 70% of patients¹⁹. Patients with infiltrating ductal carcinomas generally have poorer clinical outcomes than patients with the less common types of infiltrating tumours^{20,21}.

Histological grade has been proposed as a powerful prognostic factor²². However, the primary difficulties with tumour grading are poor reproducibility and lack of agreement among different investigators²³⁻²⁶, although not all authors agree with this^{27,28}. One of the problems is the different methodologies utilized being either the histological evaluation of tumour grade, nuclear pleomorphism and mitotic count or an assessment of nuclear features alone. The most widely used grading system for breast cancer is the Scarff-Bloom-Richardson classification^{29,30}. Probably a standardized and reproducible grading system could be one of the most powerful prognostic factors for breast cancer.

Tumour size is a powerful and consistent predictor of breast cancer recurrence. Several large studies have shown that the risk of disease recurrence increases as tumour size increases³¹⁻³⁴.

No **other histological factor** (e.g. extensive ductal carcinoma in situ, lymphatic vessel invasion) has been fully validated as prognostic.

Steroid hormone receptors

Most studies and especially the larger studies with longer follow-up demonstrate that patients with **oestrogen-receptor (ER)**-positive tumours (measured by biochemical assays) have a longer disease-free interval than patients with ER-negative tumours³⁵⁻⁴⁰. The disease-free survival advantage, however, is only about 10% at 3-5 years^{37-39,41} and with longer follow-up disease-free intervals tend to converge⁴¹⁻⁴⁴. Traditionally an ER level of <10 fmol/mg cytosol protein has been used for ER-negative tumours. This cut-off point has however been questioned, while it has also been suggested that patients with a very high ER level have a worse prognosis^{45,46}. The additional value of measuring the **progesterone-receptor (PR)** is uncertain. In some studies it gives no additional information to the ER-status^{37,43,47}, but in other studies it is a stronger predictor of survival^{38,39,48}.

Using immunohistochemical techniques a good correlation with the standard biochemical technique was found⁴⁹⁻⁵². Theoretically this is a better method especially because direct visualization of tumour cells is possible. However, the optimal cut-off values still remain to be determined. Steroid hormone receptors are more important as predictive factors for response to hormonal therapy. Initial response rate for patients with ER- and PR-positive tumours is 60 - 75%, whereas in patients with ER- and PR-negative tumours the response rate is less than 10%⁵³. Patients with a tumour that is ER-positive or PR-positive have an intermediate response rate of 25-45%, whereby PR is more important than ER⁵³. Consequently ER and PR are also predictive factors for survival after detection of the first metastasis⁵⁴. PR-status has also been suggested as a powerful predictor for response to adjuvant chemotherapy⁴⁷.

The **pS2** gene was first identified in the human breast cancer cell line MCF-7 in response to oestrogen stimulation⁵⁵. The pS2 protein is a small secreted protein with unknown function. It has been suggested that pS2 expression reflects the functional status of ER. PS2 can be determined by radioimmunoassay and by immunohistochemistry. The results of cytosolic assays tend to correlate with a good prognosis for pS2 positivity^{56,57}, although this is not confirmed in all studies⁵⁸. However, in immunohistochemical studies pS2 is not a prognostic factor^{59,60}. At present pS2 might be useful as a predictive factor for response to hormone therapy⁶¹.

Measurements of proliferation

As in other tumours, proliferative capacity is important in breast cancer. There are many methods to measure this proliferative capacity.

The **mitotic index (MI)** or mitotic activity index (MAI) is determined by counting

the number of mitoses on paraffin-embedded tumour samples stained with hematoxylin-eosin, and is usually expressed as the number of mitoses per (10) high-power field(s).

The MI is probably the oldest method of assessing proliferation, and is reproducible as shown in the Multicenter Morphometric Mammary Carcinoma Project⁶². In a few studies it was demonstrated that the MI is a prognostic factor even in multivariate analysis both for disease-free survival and overall survival^{63,64}. The MI is also a component of several prognostic indices like the Nottingham Prognostic Index⁶⁵.

The **thymidine labelling index (TLI)** is determined by counting the number of labelled nuclei on autoradiographed microsections following incubation of the tumour specimen with tritium-labelled thymidine. In a recent review article it was concluded that in most studies a better relapse-free and overall survival is found in patients with a slowly proliferating tumour⁶⁶. Although the methods were not standardized in these studies and questions about reproducibility have been raised, a good correlation within and between laboratories is possible⁶⁶. Recently a large study of 1800 node-negative breast cancer patients has confirmed an independent prognostic role for TLI⁶⁷. However, the role of TLI as a predictive factor especially for response to chemotherapy has as yet not been fully demonstrated⁶⁸⁻⁷⁰. Although **bromodeoxyuridine (BrdU)-labelling** has been shown to give results equivalent to thymidine labelling⁷¹, formal studies on its use as a prognostic factor are lacking.

DNA flow cytometry produces a DNA histogram from which a measure of DNA content (**DNA ploidy**) and a measure of proliferative activity (**S-phase fraction**) is provided. Analysis of more than 127,000 breast cancer patients demonstrated an increased incidence of higher S-phase fractions (SPF) and aneuploidy in oestrogen- and progesterone-receptor negative tumours, larger tumours, tumours with positive axillary lymph nodes and in patients younger than 35 years of age⁷². In a consensus conference in 1992 it was concluded that the literature supports an association between high SPF and increased risk of recurrence and mortality⁷³. The authors advised each laboratory to establish its own distribution of diploid and aneuploid tumours rather than use published cut-off points and to use 3 rather than 2 risk groups, to reduce the chance of misclassifying tumours with near-borderline SPF values. The prognostic significance of SPF in breast cancer can probably be improved by performing it on cytokeratin-gated cell populations⁷⁴. As a predictive factor for response to adjuvant chemotherapy SPF was not useful in several studies^{75,76}, while in the metastatic setting a correlation between high SPF and a higher response rate has been reported⁷⁷.

There are also immunohistochemical methods which are easier and faster in assessing the proliferative activity. **Ki67** is a monoclonal antibody that is expressed only in proliferating cells (late G₁, S, M and G₂ phases of the cell cycle). Origin-

ally the antibody could only be used in fresh or frozen sections. Newer antibodies like MIB-1 are effective in fixed, archival sections after microwave irradiation. In many studies Ki67 correlates with histological grade and inversely with steroid receptors. Most studies have found in univariate analysis a correlation between a higher number Ki67-positive cells and the risk of recurrence or death⁷⁸⁻⁸³. However, many studies have relatively few patients, have short follow-up and/or are composed of heterogeneous groups of node-negative and node-positive patients. More recently it has been shown that high proliferative activity as measured by Ki-67 or MIB-1 antigen expression was an independent prognostic factor for survival^{83,84}. Additional studies are clearly needed in which especially standardization of the technique is necessary.

PCNA is a nuclear protein associated with DNA polymerase delta which is present throughout the cell cycle in proliferating cells. In general, correlations with other prognostic factors are weak. Some studies have not found a relationship of PCNA positivity and poor prognosis^{85,86}, while others have reported such a relationship^{87,88}.

Not many studies have compared different methods of measuring proliferation^{83,89}. Thereby it has to be kept in mind that the above-mentioned methods reflect different parts of the cell cycle. For example the correlation between SPF and Ki-67 is weak at best⁸³.

It is hard to draw definite conclusions from these comparative studies. In general an immunohistochemical marker for proliferation has many advantages: it can be carried out on small histological sections, it allows correlation with morphological features and no sophisticated technology is needed. However, more studies with standardized methods and long follow-up have to be done.

Measurement of invasion/metastatic potential

Cellular adhesion molecules

The metastatic process involves multiple steps including cell detachment from the primary tumour, degradation of the basement membrane and extracellular matrix, migration into surrounding connective tissue, entry into the vascular or lymphatic circulation, attachment to the endothelial cells in other organs, extravasation from the circulation and colony formation at secondary sites. This process may be mediated by a variety of adhesion molecules. The role of cellular adhesion molecules was reviewed in 1994⁹⁰. On most of these adhesion molecules insufficient numbers of studies have been performed to establish their usefulness as a prognostic factor. Only of some of the factors involved in invasiveness are sufficient data available to draw some preliminary conclusions.

Cathepsin D can be measured in tissue sections by immunohistochemistry and in tissue extracts by Western blotting, ELISA and IRMA assays. As reviewed recently, in 12 out of 13 studies measuring cytosolic cathepsin D by IRMA a significantly worse prognosis was found for women with higher cathepsin D levels in either disease-free survival or overall survival or both⁹¹. The prognostic value of cathepsin D expression within clinical subgroups is more complex, but current studies suggest that cathepsin D is of value in node-positive as well as node-negative patients and may be of greater value in node-positive tumours⁹¹.

The results of immunohistochemical studies are much more variable⁹¹. This is caused by using different antibodies, different methods of scoring and different cut-off levels. Only one study looked at more than one antibody and showed that 3 different antibodies recognized overlapping subsets of cathepsin-D-positive tumours with a different association with metastasis-free survival⁹². Furthermore, there are 3 studies that show that the number of stromal macrophages is prognostically more important than staining of tumour cells⁹³⁻⁹⁵. This could suggest that stromal macrophages make a major contribution to the cathepsin D measured in cytosolic assays. However, other studies have demonstrated good correlations between staining of tumour cells and cytosol levels of cathepsin D measured by IRMA^{96,97}. In conclusion, also for cathepsin D large prospective studies are required to look for the most appropriate assay to define its prognostic value.

Three studies have investigated the prognostic value of the 67 kD **laminin receptor** expression, with contradictory results⁹⁸⁻¹⁰⁰. The by far largest study looked at the prognostic value by immunohistochemistry in 1160 breast cancer patients that received no adjuvant therapy and had a follow-up of at least 20 years. The investigators found a small, but significantly increased relative risk of death of 1.33 among patients whose tumours expressed laminin receptors¹⁰⁰. The same group reported recently that the association between expression of the laminin receptor and poor prognosis is limited to laminin-producing tumours¹⁰¹. This supports the results of in vitro experiments that laminin and the 67 kDa laminin receptor are associated with the metastatic process, but makes it unlikely that the 67 kDa laminin receptor will ever be a prognostic factor with great clinical impact.

The **urokinase-type plasminogen activator (uPA)** is a serine protease catalysing the conversion of plasminogen into the active enzyme, plasmin. Plasmin can activate type IV collagenase which subsequently degrades collagen and proteins of the basement membranes. The binding of uPA to its receptor (uPAR) is controlled by naturally occurring inhibitors, plasminogen-activator inhibitor-1 (PAI-1) and PAI-2. uPA, PAI-1 and PAI-2 have all been studied as prognostic markers. Because of slightly different cytosolic assays with different antibodies the studies had varying cut-off points to define assay positivity. However, in general it can be concluded that uPA and PAI-1 give similar prognostic information: i.e., uPA-positive and PAI-1-positive patients have a worse prognosis¹⁰²⁻¹⁰⁵. Conversely, high

PAI-2 seems to be associated with longer disease-free survival in node-negative women¹⁰⁶ and in patients with high uPA values¹⁰⁷. Furthermore, it has recently been reported that uPA-positive tumours more often are resistant to tamoxifen treatment than uPA-negative tumours¹⁰⁸.

Microvessel density

There is considerable experimental evidence that tumour growth is dependent on the induction of new capillary blood vessels (angiogenesis)¹⁰⁹. Expansion of a tumour beyond a few millimeters in diameter requires neovascularisation. Investigators using a polyclonal antiserum against factor-VIII-related antigen or a monoclonal antibody against the cell adhesion molecule, CD31, have proposed that counting microvessel density in tumours might provide prognostic information for predicting distant disease recurrence.

Since the original publication of Weidner and colleagues in 1991¹¹⁰ several studies have investigated possible correlations between microvessel density and clinical outcome in primary breast cancer. As stated in review articles in 1994 and 1995, the majority of the studies found correlations between microvessel density and relapse-free survival, overall survival or both^{111,112}. Despite a correlation with tumour grade, tumour size and lymph node status in most studies the microvessel density was still a prognostic factor in multivariate analysis. Negative studies were criticised for methodological problems. However, recently the importance of microvessel density as a prognostic factor has been questioned¹¹³⁻¹¹⁵. Several reasons were suggested for these varying results: high intrinsic variability of the microvessel count¹¹³, the subjectivity of the process of selection and counting¹¹³ and relatively small numbers of patients with relatively short follow-up in most "positive" studies. In addition, it seems clear that the ideal endothelial marker has not yet been found. Most studies used anti-f-VIII RA or anti-CD31 but other markers like anti-CD34, anti-VEGF (vascular endothelial growth factor)-receptor antibodies or Mab E9¹¹⁶ may be better endothelial markers. Clearly more studies are needed, especially because angiogenesis is more a marker of invasion in contrast to other currently used biomarkers which are related to differentiation or proliferation¹¹⁷.

Molecular markers

Oncogenes, growth factors and their receptors

Several oncogenes may play a role in the development and progression of breast cancer and might therefore be correlated with prognosis. The *erb-B* oncogene family codes for the type I growth factor receptors of which only the epidermal growth factor receptor (EGFR=c-*erbB1*) and HER-2/Neu (c-*erbB-2*) have been

extensively studied.

Overexpression of **EGFR** is found in 35-60% of breast cancers irrespective of the method employed whether radioligand-binding assays, autoradiography, immunocytochemistry, immunoenzymatic assays, immunohistochemistry or measurement of EGFR transcripts¹¹⁸. Nearly all studies have reported a negative correlation between EGFR and steroid receptor status^{118,119}, and most studies have found an association with higher tumour grade, increased proliferative activity and aneuploidy. Several reviews summarizing the results of thousands of patients have been published in recent years^{118,119}. Most of the reviewed studies show a significant relation between EGFR status and disease-free or overall-survival in univariate analyses, sometimes only in either node-negative or node-positive patients. In studies with longer follow-up the initial differences tend to diminish with time. In only a proportion of the studies was a multivariate analysis performed^{118,119}. Therefore, at the present time it is impossible to draw firm conclusions about the usefulness of EGFR as a prognostic factor, which is at least partly caused by lack of standardization of assay methods, short follow-up etc.^{118,120}. It might be that EGFR is useful as a predictive factor: EGFR-positive tumours tend to be resistant to endocrine therapy¹²¹.

The role of HER-2/Neu (c-erbB2) as a prognostic factor has recently been reviewed¹²². The reviewers found multiple technical methods used and other difficulties as mentioned before for other prognostic factors. The larger studies do not support the use of HER-2/Neu as a prognostic factor in primary breast cancer, especially in node-negative patients. Looking at 18 studies using immunohistochemistry and including more than 100 patients with more than 3 years follow-up, the reviewers concluded that overexpression of HER-2/Neu adds little to the prediction of disease-free survival but may help predict overall survival, suggesting that HER-2/Neu could be a predictive factor¹²². Two studies found that CMF(P) adjuvant chemotherapy was not effective when the tumour was overexpressing HER-2/Neu^{123,124}, although these studies were not designed to compare differences in response to adjuvant chemotherapy. However, in a study randomizing node-positive patients to different intensities of adjuvant CAF chemotherapy a significant dose-response effect was only found in patients with tumours overexpressing HER-2/Neu⁷⁶. This discrepancy could be explained by study design, different types of chemotherapy and other factors. HER-2/Neu overexpression was in several studies also associated with resistance to tamoxifen^{125,126}. HER-2/Neu may have value in predicting response to a specific therapy, but the studies so far have been too few and too small to confirm the use of HER-2/Neu for this purpose.

Some studies have also been performed on amplification of other oncogenes in breast cancer. The proto-oncogene **c-myc** encodes a nuclear protein with sequence-specific DNA-binding activity. Amplification of c-myc is found in about 20% of

breast cancers^{127,128} and is a predictor of early relapse¹²⁷⁻¹²⁹ sometimes even in multivariate analysis¹²⁸.

The **int-2** gene is a member of the fibroblast growth factor family and is shown to be amplified in about 15% of primary breast cancers^{128,130}. Amplification is a prognostic factor for early relapse but for overall survival results are contradictory^{128,130,131}. In most^{128,130} but not all¹³¹ studies the prognostic power was lost in multivariate analysis.

Tumour-suppressor genes

The **p53** tumour-suppressor gene is located on chromosome 17 and mutations are found in a wide range of malignancies¹³². Its relation to the Li-Fraumeni syndrome makes it attractive to analyze its contribution to non-inherited breast cancer. Many studies have been published especially in the last few years about p53 and breast cancer. Most used immunohistochemical methods detecting the accumulated mutant protein and only a few used DNA-based methods such as single-strand conformation polymorphism (SSCP) analysis or DNA-sequence analysis. Only one study using SSCP found p53 mutations to have significant correlations with disease-free survival in multivariate analysis¹³³.

Recently it was suggested that use of a cDNA-based sequencing method yielded better prognostic information than an immunohistochemical method¹³⁴. However, when a higher and more stringent immunohistochemical cutpoint was used, the differences almost disappeared¹³⁴. Furthermore, DNA-sequencing is at present unsuitable for routine practice.

Looking at the larger studies using immunohistochemical methods¹³⁵⁻¹⁴⁶ only two found p53 to have no prognostic value^{141,146} despite the use of many different monoclonal antibodies. All studies using multivariate analysis found p53 to be an independent prognostic factor^{136-138,140,142-144}. Therefore, it can be concluded that there is a lot of evidence linking abnormalities of p53 with a poor prognosis. However, as stated in a recent editorial any adverse prognostic effect is probably relatively small¹⁴⁷. Also standardization of assays and guidelines for interpretation of the results need to be developed.

The **bcl-2** protein encoded for by the bcl-2 proto-oncogene is thought to prevent apoptosis. Therefore, an inverse correlation with p53 expression is not surprising¹⁴⁸. In general bcl-2 expression as measured by immunohistochemistry is associated with features of differentiation and good prognosis, like ER, PR and low proliferative capacity¹⁴⁸⁻¹⁵². In multivariate analyses bcl-2 expression seems to have no prognostic value^{148,149}, although one study did find this for the node-positive subgroup whereby p53 was not studied¹⁵². Bcl-2 expression increases also after tamoxifen treatment in ER-positive tumours¹⁵¹ and is correlated with response to endocrine therapy¹⁵³.

The **nm23** gene was originally identified in a melanoma cell line and the presence

of nm23 mRNA correlated with low metastatic potential¹⁵⁴. Although several small studies reported a significant correlation between nm23 mRNA expression and longer disease-free or overall survival without performing multivariate analysis, a larger, more recent study found no relation between clinical outcome and NDP kinase/nm23 expression¹⁵⁵.

Conclusion

Dozens of prognostic indicators have been investigated in breast cancer, including markers for tumour proliferation, oncogene and tumour suppressor gene expression, growth factors and their receptors, tumour angiogenesis and local invasiveness. Most of the information mentioned above is derived from retrospective studies with often relatively few patients and generally with short follow-up. Results are often contradictory and prognostic factors are correlated, in a different way in different studies. Certainly at present no specific prognostic factor or even a prognostic factor model can be used for treatment decisions in an individual patient.

In table I.1 an attempt has been made to summarize current knowledge. To our opinion at present only classical prognostic factors like axillary lymph node status, tumour size, proliferative activity and probably tumour grade can be considered as established. The value of the steroid hormone receptors is mainly time-dependent and they are more meaningful as predictive factors for response to adjuvant/palliative hormonal therapy. Of the newer prognostic factors, only p53 and probably microvessel density can be considered as established prognostic factors. The data on the predictive value of a certain factor are generally based on limited studies.

At this moment it still is necessary to perform studies with large numbers of patients followed for long periods of time and using standardized assays to define useful prognostic factors. In this respect it is important to mention the activities of the Receptor and Biomarker Study Group of the EORTC. From a theoretical point of view, especially adhesion molecules and factors associated with the formation of new blood vessels need to be studied because of their involvement in the metastatic process. Ultimately the prognosis of the patient is determined by whether metastases develop or not.

It should be stressed that multivariate analyses which are often lacking in many studies are still necessary. Validation of results on a truly independent external population of patients is also a prerequisite. Although the studies published in the last decade have not changed the list of established prognostic factors and therefore not clinical practice, they have shed new light on the complex system of pathways that regulate human breast cancer. This has led to a newly developing field of

potentially new therapeutic approaches. Hopefully it will be possible in the future to decide on the optimal therapy for a specific patient using prognostic factors and new therapeutic approaches derived from studies on prognostic factors.

Table 1.1. Prognostic and predictive value of some factors in women with breast cancer.

Factor	Prognostic value	Predictive value	
		Chemotherapy	Hormonal therapy
Axillary lymph node status	+	-	-
Histological type	-	-	-
Histological grade	+	?	?
Tumour size	+	-	-
Age (<40 yr)	+	?	?
Race (black)	+	?	?
ER ¹	±	-	+
PR	±	-	+
pS2	-	-	+
Proliferation ²	+	±	?
EGFR ¹	±	?	+
HER-2/Neu	-	+	+
p53	+	+	?
Bcl-2	-	?	+
Microvessel density	+	-	-
Cathepsin D ³	±	-	+
67 kD laminin-receptor	±	?	?
uPA/PAI-1/PAI-2	+	?	+

- = no; + = yes; ± = weak or doubtful, ? = unknown

¹ Time-dependent; ² Optimal technique to be determined; ³ Cytosolic assay

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Chapter IB

This thesis

RLH Jansen

This thesis

The work described in this thesis, tried to overcome some of the problems which are inherent to many of the previous studies on prognostic factors in breast cancer as mentioned in chapter 1A. We have investigated the prognostic value of several factors in all known patients, diagnosed with primary breast cancer over a period of about five years in the University Hospital Maastricht. The median follow-up period was more than 10 years. Furthermore, during this period adjuvant treatment after surgery was uniform as much as possible. Axillary node-positive patients below the age of 70 years were treated with chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide. The majority of these patients were treated according to a protocol for concomitant treatment with medroxyprogesterone-acetate (MPA) or no MPA. Axillary node-positive patients of 70 years and older were treated with adjuvant tamoxifen. Axillary node-negative patients received no adjuvant treatment.

This cohort of patients was used to investigate several possible prognostic factors that are involved in tumor growth, apoptosis and the process of metastasis.

In Chapter II the prognostic significance of the pS2 protein was studied. pS2 has been associated with the functional status of the oestrogen receptor but is probably also involved in growth regulation. Furthermore the prognostic value of the adhesion molecule CD44v6 (Chapter III), the proliferation marker MIB-1 (Chapter IV), the proto-oncogene bcl-2 and the tumour suppressor gene p53 (Chapter V) and of microvessel density (Chapter VI) were analyzed. All factors were studied by immunohistochemical methods.

Chapter II

**pS2 is an independent
prognostic factor for post-
relapse survival in primary
breast cancer**

RLH Jansen, PSGJ Hupperets, JW Arends, SR Joosten-
Achjanie, A Volovics, HFP Hillen, HC Schouten

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Abstract

Background

The pS2 protein is involved in the maintenance of the integrity of the gastrointestinal tract. In breast cancer pS2 can be demonstrated in at least half of the tumours and probably reflects the functional status of ER. Several features make it likely that pS2 is involved in growth regulation.

Patients and Methods

We have investigated the value of immunohistochemical pS2 determination as a prognostic factor in 339 breast cancer patients with long follow-up from one hospital.

Results

A prognostic role for pS2 could not be demonstrated considering disease-free and overall survival, although in pS2-negative tumours a trend for less locoregional relapse was found. However, in multivariate analysis pS2 showed independent prognostic value for post-relapse survival.

Conclusions

PS2 is an independent prognostic factor for post-relapse survival, most likely because it is a predictive factor for response to systemic therapy.

Introduction

Twenty years ago the first of many reports on the prognostic significance of the oestrogen-receptor (ER) in patients with breast cancer was published¹. The larger studies with longer follow-up consistently demonstrate that disease-free survival in patients with ER-positive tumours is longer than in patients with ER-negative tumours¹⁻⁶. The disease-free survival advantage is 8-10% at 5 years³⁻⁵, but the difference tends to decrease with time⁷⁻⁹. A variety of proteins functionally related to the oestrogen-receptor have been reported to have prognostic significance in breast cancer, including the progesterone receptor (PR), cathepsin D and the pS2 protein. The pS2 protein was first identified in the human breast cancer cell line MCF-7 in response to oestrogen stimulation¹⁰. The pS2 protein is a small (6.45 kD) protein of repair function, and belongs to the trefoil peptides, that are mainly expressed in the gastrointestinal mucosa¹¹. The expression of pS2 and other trefoil peptides like pancreatic spasmic polypeptide is induced in inflammatory and ulcerative settings in the gastrointestinal tract and they have probably an important function in the maintenance of the gastrointestinal integrity¹². In tumours of the stomach¹³, biliary tract¹⁴, pancreas¹⁵, colon and rectum¹⁶ the pS2 protein has also been demonstrated. In normal breast tissue pS2 expression is lower than in uninvolved breast tissue from mastectomies for breast carcinomas¹⁷. This could indicate that some biochemical changes have taken place in the apparently uninvolved breast tissue during the development of the carcinoma. These changes could be hormone dependent because it has been suggested that pS2 expression reflects the functional status of ER. The fact that the pS2 gene has an upstream enhancer region that is responsive to oestrogen, plasminogen activator, activated Fos and Jun oncoproteins, the phorbol ester tetradecanoyl phorbol acetate (TPA) and epidermal growth factor (EGF) make it likely that pS2 is involved in growth regulation. This makes it attractive to investigate pS2 as a prognostic factor in breast cancer.

In human breast cancer associations have been demonstrated between pS2 and ER and PR status¹⁸⁻²⁰. Most studies, however, have found no significant associations between pS2 and nodal status or tumour size^{19,20}. First studies investigating the prognostic significance have used radioimmunoassays, and generally it has been reported that pS2 positivity is associated with a better relapse-free and overall survival^{19,21,22}. Sometimes, this has been observed, however, only for overall survival²³, in a small group of patients²⁰ or not at all²⁴. Several other investigators have determined the pS2 status by immunohistochemical techniques. Although similar rates of pS2 positivity have been observed compared with studies using radioimmunoassays, generally an association between immunohistochemical pS2 status and clinical outcome has not been found^{25,26,27}. However, in some investigations trends for better overall survival have been demonstrated for pS2 positive patients^{27,28}. These results could suggest that pS2 merely is a predictive

factor for response to hormonal therapy for both primary²³ and advanced breast cancer²⁹.

In order to elucidate the prognostic significance and also the predictive value of pS2 status as determined by immunohistochemical techniques we investigated a group of 339 patients from one hospital with a long follow-up period.

Patients and Methods

Patients

The 339 patients were treated at the University Hospital of Maastricht, between May 1982 and August 1987. Patients were selected according to the following criteria: 1. primary unilateral breast cancer without distant metastases, 2. no other primary tumour, 3. histological material available.

The clinical, histological and biological information on all patients were entered on a computerized database. All patients were staged at the time of diagnosis according to the International Union Against Cancer TNM Classification³⁰. The median age was 57 years (range 25-87 years). Two hundred and twenty-two patients (65.5%) had undergone a modified radical mastectomy, 98 patients (28.9%) a lumpectomy with axillary lymph node dissection and in 19 patients (5.6%) only a biopsy was performed either because of T₄ stage or high age, in two cases with an axillary lymph node dissection. One hundred eighty-three patients (54.0%) had no axillary lymph node metastases, while in 139 patients (41.0%) metastases were found in one or more axillary lymph nodes. Axillary lymph node-negative patients received no adjuvant systemic therapy. Axillary lymph node-positive patients below the age of 70 years were treated with adjuvant chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide. If these patients agreed, in addition they were randomized for concomitant treatment with MPA or no MPA. Clinical results of this multicenter trial have been described more in detail elsewhere^{31,32}. Axillary node-positive patients older than 69 years were treated with tamoxifen. The median follow-up was 128 months (range 61-170 months). One hundred forty-seven patients experienced a relapse during the follow-up period: 45 had a locoregional relapse, 85 distant metastases and 17 both simultaneously at the moment of first relapse.

Methods

ER and PR-status

The ER and PR assays were all performed on histologically proven breast cancer tissues using the dextran-coated charcoal method with multiple-point scatchard-plot

analysis³³.

For all the assays the minimum cytosol protein concentration was 2 mg/ml cytosol. PR status was determined only in patients entered from August, 1983.

Tumours were considered ER- or PR-positive when containing > 10 fmol/mg protein.

Immunohistochemical pS2-assay

Three μm sections were cut from routinely formalin-fixed and paraffin-embedded archival tumour samples. Deparaffinization in xylene and washes in 100% ethanol were followed by removing endogenous peroxidase in 0.3% H_2O_2 in methanol during 30 minutes at room temperature. After washing away the excessive amount of methanol in demineralized water trypsin digestion was performed. For this purpose the slides were incubated at 37°C for 10 minutes in 0.1% trypsin (pH=7.6)³⁴. Then the primary antiserum, a rabbit polyclonal antibody (NCL-pS2, Novacastra Laboratories Ltd (Sanbio)) was applied to the sections at a 1:400 dilution. Nonspecific binding of the antibody was blocked with 5% bovine serum albumin (BSA, Ria Grade, Sigma) in phosphate buffered saline (PBS, pH=7.4). Then the primary antibody was applied to the tumour sections. Excessive amounts of the antibody were washed away in PBS. The avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories, CA, USA) method was used to obtain amplification of the primary antigen-antibody bindings³⁵. These bindings were highlighted with DAB (di-amino-benzidin, Sigma). Finally counterstaining with hematoxylin completed the procedure.

The percentage of breast cancer cells showing a positive immunohistochemical reaction in a representative section of each tumour was determined by counting the number of stained cells in 500 cancer cells. The results were then classified into the following groups, comparable to previous described classifications^{27,28}: 0 if staining was negative, 1 if 1-10% of the cancer cells showed positive staining, 2 if 11-20% of the cancer cells showed positive staining and 3 if more than 20% of the cancer cells were positively stained.

Flow cytometric evaluation of ploidy status and S-phase fraction

Flow cytometric determination of DNA levels was performed in nuclei isolated from paraffin-embedded tissue^{36,37}. Fifty μm sections were cut from formalin-fixed paraffin-embedded tissue blocks of the primary tumours. An adjacent 5 μm section was cut for histological control. DNA content was measured by the method of Vindeløv³⁸. Tumours with a single G_1 peak were considered to be diploid, whereas evidence of an additional peak indicated aneuploidy. DNA index (DI) was calculated as the ratio of aneuploid to diploid G_1/G_0 peak level. Histograms with coefficients of variation less than 8% were considered of good quality. The

proliferative activity (SPF) was calculated by counting the number of cells between the inclination points of the descending G₁ peak and the ascending G₂/M peak^{39,40}. In cases of less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated without corrections for the presence of diploid S- and G₂/M-phase cells. In case of more than 30% admixture of diploid cells in overlap of diploid and hyperdiploid histograms the percentage of S-phase cells could not be calculated.

Statistical Analysis

Disease-free survival was defined as the time from the day of diagnosis till the time of first relapse, death or last follow-up.

Overall survival was defined as the time from the day of diagnosis till the day of death or last follow-up.

Statistical analysis was done using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-plus (Statistical Sciences Europe, Oxford, UK). The association between the expression of pS2 and other possible prognostic factors was analyzed by the chi-square test. Curves for disease-free survival and overall survival were estimated by the Kaplan-Meier method. Differences were analyzed using the logrank test.

Finally, prognostic variables were included in a Cox regression analysis.

For further survival analysis all patients showing any degree of pS2-positivity were considered as one group, as no difference between pS2-positive subgroups was demonstrated.

Results

From the 339 patients 162 (47.8%) were negative for pS2. In the 177 pS2-positive patients (52.2%) the majority (140 patients=41.3%) showed staining in only 1-10% of cells, while in 21 patients (6.2%) staining was seen in 11-20% of cells and in 16 patients (4.7%) in more than 20% of cells.

A significant association was found between pS2 expression and ER-status ($p < 0.05$), however, this was not found with PR. No relationship was demonstrated between pS2-positivity and age, histological type, tumour-size, S-phase fraction, ploidy status and axillary lymph node status (table II.1).

Table II.1. Correlation between pS2 status and other clinical, pathological and biological factors.

	pS2-negative	pS2-positive	p-value
ER (n=328)			
≤ 10 fmol/mg protein	60	32	0.0004
> 10 fmol/mg protein	97	139	
PR (n=263)			
≤ 10 fmol/mg protein	56	65	NS*
> 10 fmol/mg protein	63	79	
Age (n=339)			
≤ 50 years	50	61	NS
> 50 years	112	116	
Histological type (n=339)			
ductal carcinoma	120	130	NS
other types	42	47	
Tumour size (n=339)			
T ₁	71	78	NS
T ₂ , T ₃ , T ₄	91	99	
Node status (n=322)			
negative	86	97	NS
positive	66	73	
SPF (n=220)			
≤ 8.0	45	57	NS
> 8.0	55	63	
Ploidy status (n=318)			
diploid	61	67	NS
aneuploid	92	98	

* NS=not significant

No difference in both disease-free and overall survival for pS2-positive and pS2-negative patients was found. This was the case for both the axillary node-positive and node-negative subgroups. Only when prognosticating the risk for locoregional recurrence there was a trend for better outcome in pS2-negative patients ($p=0.08$, see figure II.1). Looking at ER-positive and ER-negative subgroups the pS2-status also had no additional prognostic value with respect to disease-free and overall survival. We also have investigated the possible role of pS2 in predicting the response to adjuvant therapy with MPA. Such a role could not be demonstrated whereby it has to be kept in mind that the number of patients was small (30 patients in both groups).

Figure II.1 Locoregional relapse specific cumulative incidence curves for pS2-negative and pS2-positive patients ($p=0.08$)

In pS2-positive patients the survival time after the first locoregional relapse and/or distant metastasis was found to be significantly longer than in pS2-negative patients ($p=0.002$, see figure II.2). In a multivariate analysis including lymph node status, ER, PR, age, ploidy status and S-phase, pS2 positivity was the only predictive factor for postrelapse survival ($p=0.006$, see table II.2). After excluding pS2 from this multivariate analysis, ER showed a significant association with post-relapse survival ($p=0.04$).

Figure II.2 Survival after first relapse (locoregional and/or distant metastasis) for pS2-negative and pS2-positive patients ($p=0.002$)

Table II.2. Cox multivariate analysis for post-relapse survival.

	RHR ¹	p-value
pS2 (positive vs. negative)	0.574	0.006
Node status (negative vs positive)	1.226	0.330
ER (≤ 10 vs > 10 fmol/mg protein)	1.438	0.170
PR (≤ 10 vs > 10 fmol/mg protein)	1.423	0.140
Ploidy status (diploid vs aneuploid)	1.171	0.510
SPF (≤ 8.0 vs $> 8.0\%$)	0.664	0.120
Age (≤ 50 vs > 50 years)	0.855	0.490

¹ relative hazard rate

Discussion

We have investigated the relationship between immunohistochemical pS2 expression and a number of other possible prognostic factors, and the possible influence of pS2 positivity on disease-free, overall and post-relapse survival, in patients with primary breast cancer.

This is the first study demonstrating a significant difference in post-relapse survival between pS2-positive and pS2-negative patients, also in multivariate analysis. This probably implies that pS2 may be a predictive factor for response to systemic therapy, although this issue was not formally addressed in this study. The same has been found in two other studies in univariate analysis^{27,28}. In one of these studies a significant difference was only found in the subgroup of patients treated by chemotherapy or local therapy²⁷. Altogether the data suggest that pS2 is more a predictive than a prognostic factor, which has been supported by a study investigating the value of pS2 for predicting the response to tamoxifen therapy²⁹.

Slightly more than half of the tumours were pS2-positive, which is in the range of 49-73% reported in other studies that used immunohistochemical methods, although with slightly different cut-off points^{25,27,28}.

In our study we have found an association of pS2 with ER-status, which is in accordance with all other studies¹⁹⁻²⁹. However, in contrast to the other studies^{19-24,26-29} no association with PR status has been found. In an earlier study PR was also not a prognostic factor using a cut-off point of 10 fmol/mg protein⁴¹.

Correlations between S-phase fraction and pS2 status have not been demonstrated in our study, neither between ploidy status and pS2 status. These correlations have not been investigated in the literature before. One study has reported a negative correlation between pS2 status and another proliferation marker²⁶, the thymidine

labelling index. Such a negative correlation seems, however, consistent with the fact that pS2-deficient mice develop large adenomas in the stomach by five months of age and in 30% even intra-epithelial and intramucosal carcinomas¹¹. Whether pS2 has a comparable role in the development of breast cancer is unknown.

Data on the prognostic significance of pS2 in breast cancer are certainly not consistent. We could not demonstrate a relation between pS2-status and disease-free or overall-survival. Also, no other study using the easier immunohistochemical method has found prognostic significance for pS2 status in multivariate analysis²⁵⁻²⁸, although in univariate analysis this was sometimes demonstrated for node-negative patients²⁵, for node-positive patients²⁸ or for overall survival only²⁷. Most studies have a median follow-up of at least 70 months²⁵⁻²⁸.

On the contrary the majority of studies using cytosol assays have found a better outcome for both disease-free and overall survival in patients with pS2-positive tumours even in multivariate analysis¹⁹⁻²². However, in one study only a relation with overall survival has been demonstrated²³, while in another study the prognostic significance of pS2 was lost in multivariate analysis²⁴. Of importance is that the median follow-up in all these studies was 6 years at most.

It cannot be excluded that the prognostic significance of pS2 is time-dependent, although this is not very likely in view of the sometimes large differences in the studies using cytosolic assays. Probably cytosolic assays and immunohistochemical methods are measuring different aspects of these proteins. The immunohistochemical method is thereby easy to perform in a routine laboratory and not time-consuming in contrast to the cytosolic assay.

In conclusion, pS2 measured by immunohistochemistry has no role as a prognostic factor in breast cancer. However, pS2 is an independent prognostic factor for post-relapse survival. The most likely explanation for this phenomenon is that pS2 is a predictive factor for response to systemic therapy.

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Chapter III

CD44v6 is not a prognostic factor in primary breast cancer

RLH Jansen, SR Joosten-Achjanie, JW Arends,
A Volovics, PSGJ Hupperets, HC Schouten, HFP Hillen

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Summary

Background

CD44 is an adhesion molecule and represents a highly variable family of isoforms. The isoform CD44v6 has been associated with metastasis formation and poor prognosis in animal models and human colon cancer. Results of studies in primary breast cancer are relatively small and contradictory.

Patients and Methods

The immunohistochemical expression of CD44v6 was studied in a series of 338 patients with primary breast tumours, uniformly staged and treated in a single center with a long median follow-up of 128 months. The prognostic significance of CD44v6 as well as the correlation with several clinicopathological features were analysed.

Results

Two hundred nineteen of 338 (64.8%) of the breast cancers were CD44v6-positive (>5% of tumour cells with positive staining). CD44v6 expression had no value for prognosticating disease-free or overall survival at this or any other cut-off point.

Conclusion

CD44v6 expression is not a prognostic factor in primary breast cancer.

Introduction

The hyaluronate receptor CD44 is an adhesion molecule and represents a highly variable family of isoforms, with putative functions in development, somatic cell functions and metastatic disease¹. Many variant isoforms exist characterized by the insertion of additional amino acids in the extracellular domain of the protein (CD44v1-CD44v10)^{2,3}. Isoforms containing the exon v6 encoded domains seem to promote tumour dissemination in rat pancreas carcinoma⁴ and have been associated with poor prognosis in human colon carcinoma⁵. The first study on the prognostic value of CD44v6 in 100 patients with primary breast cancer has shown it to be an independent prognostic marker⁶. This result could not be confirmed in a second study with 108 node-negative and 119 node-positive patients⁷. Discrepancy could be due to the different patient numbers and relatively short follow-up. Therefore, we have performed a study on the possible use of CD44v6 as a prognostic factor in a large group of 338 patients with primary breast cancer and a median follow-up of more than 10 years.

Patients and Methods

Patients

In the period May 1982-August 1987 the 338 patients were treated for primary breast cancer in the University Hospital Maastricht. All patients were staged at the time of diagnosis according to the TNM classification. The median age was 57 years (range 25-87 years). Two hundred eighteen patients (64.5%) had undergone a modified radical mastectomy, 97 patients (28.7%) a lumpectomy with axillary lymph node dissection, whereas in 23 patients (6.8%) only a biopsy was done either because of T₄ stage or high age, in 4 patients with an axillary lymph node dissection. One hundred eighty-three patients (54.2%) had no axillary lymph node metastases, 136 patients (40.2%) had metastasis in one or more axillary lymph nodes, while in 19 patients (5.6%) the axillary lymph node status was unknown. Axillary lymph node-positive patients younger than 70 years were treated with adjuvant chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide. If they agreed to participate in a multicenter clinical trial, they were randomized for concomitant treatment with medroxyprogesterone acetate (MPA) or no MPA. The results of this clinical trial have been described more in detail elsewhere⁸. The median follow-up was 128 months (range 61-170 months). One hundred forty-six patients experienced a relapse: 44 had a locoregional relapse, 86 distant metastases and 16 patients both simultaneously at the moment of first relapse.

Methods

Steroid receptors

The oestrogen-receptor (ER) and progesterone-receptor (PR) assays were all performed on histologically proven breast cancer tissues using the dextran-coated charcoal method with multiple-point scatchard-plot analysis.

For all the assays the minimum cytosol protein concentration was 2 mg/ml cytosol. PR status was determined only in patients entered from August 1983 (n=261).

Immunohistochemistry

Three μm sections were cut from routinely formalin-fixed and paraffin-embedded archival tumour blocks. Immunohistochemical staining was performed using the mouse monoclonal antibody against CD44v6 (R&D Systems, Abingdon, UK; 1:1000; one hour, room temperature). Microwave antigen retrieval with citric buffer (ph=6.0) was administered. The three-stage avidin-biotin-peroxidase complex method was used (Vectastain ABC Kit, Vector Laboratories, CA, USA).

The antibodies were diluted in PBS containing 0.5% BSA. The staining was highlighted with DAB (di-amino-benzidin, SIGMA) and counterstained with hematoxylin. If more than 5% of tumour cells showed CD44v6 expression, the tumour was considered CD44v6-positive. At least 1000 tumour cells were counted. However, also other cut-off points as well as the intensity of staining in combination with a cut-off of 5% positive tumour cells were analysed^{6,7}.

For other immunohistochemical staining procedures the mouse monoclonal antibody MIB-1 (Dianova, Hamburg, Germany; 1:100, one hour incubation at room temperature) and rabbit polyclonal antibody NCL-pS2 (Novacastra Laboratories Ltd, Sanbio, Newcastle, UK; 1:400, one hour incubation at room temperature) were used. Antigen unmasking by microwave antigen retrieval was necessary for MIB-1 staining. Tumours were considered pS2-positive in the case of $\geq 1\%$ of tumour cells staining.

Flow cytometric evaluation of ploidy status and S-phase fraction

Flow cytometric evaluation of ploidy status and S-phase fraction was done as described previously⁹.

Statistical Analysis

Disease-free survival was defined as the time from the day of diagnosis till the time of first relapse, death or last follow-up.

Overall survival was defined as the time from the day of diagnosis till the day of death or last follow-up.

Statistical analysis was done using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-plus (Statistical Sciences Europe, Oxford, UK). The association between the expression of CD44v6 and other possible prognostic factors was analyzed by the chi-square test. Curves for disease-free survival and overall survival were estimated by the Kaplan-Meier method. Differences were analyzed using the logrank test.

Results

CD44v6 staining was membranous, and mostly heterogeneous. Staining was seen in 0-100% (median 13%) of tumour cells. In most cases moderate to strong staining intensity was seen. With a cut-off point of 5% 219 of 338 (64.8%) of tumours were positive. CD44v6 expression was associated with smaller tumour size ($p < 0.01$) and the absence of axillary lymph node metastases ($p < 0.001$) (table III.1). No correlation could be demonstrated with ER, PR, histology, pS2, S-phase fraction, ploidy, MIB-1, and age (table III.1). In univariate analysis CD44v6-positivity did not influence the disease-free survival ($p = 0.19$) (Figure III.1). Also no correlation with overall survival was demonstrated. Furthermore, looking at node-positive and node-negative subgroups no difference in disease-free or overall survival was found between CD44v6-positive and negative patients. When CD44v6 was analyzed as a continuous variable, then it showed no risk differentiation at any cut-off point both for disease-free and overall survival. When staining intensity was taken into account (positive = $> 5\%$ positive tumour cells and at least moderate staining) the association with nodal status was only borderline ($p = 0.06$) and the association with T stage was lost. With this cut-off point also no difference between CD44v6-positive and CD44v6-negative patients was seen in disease-free and overall survival. Furthermore, CD44v6 did not influence post-relapse survival.

Discussion

Our results do not confirm the results of the first published study on the prognostic value of CD44v6⁶. These first data have, however, been questioned for several reasons, e.g. small numbers of patients, relatively short follow-up and the use of a polyclonal antibody¹⁰. The results of our study are, however, quite similar to those of another quite large study with 227 patients⁷. In this study 56% of breast cancers were CD44v6-positive and as in our study a slightly better disease-free survival has been found for CD44v6-positive patients although this trend did not reach statistical significance⁷. A smaller study has also not been able to demonstrate a prognostic value for CD44v6¹¹ while another reports a prognostic impact only in univariate

analysis¹². In the published studies quite different cut-off points are used, varying from all positive staining¹¹ to a multiplication of percentage positive cells and staining intensity¹³ or only strong and/or widespread staining¹². Furthermore, different antibodies were used. These facts could explain the varying results with respect to the prognostic value of CD44v6. We have also looked at CD44v6 as a continuous variable, but we were not able to demonstrate a prognostic significance with any cut-off point for both disease-free and overall survival, using a specific monoclonal antibody.

Besides the results of our and several other studies the fact that CD44v6 is often expressed in normal tissues¹⁴ including normal breast tissue^{7,13,14} argue against an important role of CD44v6 in metastasis formation.

The expression of CD44 isoforms in tumour cells is much more complicated than simple the expression of a metastasis promoting factor. Isoforms of CD44 have different functions, and possibly different ligands depending on the microenvironment. As suggested recently the measurement of specific ligand binding may be more informative than the single determination of CD44 isoform expression to elucidate the role of CD44 in the progression of cancer¹⁵.

So in conclusion, despite the important role of CD44v6 in an animal model system, the results of this as well as most other smaller studies make it unlikely that CD44v6 is an important prognostic factor in breast cancer.

Figure III.1 Disease-free survival curves for the CD44-positive (>5% staining) and CD44-negative ($\leq 5\%$ staining) patients

Table III.1 Correlation between CD44v6 and other prognostic factors

	CD44 negative ¹	CD44 positive ²	n (number)	p-value
ER ≤ 10 fmol/mg protein	36	59	327	0.46
ER > 10 fmol/mg protein	78	154		
PR ≤ 10 fmol/mg protein	45	76	261	0.62
PR > 10 fmol/mg protein	48	92		
Ductal carcinoma	93	159	338	0.26
Other histological types	26	60		
Tumour size: T=1	40	107	338	0.007
T>1	79	112		
Axillary node-negative	47	136	319	<0.001
Axillary node-positive	67	69		
pS2 negative	59	99	329	0.34
pS2 positive	56	115		
MIB-1 > 7%	63	97	335	0.12
MIB-1 ≤ 7%	55	120		
S-phase fraction > 8%	37	84	220	0.93
S-phase fraction ≤ 8%	32	67		
Diploid	38	88	317	0.14
Aneuploid	73	118		
Age > 50 years	34	78	338	0.19
Age ≤ 50 years	85	141		

¹ ≤5% of tumour cells with positive staining; ² >5% of tumour cells with positive staining

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Chapter IV

MIB-1 labelling index is an independent prognostic marker in primary breast cancer

RLH Jansen, PSGJ Hupperets, JW Arends,
SR Joosten-Achjanie, A Volovics, HC Schouten,
HFP Hillen

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Summary

The proliferative activity of a tumour is considered to be an important prognostic factor in primary breast cancer. We have investigated the prognostic value of the MIB-1 labelling index in 341 patients with primary breast cancer and compared the results with the S-phase fraction in 220 patients of the same cohort. All patients were treated in one hospital and had a median follow-up of 128 months. No correlation between MIB-1 labelling and S-phase fraction could be demonstrated. MIB-1 had prognostic value for disease-free survival in the whole group of patients ($p < 0.001$) and in the node-negative subgroup ($p < 0.001$). In multivariate analysis MIB-1 was an independent prognostic factor ($p = 0.004$) besides axillary lymph node status ($p = 0.001$). In univariate analysis high S-phase fraction was associated with decreased overall survival ($p = 0.04$); however, not in multivariate analysis. Moreover, S-phase fraction had a borderline prognostic significance for post-relapse survival in multivariate analysis ($p = 0.08$). Thus, in conclusion the growth fraction of a tumour as determined by the MIB-1 labelling index is an important prognostic factor in patients with primary breast cancer.

Introduction

In primary breast cancer the axillary lymph node status is still the most important prognostic factor and is used for deciding on adjuvant treatment. However, an axillary lymph node dissection itself has no or at best a very limited influence on disease-free survival and it causes substantial morbidity^{1,2}. Moreover, the prognostic value of the axillary lymph node status is not absolute, as 30% of node-negative patients die within 10 years because of recurrent disease and 30% of node-positive patients survive 10 years without disease³. Therefore, routine axillary lymph node dissection has recently become a matter of debate² and search for other factors to identify patients at high risk of (early) relapse is thus needed. Many prognostic factors have been investigated, but so far no single factor or combination of factors can be used for treatment decisions in an individual patient. The proliferative activity of a tumour is, however, an important prognostic factor known to have an inverse relationship with the survival of patients with breast cancer. It can be measured by different methods, which all have their own advantages and disadvantages.

First, counting the number of mitoses in a hematoxylin- and eosin-stained slide is still an inexpensive method for the assessment of tumour cell proliferation. The mitotic index (MI) may be reproducible as shown in one study⁴, but there is no general agreement on this. The independent prognostic value of the MI has been shown in a few studies⁵.

Second the thymidine labelling index (TLI), reflecting the proliferative activity, has been claimed to be a strong and independent prognostic factor^{6,7}. However, like the bromodeoxyuridine labelling index, viable tissue is needed, which make these methods hard to apply in a routine setting.

Third, DNA flow cytometry can be used to measure the percentage of cells in the S-phase in the cell cycle. In 1992 the DNA Cytometry Consensus Conference concluded that the literature supported a clear association between high S-phase fraction and an increased risk of recurrence and mortality for both axillary node-negative and node-positive breast cancer patients⁸. A disadvantage of this technique, however, is that tumour heterogeneity cannot be assessed, and 10-20% of specimens are not evaluable because of a large coefficient of variation or admixture of stromal cells.

Finally, in the evaluation of proliferative activity there are also immunohistochemical methods using antibodies directed against nuclear antigens expressed during the cell cycle. Mainly applied is the Ki-67 antibody which binds to a large, non-histone nuclear protein that is expressed in the late G₁, S, G₂ and M phase of the cell cycle. Originally the antibody could only be used on fresh or frozen sections. More recently monoclonal antibodies such as MIB-1, raised against parts of Ki-67 antigen, have become available that can be used on formalin-fixed and routinely

processed archival tissue⁹. Several studies have shown a close correlation between the Ki-67 results on frozen sections and the MIB-1 findings on paraffin sections⁹⁻¹¹, but the prognostic value of both antibodies is not necessarily the same. Many studies have been performed using Ki-67 in breast cancer and at least 9 of these studies reported on correlations between Ki-67 and disease-free survival (DFS) and/or overall survival (OS)¹²⁻¹⁹. Seven of these studies demonstrated significant difference with respect to prognosis in patients with high and low Ki-67 labelling^{12-16,18,20}. However, follow-up was mostly short and, in addition, in only four of these studies multivariate analyses has been performed^{14,15,17,20}. Three of these studies showed significant correlations between high Ki-67 labelling and shorter disease-free survival^{14,15,20}.

Fewer studies have been performed with the MIB-1 antibody. In general, the MIB-1 index also seems to be a prognostic factor sometimes even in multivariate analyses, but follow-up is generally rather short and/or the number of patients is low^{11,21-24}.

Not many studies have been performed comparing the different methods of assessing the proliferative activity of a tumour. Because immunohistochemical methods seem most suitable in a routine setting, we investigated MIB-1 immunoreactivity in a group of 341 patients uniformly treated in one hospital with a median follow-up of 128 months, have compared the results with the S-phase fraction in 220 patients of the same cohort, and examined correlations with other clinicopathological factors previously studied by our group.

Patients and Methods

Patients

The 341 patients were treated at the University Hospital Maastricht in the period May 1982 - August 1987. Patients were selected according to the following criteria: 1. primary unilateral breast cancer without distant metastases; 2. no other primary tumour; 3. histological material available. All patients were staged at the time of diagnosis according to the International Union Against Cancer TNM Classification. The median age was 57 years (range 25-87 years). A total of 220 patients (64.5%) had undergone a modified radical mastectomy, 97 patients (28.5%) a lumpectomy with axillary lymph node dissection and in 24 patients (7.0%) only a biopsy was performed, either because of T₄ stage or advanced age, in 4 patients with an axillary lymph node dissection. A total of 183 patients (53.7%) had no axillary lymph node metastases, 138 patients (40.4%) had metastases in one or more axillary lymph nodes, whereas in 20 patients (5.9%) the axillary lymph node status was unknown. Axillary lymph node-positive patients

younger than 70 years were treated with adjuvant chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide. If they agreed to participate in a clinical trial, they were randomized for concomitant treatment with or without medroxyprogesterone acetate. The results of the clinical trial have been described elsewhere^{25,26}. Axillary lymph node-negative patients received no adjuvant systemic therapy. The median follow-up of all patients was 128 months (range 61-170 months).

Methods

Steroid-receptors

The oestrogen receptor (ER) and progesterone receptor (PR) assays were all performed on histologically proven breast cancer tissues using the dextran-coated charcoal method with multiple-point scatchard-plot analysis.

For all the assays the minimum cytosol protein concentration was 2 mg/ml cytosol. PR status was determined only in patients entered from August 1983. Tumours with ER or PR > 10 fmol/mg protein were considered ER- or PR-positive.

Flow cytometric evaluation of ploidy status and S-phase fraction

Flow cytometric determination of DNA levels was performed on nuclei isolated from paraffin-embedded tissue^{27,28}. Sections (50 μ m) were cut from formalin-fixed paraffin-embedded tissue blocks of the primary tumours. An adjacent 5 μ m section was cut for histological control. DNA content was measured by the method of Vindeløv²⁹. Tumours with a single G₁ peak were considered to be diploid, whereas evidence of an additional peak indicated aneuploidy. DNA index (DI) was calculated as the ratio of aneuploid to diploid G₁/0 peak level. Histograms with coefficients of variation less than 8% were considered of good quality. The S-phase fraction (SPF) was calculated by counting the number of cells between the inclination points of the descending G₁ peak and the ascending G₂/M peak³⁰. In cases of less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated without corrections for the presence of diploid S- and G₂/M-phase cells. In case of more than 30% admixture of diploid cells in overlap in diploid and hyperdiploid histograms the percentage of S-phase cells was not calculated. After descriptive analysis the cut-off levels for the proportion of S-phase cells were set at $\leq 8\%$ and $> 8\%$ in order to define two groups, with low and high SPF respectively.

Immunohistochemistry

Staining was performed using the mouse monoclonal antibody MIB-1 (Dianova,

Hamburg, Germany; 1:100, 1 hour incubation at room temperature) and rabbit polyclonal antibody NCL-pS2 (Novocastra Laboratories Ltd, Sanbio, Newcastle, UK; 1:400, 1 hour incubation at room temperature). All antibodies were diluted in 0.5% BSA (bovine serum albumin, Sigma) containing PBS (phosphate buffered saline, pH=7.4). To reach optimal staining results for MIB-1 antigen unmasking was necessary by microwave antigen retrieval with citric buffer (0.01 M, pH=6.0)³¹. All staining procedures were done using a standard method. In short: 3 μ m sections were cut from routinely formalin-fixed and paraffin-embedded archival tumour samples. The MIB-1 sections were incubated overnight in a 60°C oven on APS (3-aminopropyltriethoxysilane, Sigma) coated glass slides, to obtain optimal fixation. Deparaffinization in xylene and washes in 100% ethanol were followed by removing endogenous peroxidase in 0.3% H₂O₂ containing methanol (30 minutes, room temperature). After washing away the excessive amount of methanol in demineralized water, the necessary antigen unmasking procedure was performed. Non-specific binding of the antibodies was blocked with 5% BSA containing PBS. Then, the primary antibodies were applied to the tumour sections. Excessive amounts of antibodies were washed away in PBS. The avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories, CA, USA) method was used to obtain amplification of the primary antigen-antibody bindings. These bindings were highlighted with DAB (di-amino-benzidin, Sigma). Finally, counterstaining with hematoxylin completed the procedure.

The percentage of breast cancer cells showing a positive immunohistochemical reaction in a representative section of each tumour was determined by counting the number of positively stained cells in 1000 cancer cells for MIB-1 and 500 tumour cells for pS2. For pS2 tumours were considered positive if at least 1% of tumour cells showed staining comparable to previously described classifications³².

Statistical Analysis

Disease-free survival was defined as the time from the day of diagnosis till the time of first relapse, death or last follow-up.

Overall survival was defined as the time from the day of diagnosis till the day of death or last follow-up.

Statistical analysis was performed using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-plus (Statistical Sciences Europe, Oxford, UK). The association between the expression of MIB-1 and other possible prognostic factors was analyzed by the chi-square test. Curves for disease-free survival and overall survival were estimated by the Kaplan-Meier method. Differences were analyzed using the logrank test. Finally, prognostic variables were included in a Cox regression analysis.

Results

MIB-1 labelling index

Of the 341 tumours 14 (4.1%) showed less than 1% positive tumour cells. The distribution of the percentage of staining tumour cells was asymmetric (range 0-71%; mean 11.0%, median 7.0%). The relationship between MIB-1 labelling index when dichotomized at the median value ($\leq 7\%$ versus $> 7\%$) and other clinical and histological variables is shown in table IV.1. High MIB-1 was associated with aneuploidy ($p=0.005$), ER-negativity ($p<0.001$), PR-negativity ($p=0.01$), the presence of axillary lymph node metastases ($p<0.001$) and larger tumour size ($p=0.02$). MIB-1 staining showed no relation with age, histology or pS2 status. Concerning histology, however, all 9 tubular carcinomas had a low MIB-1 labelling index, while medullary carcinomas had a significantly higher MIB-1 labelling index than ductal carcinomas ($p<0.05$). No correlation at all was shown between high MIB-1 and high S-phase fraction ($p=0.50$), while the Pearson correlation coefficient was 0.15. For diploid tumours the Pearson correlation coefficient was -0.09 and for aneuploid tumours 0.25.

Table IV.1 Correlation between MIB-1 and other factors.

	MIB-1 $\leq 7\%$	MIB-1 $> 7\%$	number	p-value
ER ≤ 10 fmol/mg protein	35	60	330	<0.001
ER > 10 fmol/mg protein	134	101		
PR ≤ 10 fmol/mg protein	55	67	264	0.01
PR > 10 fmol/mg protein	86	56		
Age ≤ 50 years	59	52	341	0.74
Age > 50 years	118	112		
Ductal carcinoma	126	127	341	0.18
Other histological types	51	37		
Node-negative	111	72	321	<0.001
Node-positive	57	81		
Tumour size: T=1	88	61	341	0.02
T>1	89	103		
Diploid	76	50	320	0.005
Aneuploid	86	108		
S-phase fraction $\leq 8\%$	57	43	220	0.50
S-phase fraction $> 8\%$	63	57		
pS2 negative*	85	75	332	0.72
pS2 positive*	88	84		

* by immunohistochemistry

In univariate analysis it was demonstrated that high MIB-1 ($>7\%$) was associated significantly with shorter disease-free survival ($p<0.001$, see figure IV.1). Looking at subgroups, the same was found for node-negative patients ($p<0.001$; see figure IV.2) while in node-positive patients no significant difference in disease-free survival was demonstrated ($p=0.24$, see figure IV.2). Furthermore, every cut-off point of MIB-1 from 2% to 25% showed a significant effect on disease-free survival in univariate analysis.

Figure IV.1 Disease-free survival curves for the whole group of 341 patients with low MIB-1 labelling index ($\leq 7\%$) and high MIB-1 labelling index ($> 7\%$)($p<0.001$).

Figure IV.2 Disease-free survival curves for the group of 183 node-negative patients and the group of 138 node-positive patients with low MIB-1 labelling index ($\leq 7\%$) and high MIB-1 labelling index ($> 7\%$)($p<0.001$ and $p=0.24$)

In multivariate analysis high MIB-1 ($>7\%$) was, besides the presence of axillary lymph node metastases the only independent prognostic factor for shorter disease-free survival ($p=0.004$; see table IV.2). The same was true when MIB-1 was analysed as a continuous variable ($p=0.005$, data not shown). High MIB-1 ($>7\%$) showed a borderline significant association with overall survival in the whole group of patients ($p=0.05$, see figure IV.3) but not in the node-negative and node-positive subgroups.

In multivariate analysis for overall survival only the presence of axillary lymph node metastases, T-stage more than T_2 and age above 50 years, but not MIB-1 labelling index were independent prognostic factors (see table IV.3). Furthermore, MIB-1 was not associated with post-relapse survival.

Table IV.2 Multivariate analysis for disease-free survival.

	RHR*	p-value
MIB-1 (> 7 vs $\leq 7\%$)	1.668	0.004
T-stage (T2 vs T1)	1.311	0.154
T-stage (T3 vs T1)	1.760	0.054
Node status (positive vs negative)	1.839	0.001
Ploidy status (aneuploid vs diploid)	1.412	0.122
SPF (> 8 vs $\leq 8\%$)	1.281	0.287
ER (> 10 vs ≤ 10 fmol/mg protein)	1.430	0.089
PR (> 10 vs ≤ 10 fmol/mg protein)	0.876	0.500
Age (> 50 vs ≤ 50 years)	1.007	0.971

* relative hazard rate

Table IV.3 Multivariate analysis for overall survival.

	RHR*	p-value
MIB-1 (> 7 vs $\leq 7\%$)	1.091	0.598
T-stage (T2 vs T1)	1.262	0.205
T-stage (T3 vs T1)	2.001	0.014
Node status (positive vs negative)	1.595	0.008
Ploidy status (aneuploid vs diploid)	0.933	0.741
SPF (> 8 vs $\leq 8\%$)	1.451	0.089
ER (> 10 vs ≤ 10 fmol/mg protein)	1.194	0.370
PR (> 10 vs ≤ 10 fmol/mg protein)	0.802	0.241
Age (> 50 vs ≤ 50 years)	1.827	0.002

Figure IV.3 Overall survival curves for the whole group of 341 patients with low MIB-1 labelling index ($\leq 7\%$) and high MIB-1 labelling index ($> 7\%$)($p=0.05$)

Figure IV.4 Overall survival curves for the group of 220 patients with low S-phase fraction ($\leq 8\%$) and high S-phase fraction ($> 8\%$)($p=0.04$)

S-phase fraction

High S-phase fraction ($> 8\%$ vs $\leq 8\%$) was related with a significantly decreased overall survival in univariate analysis ($p=0.04$; see figure IV.4). The effect of high S-phase fraction on overall survival was lost in multivariate analysis (see table IV.3). Concerning disease-free survival the effect of high S-phase fraction was borderline significant ($p=0.06$). With respect to post-relapse survival high S-phase fraction was also an unfavourable prognostic factor ($p=0.03$). In multivariate analysis S-phase fraction was a borderline prognostic factor looking at post-relapse survival ($p=0.08$; see table IV.4).

Table IV.4 Multivariate analysis for post-relapse survival.

	RHR*	p-value
MIB-1 (>7 vs $\leq 7\%$)	0.959	0.838
T-stage (T2 vs T1)	1.328	0.261
T-stage (T3 vs T1)	2.417	0.019
Node status (positive vs negative)	1.202	0.428
Ploidy status (aneuploid vs diploid)	0.950	0.841
SPF (>8 vs $\leq 8\%$)	1.585	0.083
ER (>10 vs ≤ 10 fmol/mg protein)	0.624	0.073
PR (>10 vs ≤ 10 fmol/mg protein)	0.768	0.263
Age (>50 vs ≤ 50 years)	1.300	0.242

* relative hazard rate

Combined prognostic value of MIB-1 and S-phase fraction

Looking at a possible combined prognostic value of MIB-1 and S-phase fraction only with respect to disease-free survival MIB-1 labelling index was a significant prognostic factor both for patients with tumours with low and high S-phase fraction ($p < 0.01$ in both cases). Concerning overall survival MIB-1 labelling index had an additional prognostic value only in case of a high S-phase fraction ($p = 0.03$). With respect to post-relapse survival no differences between subgroups could be demonstrated.

Discussion

In this study with long follow-up, MIB-1 expression was a significant prognostic factor for disease-free survival both in univariate and multivariate analysis, for both the whole group of patients and the 183 node-negative patients. MIB-1 expression had borderline significant influence on overall survival. Not many studies have as yet investigated MIB-1 as a prognostic factor. Three studies have demonstrated a significant influence of MIB-1 on overall survival but have not mentioned disease-free survival^{21,22,33}. The largest study on MIB-1 has shown a significant influence of MIB-1 on both disease-free and overall survival in multivariate analysis²³ after a follow-up of 66 months. Remarkably, however, the by far largest study on Ki-67 in 674 node-negative breast cancer patients has found a significant influence on disease-free but not overall survival as in our study²⁰. Several other studies have also shown a significant association of Ki-67 and disease-free survival^{12,16,18} and Ki-67 and overall survival^{16,18}. Only two studies have not found such an association

between Ki-67 and disease-free survival^{17,19}. The results of the latter two studies could be explained by dividing the patients in three groups¹⁷ and by a relatively short follow-up period¹⁹ respectively. However, multivariate analysis was not often performed. In our study we have found a significant influence of MIB-1 on disease-free survival in node-negative, but not node-positive patients. Comparable results have been found in two other studies^{24,34}, while one study has demonstrated an effect of MIB-1 on disease-free survival for both groups of patients²³. A difference in the prognostic value of MIB-1 for node-negative and node-positive patients could be explained by more susceptibility for adjuvant (chemo)therapy in node-positive patients with high MIB-1 labelling index, whereas node-negative patients were not treated by adjuvant systemic therapy.

The median percentage of MIB-1 positive cells in our patients (7%) tended to be lower than the median value of 16-20% reported from several other studies^{11,21,22}, but is comparable with the median value of two other reports^{19,35}. For Ki-67 as measured in frozen sections also quite different median values varying from 2%²⁰ to 12%¹² are reported. The reason for the considerable variability in Ki-67 and MIB-1 scores in the different studies is not directly clear. It seems likely that these differences can be explained at least partly because of different methodology (different antibodies, different staining methods, different ways of counting). Therefore, it is certainly possible that comparable results of the MIB-1 (and Ki-67) labelling index will be found in different laboratories, when an uniform methodology is applied. In general, in comparative studies a good statistical correlation is found between Ki-67 and MIB-1^{10,11}.

In this study a low correlation coefficient of 0.15 between MIB-1 and SPF was found, which seems in line with the result of another study reporting on low correlation coefficients between Ki-67 and SPF²⁰ (Spearman rank correlation of 0.15). Several other studies reported higher correlation coefficients between Ki-67 and SPF¹⁹ and between MIB-1 and SPF³⁵. In general, correlations are based on aneuploid tumours as in our study^{35,36}. However, it has to be kept in mind that the Ki-67/MIB-1 nuclear antigen is present in all parts of the cell cycle whereas S-phase only relates to one specific stage in the cell cycle.

In the literature, data on the prognostic significance of SPF are conflicting and sometimes based on a low number of patients. When comparing MIB-1 and SPF in multivariate analysis one study has found SPF to be better than MIB-1³⁶ contradictory to our results. This could be explained by different methodology and patient selection. SPF is, however, not a method that is easily applied in routine practice.

Therefore, at this moment Ki-67 as measured on frozen sections or MIB-1 as measured on paraffin sections is probably the best method for assessing proliferation in a routine setting. This and several other studies have demonstrated the independent prognostic value of Ki-67 and MIB-1 in breast cancer patients. As

with most other newer prognostic markers, however, at this moment it is not possible to use them for clinical decision-making. For that purpose it is at least necessary that a uniform methodology is developed.

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Chapter V

Relevance of the expression of bcl-2 in combination with p53 as a prognostic factor in breast cancer

RLH Jansen, SR Joosten-Achjanie, A Volovics,
JW Arends, PSGJ Hupperets, HFP Hillen,
HC Schouten

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Abstract

Background

Both the proto-oncogene *bcl-2* and the tumour suppressor gene *p53* are involved in the regulation of apoptosis.

Patients and methods

We have investigated the prognostic value of the immunohistochemical expression of *p53* and *bcl-2* separately and in combination in a group of 345 breast cancer patients from one hospital with a long median follow-up of more than 10 years.

Results

Bcl-2 expression was not a prognostic factor. *p53* was an independent prognostic factor for overall survival ($p=0.005$) and for post-relapse survival ($p=0.006$). Looking at *bcl-2/p53* subgroups in the *bcl-2* positive subgroups there was a large difference in both disease-free and overall survival between *p53* negative and *p53* positive patients. In the *bcl-2* negative subgroup the *p53* status was not a prognostic factor at all.

Conclusion

p53 is an independent prognostic factor for overall survival and post-relapse survival. However, *p53* status is only important in the *bcl-2* positive subgroups.

Introduction

Several genes are involved in the regulation of programmed cell death (apoptosis). One of these genes is the *bcl-2* proto-oncogene, encoding for a protein that may block apoptosis when prevailing over the *bax* gene product. *Bcl-2* overexpression was first identified in a high percentage of follicular B-cell lymphomas, associated with a t(14;18) translocation¹.

Bcl-2 is, however, much more widely expressed than the lymphoid and hemato-poietic system. It is present in glandular epithelium regulated by hormones, complex epithelia such as skin and the gastro-intestinal system and long living post-mitotic cells such as neurons². It has also been demonstrated in normal breast epithelium^{2,3} and in preinvasive breast lesions^{3,4}.

Several studies have investigated the prognostic value of *bcl-2* expression in primary breast cancer^{3,5-9}. Generally high *bcl-2* is associated with features of differentiation and good prognosis like low tumour grade^{3,5,7,9}, low proliferative activity^{3,6,7,9}, oestrogen receptor (ER-)positivity⁵⁻⁹ and progesterone receptor (PR-)positivity^{5,7-9}. Looking at disease-free survival and overall survival the expression of *bcl-2* is associated with a better prognosis in univariate analysis in most studies^{3,6,8,9}. This association is, however, lost in most^{3,6,9}, but not all⁸ studies in multivariate analysis. The latter study suggests that the difference is only present in the node-positive subgroup⁸. Two of the studies in which prognostic significance was lost in multivariate analysis, were confined to node-negative patients^{6,9} while the other study also found a favourable outcome for high *bcl-2* only in node-positive patients³. A possible explanation for this phenomenon is that *bcl-2* is more a predictive factor for response to systemic therapy than a prognostic factor in itself. This has been demonstrated for the response to adjuvant treatment in node-positive patients¹⁰ and the response to tamoxifen in ER-positive patients with metastatic breast cancer¹¹.

The tumour suppressor gene *p53* also plays a role in apoptosis, whereby it induces this process, in response to irreparable DNA damage. Cells deficient of normal ("wild-type") *p53* are resistant to the induction of apoptosis¹². Loss of *p53* function allows the survival of cells with DNA-damage and is therefore associated with tumour progression. Mutation or inactivation of wild-type *p53* could also block apoptosis, which is necessary for a response to hormonal or chemotherapy.

The prognostic role of *p53* in breast cancer has been elucidated in numerous studies mostly by immunohistochemistry. Almost invariably *p53* was found to be an independent prognostic factor in studies with multivariate analysis¹³⁻¹⁸. The prognostic effect of *p53*, however, is probably small¹⁹.

Bcl-2 and *p53* can also influence each other's effects. It has been demonstrated that *bcl-2* is able to inhibit the apoptosis induced by *p53*²⁰. However, also it has been shown that wild-type as well as mutant *p53* proteins can down-regulate *bcl-2*

expression²¹.

We have investigated the prognostic value of *bcl-2* and *p53* separately and in combination in a group of 345 patients with primary breast cancer treated in a single hospital and with a long median follow-up of more than 10 years.

Patients and Methods

Patients

In the period May 1982-August 1987 the 345 patients were treated for primary breast cancer in the University Hospital Maastricht. Patients were selected according to the following criteria: 1. primary breast cancer without distant metastases, 2. no other primary tumour, 3. histological material available. All patients were staged at the time of diagnosis according to the TNM classification²². The median age was 57 years (range 25-87 years). Two hundred and twenty-three patients (64.6%) had undergone a modified radical mastectomy, 98 patients (28.4%) a lumpectomy with axillary lymph node dissection, whereas in 24 patients (7.0%) only a biopsy was done either because of T₄ stage or high age. In 4 of these 24 patients an axillary lymph node dissection was done. One hundred eighty-five patients (53.6%) had no axillary lymph node metastases, 140 patients (40.6%) had metastasis in one or more axillary lymph nodes, while in 20 patients (5.8%) the axillary lymph node status was unknown. Axillary lymph node-positive patients younger than 70 years were treated with adjuvant chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide. If they agreed to participate in a multicenter clinical trial, they were randomized for concomitant treatment with medroxyprogesteron acetate (MPA) or no MPA. The results of this clinical trial have been described in more detail elsewhere^{23,24}. Axillary lymph node positive patients of 70 years and older were treated with tamoxifen. Axillary lymph node-negative patients received no adjuvant systemic therapy. The median follow-up is 128 months (range 61-170 months). During the follow-up 152 patients experienced a relapse: 88 had distant metastases, 46 a locoregional relapse and 18 both simultaneously at the time of first relapse.

Methods

Immunohistochemistry (IHC)

Bcl-2 immunostaining

Three μ m sections were cut from routinely formalin-fixed and paraffin-embedded archival tumour blocks. Good fixation of the tumours on glass slides was obtained by overnight incubation of the tumour-slides in an 60°C oven on APS (3-aminopropyl triethoxysilane) (Sigma) coated glass-slides.

Staining was performed using the mouse monoclonal anti-bcl-2 antibody (clone 124, DAKO, Denmark; 1:100, two hours incubation at room temperature).

To reach optimal staining results microwave antigen retrieval with citric buffer (0.01M, pH=6.0) was administered. A standard staining procedure was performed. In short: deparaffination in xylene and washes in 100% ethanol were followed with removing endogenous peroxidase in 0.3% H₂O₂ containing methanol (30 minutes, room temperature). After washing away the excessive amount of methanol in demineralized water, the antigen unmasking procedure was performed. Non specific bindings were blocked with 5% BSA-containing PBS (phosphate buffered saline, pH=7.4). Then, the mouse anti bcl-2 was applied to the tumour sections. Excessive amounts of antibodies were washed away in PBS. The avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories, CA, USA) method was used to obtain amplification of the primary antigen-antibody bindings. These bindings were highlighted with DAB (di-amino-benzidin, Sigma). Finally, counterstaining with hematoxylin completed the staining.

p53 immunostaining and other immunohistochemical determinations

For other immunohistochemical staining procedures the anti-p53 mouse monoclonal antibody DO7 (DAKO A/S, Denmark, 1:200, two hours incubation at room temperature), the mouse monoclonal antibody MIB-1 (Dianova, Germany, 1:100, one hour incubation at room temperature), rabbit polyclonal antibody NCI-pS2 (Novacastra Laboratories, UK, 1:400, one hour incubation at room temperature), and mouse monoclonal antibody against CD44v6 (R & D Systems, UK, 1:1000, one hour incubation at room temperature) were used. Antigen unmasking by microwave antigen retrieval was done for p53, MIB-1 and CD44v6-staining.

IHC-score

For bcl-2 tumours were considered positive if more than 10% of cells showed positive staining, based on prior studies⁸ (only for bcl-2 tumours were scored semiquantitatively: negative; 0-10%; 10-25%; 25-50%; 50-75% and >75% of tumour cells staining). The same cut-off point was used for p53^{25,26}. For MIB-1 the median value of 7% was used. Tumours were considered pS2-positive in case of any positive staining, while for CD44v6 5% was used as cut-off point.

Steroid receptors

The ER and PR assays were all performed on histologically proven breast cancer tissues using the dextran-coated charcoal method with multiple-point scatchard-plot analysis.

For all the assays the minimum cytosol protein concentration was 2 mg/ml cytosol. PR status was determined only in patients entered from August, 1983 (n=252).

Flow cytometric evaluation of ploidy status and S-phase fraction

Flow cytometric evaluation of ploidy status and S-phase fraction was done as described previously²⁷.

Statistical Analysis

Disease-free survival was defined as the time from the day of diagnosis till the time of first relapse, death or last follow-up.

Overall survival was defined as the time from the day of diagnosis till the day of death or last follow-up.

Statistical analysis was done using the statistical package Stata (Stata Corp., 1997, Stata Statistical Software: Release 5.0, College Station, TX, USA). The association between the expression of bcl-2, p53 and other possible prognostic factors was analyzed by the chi-square test. Curves for disease-free survival and overall survival were estimated by the Kaplan-Meier method. Differences were analyzed using the logrank test.

Finally, prognostic variables were included in a Cox regression analysis.

Results

Bcl-2 expression and its relation to other clinicopathological factors

Positive staining in tumour cells for the bcl-2 protein was found in 205/337 (60.8%) of the primary breast tumours. In all tumour sections lymphocytes were bcl-2 positive. Correlations between bcl-2 expression and other clinicopathological factors are shown in table V.1. No correlation was demonstrated with histology, and S-phase fraction, while a borderline correlation with lower tumour stage was found ($p=0.054$). Looking at histology, however, medullary carcinomas were mostly (15/19) bcl-2 negative. A significant association was demonstrated between bcl-2 expression and ER-positivity, PR-positivity, pS2-staining (all $p<0.001$), CD44v6-positivity ($p=0.003$), diploidy ($p=0.008$) and age >70 years ($p=0.02$). A negative association of bcl-2 expression was found with MIB-1 and p53 staining (both $p<0.001$).

Table V.1 BCL-2 immunostaining and its relationship to other possible prognostic factors.

	Bcl-2 positive	Bcl-2 negative	Number	p-value
ER $\leq$ 10 fmol/mg protein	25	70	327	<0.001
ER $>$ 10 fmol/mg protein	174	58		
PR $\leq$ 10 fmol/mg protein	60	61	261	<0.001
PR $>$ 10 fmol/mg protein	101	39		
Age $\leq$ 50 years	65	44	337	0.76
Age $>$ 50 years	140	88		
Age $\leq$ 70 years	166	119	337	0.02
Age $>$ 70 years	39	13		
Ductal carcinoma	151	97	337	0.97
Other histological types	54	35		
Node-negative	118	64	318	0.18
Node-positive	78	58		
Tumour-size: T=1	98	49	337	0.054
T>1	107	83		
Diploid	86	39	316	0.008
Aneuploid	103	88		
S-phase fraction $\leq$ 8%	70	31	218	0.09
S-phase fraction $>$ 8%	68	49		
MIB-1 $\leq$ 7%	123	52	335	<0.001
MIB-1 $>$ 7%	80	80		
p53 positive	62	68	331	<0.001
p53 negative	142	59		
pS2 negative	82	76	329	0.001
pS2 positive	118	53		
CD44v6 $>$ 5%	143	71	332	0.003
CD44v6 $\leq$ 5%	59	59		

Bcl-2 expression and survival

With respect to disease-free survival bcl-2 expression proved to have no prognostic value ($p=0.90$, see figure V.1). Also for overall survival no difference could be demonstrated between bcl-2 positive and bcl-2 negative patients ($p=0.28$, see figure V.2). The Kaplan-Meier survival curves, however, suggested some time-dependency for bcl-2 expression. After 3 years of follow-up there was a difference of about 10% between bcl-2 positive and bcl-2 negative patients both for disease-free and overall survival. These differences virtually disappeared after the whole follow-up period (see figure V.1 and figure V.2 respectively).

Looking at post-relapse survival there was a trend for a better prognosis for patients with bcl-2 positive tumours ($p=0.09$).

Figure V.1 Overall survival in 337 patients with bcl-2 negative ($\leq 10\%$ staining) and BCL-2 positive ($> 10\%$ staining) tumours ($p=0.90$).

Figure V.2 Disease-free survival in 337 patients with bcl-2 negative and bcl-2 positive ($\leq 10\%$ staining) tumours ($p=0.28$)

p53 expression in relation to other clinicopathological factors

Of the 337 breast tumours 131 (38.6%) were p53 positive. P53 positivity was significantly more often seen in case of ER-negativity, bcl-2 negativity, pS2-negativity, ductal histology and a high MIB-1 percentage (see table V.2). P53 was not associated with nodal status, T-stage, PR, S-phase fraction, ploidy, CD44v6 or age (see table V.2).

Table V.2 p53 immunostaining and its relationship to other possible prognostic factors.

	p53 positive	p53 negative	Number	p-value
ER $\leq$ 10 fmol/mg protein	55	41	329	<0.001
ER > 10 fmol/mg protein	75	158		
PR $\leq$ 10 fmol/mg protein	52	71	262	0.13
PR > 10 fmol/mg protein	46	93		
Age $\leq$ 50 years	49	61	339	0.12
Age > 50 years	82	147		
Age $\leq$ 70 years	112	174	339	0.65
Age > 70 years	19	34		
Ductal carcinoma	106	144	339	0.02
Other histological types	25	64		
Node-negative	72	111	319	0.84
Node-positive	52	84		
Tumour-size: T = 1	51	98	339	0.14
T > 1	80	110		
Diploid	43	83	319	0.11
Aneuploid	83	110		
S-phase fraction $\leq$ 8%	33	66	219	0.44
S-phase fraction > 8%	46	74		
MIB-1 $\leq$ 7%	57	117	335	0.01
MIB-1 > 7%	74	87		
bcl-2 positive	62	142	331	<0.001
bcl-2 negative	68	59		
pS2 negative	71	85	330	0.02
pS2 positive	57	117		
CD44v6 > 5%	82	132	331	0.63
CD44v6 $\leq$ 5%	48	69		

p53 expression and survival

In univariate analysis patients with p53 negative tumours had a significantly better overall survival ($p < 0.01$, see figure V.3). Generally, overall survival worsened with a higher percentage of p53 positive tumour cells.

In the node-negative group of 183 patients p53 negative tumours were also associated with a better overall survival ($p = 0.01$), while in the node-positive subgroup ($n = 136$) the difference was borderline ($p = 0.06$).

With respect to disease-free survival there was a trend for a better prognosis with p53 negative tumours, but the difference was not statistically significant ($p = 0.09$). In the node-negative and node-positive subgroups there were no statistically significant differences ($p = 0.13$ and $p = 0.23$ respectively).

We also investigated the role of p53 in post-relapse survival. Thereby it was shown that patients with p53 negative tumours had a significantly better survival after their first relapse. Remarkably this difference started to emerge only after about 2 years.

Figure V.3 Overall survival in 339 patients with p53 negative ($\leq 10\%$ staining) and p53 positive ($> 10\%$ staining) tumours ($p < 0.01$).

Table V.3 Multivariate analysis for overall survival

	RHR*	p-value	95% confidence interval
Age (> 50 vs ≤ 50 years)	1.881	0.002	1.272-2.782
Node status (positive vs negative)	1.680	0.004	1.183-2.386
ER (> 10 vs ≤ 10 fmol/mg protein)	1.265	0.293	0.817-1.960
PR (> 10 vs ≤ 10 fmol/mg protein)	0.829	0.328	0.569-1.208
pS2 (positive vs negative)	0.877	0.434	0.630-1.219
SPF ($> 8\%$ vs $\leq 8\%$)	1.412	0.124	0.910-2.191
Ploidy status (aneuploid vs diploid)	0.956	0.833	0.632-1.447
p53 ($> 10\%$ vs $\leq 10\%$)	1.621	0.005	1.155-2.275
bcl-2 ($> 10\%$ vs $\leq 10\%$)	1.136	0.521	0.770-1.676
CD44v6 (positive vs negative)	1.049	0.783	0.774-1.479
MIB-1 ($> 7\%$ vs $\leq 7\%$)	1.054	0.756	0.755-1.473
T-stage (T_2 vs T_1)	1.238	0.252	0.859-1.748
T-stage (T_3 and T_4 vs T_1)	1.949	0.020	1.112-3.414

* RHR = relative hazard rate

Multivariate analysis

In multivariate analysis p53 was an independent indicator for overall survival ($p=0.003$) besides axillary lymph node status, age and T3 tumour stage (see table V.3). Looking at disease-free survival, axillary lymph node status, tumour stage, MIB-1 and ER-status were the only prognostic factors (data not shown).

In multivariate analysis p53 status was the most important independent factor for post-relapse survival ($p=0.006$) besides pS2 ($p=0.008$) (see table V.4).

Table V.4 Multivariate analysis for post-relapse survival*

	RHR*	p-value	95% confidence interval
Age (> 50 vs ≤ 50 years)	1.566	0.061	0.980-2.502
Node status (positive vs negative)	1.213	0.421	0.758-1.941
ER (> 10 vs ≤ 10 fmol/mg protein)	0.795	0.441	0.444-1.424
PR (> 10 vs ≤ 10 fmol/mg protein)	0.681	0.120	0.420-1.104
pS2 (positive vs negative)	0.563	0.008	0.367-0.864
SPF (> 8% vs ≤ 8%)	1.465	0.160	0.860-2.496
Ploidy status (aneuploid vs diploid)	0.986	0.956	0.596-1.632
p53 (> 10% vs ≤ 10%)	1.768	0.006	1.174-2.664
Bcl-2 (> 10% vs ≤ 10%)	1.147	0.575	0.711-1.848
CD44v6 (positive vs negative)	0.828	0.385	0.540-1.269
MIB-1 (> 7% vs ≤ 7%)	1.149	0.502	0.765-1.727
T-stage (T ₂ vs T ₁)	0.916	0.738	0.549-1.529
T-stage (T ₃ and T ₄ vs T ₁)	1.885	0.093	0.899-3.951

* corrected for disease-free interval

* RHR = relative hazard rate

Subsets of bcl-2/p53 phenotypes and survival

Significant differences were found for disease-free survival between bcl-2/p53 subgroups ($p=0.01$) (figure V.4). The bcl-2 positive/p53 negative subgroup showed the best 5-year survival (77%) while in the bcl-2 positive/p53 positive subgroup the worst 5-year survival was seen (53%). Also with respect to overall survival significant differences between the bcl-2/p53 subgroups were seen ($p<0.001$) (figure V.5). In the bcl-2 positive subgroup the 5-year survival was 86% and 59% for the p53 negative and p53 positive patients respectively. Remarkably in both bcl-2 negative subgroups the p53 status did not essentially influence disease-free or overall survival.

In node-negative and node-positive subgroups comparable tendencies were found as

in the whole group for disease-free and overall survival, whereby for disease-free survival the differences between the *bcl-2*/p53 subgroups only reached borderline significance ($p=0.07$ and $p=0.06$ for the node-negative and the node-positive subgroup respectively). We also investigated post-relapse survival in the same *bcl-2*/p53 subgroups. Significant differences were demonstrated for the whole group of patients ($p=0.01$) and the node-negative patients ($p<0.05$), but not for the node-positive patients ($p=0.28$). For all patients with a relapse the longest median survival was seen in the *bcl-2* positive/p53 negative patients: 33 months, versus 17 months for *bcl-2* negative/p53 negative, 21 months for *bcl-2* positive/p53 positive and 18 months for *bcl-2* negative/p53 positive patients.

Figure V.4 Disease-free survival in *bcl-2*/p53 subgroups ($p=0.01$)

Figure V.5 Overall survival in *bcl-2*/p53 subgroups ($p<0.001$)

Discussion

Numerous prognostic factors have been investigated in primary breast cancer. Especially factors involved in proliferation and growth control have been the subject of many studies. More recently apoptosis has been recognized as an important factor, i.e. tumour growth only occurs when the rate of proliferation exceeds that of death. P53 is a factor involved in growth control and apoptosis and is often referred to as "guardian of the genome".

In our study p53 expression was associated with a poor prognosis as has been reported by many¹³⁻¹⁸ but not all^{26,28} studies in the literature. In several studies p53 was an independent prognostic factor for both disease-free and overall survival^{14,16,17}, but sometimes only for disease-free survival¹⁸ or overall survival¹³. In our study a statistically significant effect of p53 in multivariate analysis was only found for overall survival. Differences between reported studies can be explained by different cut-off points for p53 positivity, different antibodies, variance in prognostic factors investigated in multivariate analysis and loss of p53 immunostaining intensity. We also have found a clear difference in post-relapse survival depending on p53 status. In multivariate analysis p53 was even the most important factor for post-relapse survival. An effect of p53 on survival after relapse has been reported before¹⁵, but is not investigated in most studies on p53 in breast cancer. It probably implies that "wild-type" p53 is necessary for a response to systemic therapy as has been found, although we were not able to investigate this in our study. Because of these findings and the suggestion that the prognostic value of p53 is probably small¹⁹ it seems reasonable to suggest that p53 is more useful as a predictive factor for response to systemic therapy than as a prognostic factor.

Our findings on the value of bcl-2 as a prognostic factor are in concordance with the literature. The fact that bcl-2 seems to be a time-dependent factor confirms the results of the study of Joensuu et al³, while most other studies showing bcl-2 to be a prognostic factor at least in univariate analysis have shorter follow-up⁶⁻⁸. Also the relationship of bcl-2 and other clinicopathological factors shows, in general, resemblance with other reported findings.

Most interesting are the findings in bcl-2/p53 subgroups, whereby in the subgroup of bcl-2 positive patients the p53 negative patients had a much better prognosis both for disease-free and overall survival than the p53 positive patients. On the other hand in bcl-2 negative patients p53 status was not influencing prognosis anymore. Although a (negative) association between p53 and bcl-2 has been reported before^{3,6}, the combined prognostic value of both factors in subgroups has to our knowledge not been investigated so far. Recently, it has been reported that no clinically or statistically significant differences exist in response rate to tamoxifen in ER-positive metastatic breast cancer between the four possible phenotypes¹¹. However, as in our study the best post-relapse survival was found in

p53 low/bcl-2 high patients¹¹.

It is rather surprising that bcl-2 expression is associated with good prognosis in breast cancer, because it would be expected to be associated with a more aggressive behavior. It has been suggested that bcl-2 expression may produce slower growing tumours and reflects the maintenance of a more normal phenotype³⁰. If this is the case, it would be less surprising that in bcl-2 positive breast tumours p53 status is important for the prognosis of the patient but not necessarily related to apoptosis. On the other hand, if loss of bcl-2 expression is correlated with faster growth of breast cancer as suggested by the association with high MIB-1 labelling index in our study, it is conceivable that p53 then does not play a role anymore. However, this is rather speculative and more studies on the effect of bcl-2 and p53 on each other are needed.

In summary, we have found that bcl-2 is not a prognostic factor. P53 is an independent prognostic factor for overall survival. This could partly be explained by the fact that it is also an independent prognostic factor for post-relapse survival, which suggests that p53 might be more a predictive than a prognostic factor. Furthermore, in bcl-2 positive tumours p53 status is very important with respect to prognosis, whereas in bcl-2 negative tumours p53 status is irrelevant. These latter findings need confirmation in other studies.

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Chapter VI

**Microvessel density is
correlated with age, but not
with prognosis in breast cancer**

RLH Jansen, SR Joosten-Achjanie, JW Arends,
A Volovics, PSGJ Hupperets, HC Schouten,
HFP Hillen

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Summary

Many studies in patients with breast cancer have shown an association between prognosis and microvessel density, mostly as determined by anti-FVIII-RAg or anti-CD31. From a theoretical point of view anti-CD34 should be a more suitable antibody.

Therefore, we have investigated the prognostic value of microvessel density as determined by immunostaining for CD34 in a large group of 324 patients with primary breast cancer and a median follow-up of more than 10 years.

Microvessel density by FVIII-RAg and CD34 showed a moderate correlation ($r_p=0.54$ for the highest counts and $r_p=0.58$ for the mean of three hotspots, both $p<0.001$). When microvessel density as determined by CD34 was analysed as a continuous variable an increasing risk of relapse with a rising number of microvessels was demonstrated. However, no single cut-off point could be found that lead to a significant difference in disease-free survival. Microvessel density as determined by FVIII-RAg showed no prognostic value for disease-free or overall survival. Remarkably, the mean microvessel density was lower in patients of 70 years and older compared to other age categories, which reached statistical significance only for FVIII-RAg ($p<0.001$).

In conclusion: microvessel density as determined by current antibodies is not a useful prognostic factor in routine clinical practice. The association of lower microvessel density with higher age suggests a more favourable prognosis for this category of patients.

Introduction

Although many prognostic factors have been investigated in primary breast cancer, so far the axillary lymph node status is still the most important prognostic factor. About 30% of the axillary node-negative patients and 70% of the node-positive patients will develop metastases. It would be of great clinical importance to have prognostic factors that indicate more precisely the subset of patients that will develop distant metastases. Angiogenesis could be such a prognostic factor since there is solid evidence that the extent of angiogenesis is correlated with metastasis in the pathophysiology of breast cancer. The biological background of angiogenesis in human breast cancer has recently been reviewed¹. One of the most widely applied methods to assess the angiogenic activity of a tumour is the measurement of the microvessel density. The original study of Weidner² and many studies thereafter have investigated the prognostic role of microvessel density in primary breast cancer. Most of these studies demonstrate a correlation between a high microvessel density and poor outcome as has been reviewed^{1,3}. The majority of the studies used anti-FVIII-RAg antibody. Although FVIII-RAg is highly specific for the vasculature, it is absent on part of the capillary endothelium in tumour tissue⁴. Therefore, anti-FVIII-RAg antibody is probably not an optimal antibody to be used in the quantification of microvessel density, and it has been stated that anti-CD31 antibodies are superior on paraffin sections⁵. However, a good alternative could be anti-CD34-antibody. It has been found that anti-CD31 and anti-CD34 demonstrated a similar degree of specific staining with less background staining for anti-CD34⁶. Furthermore, CD34 is suggested to be a marker for the endothelial cells of newly formed vessels^{7,8}, which makes it a very suitable antigen to use in the determination of microvessel density. So far only a few studies used anti-CD34-antibodies^{9,10,11} of which only one small study with 77 patients compared different immunohistochemical methods for the assessment of angiogenesis including anti-CD34 antibodies¹⁰.

Against this background we have investigated the prognostic value of microvessel density as determined by immunostaining for CD34 in a large group of 324 patients and compared this with the results of immunostaining for FVIII-RAg. Furthermore, we have analysed possible associations with other prognostic factors previously investigated in these patients. The study was performed in patients from one hospital with a median follow-up of more than 10 years.

Patients and Methods

Patients

The 324 patients were treated for primary breast cancer in the University Hospital Maastricht between May 1982 and August 1987. Patients were included if they had primary breast cancer with no other primary tumour and without distant metastases, provided histological material was available. All patients were staged at the time of diagnosis according to the TNM classification¹².

The median age was 56 years (range 28-87 years). Surgical treatment consisted of a modified radical mastectomy in 209 patients (64.5%), and a lumpectomy in 93 patients (28.7%). In 22 patients (6.8%) only a biopsy was done either because of T₄ stage or high age, in 3 patients with an axillary lymph node dissection. In 136 patients (42.0%) metastases were found in one or more axillary lymph nodes, while in 169 patients (52.2%) no axillary lymph node metastases were found and in 19 patients (5.8%) the axillary lymph node status was unknown. Axillary lymph node-positive patients younger than 70 years were treated with adjuvant chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide. If they agreed to participate in a multicenter clinical trial, they were randomized for concomitant treatment with medroxyprogesteron acetate (MPA) or no MPA. The results of this clinical trial have been described in more detail elsewhere^{13,14}. Axillary lymph node-positive patients of 70 years and older were treated with adjuvant tamoxifen. Axillary lymph node-negative patients received no adjuvant systemic therapy. The median follow-up is 126 months (range 61-170 months). During the follow-up 145 patients experienced a relapse: 83 had distant metastases, 44 a locoregional relapse and 18 both a locoregional relapse and distant metastases at the time of first relapse.

Methods

Immunohistochemistry

FVIII-related antigen and CD34

From the routine formalin-fixed paraffin-embedded archival tumour blocks, slides of 3 μ m thickness were cut. Blood vessels were highlighted by staining endothelial cells for CD34 in one section (NCL-END mouse monoclonal antibody, Novocastra Laboratories, Newcastle, UK; 1:50) and for anti-FVIII-RAg vWF in a consecutive section (rabbit anti-human vWF, Dako, Glostrup, Denmark; 1:2000). The immunohistochemical method used was the avidin-biotin-peroxidase-complex technique (Vectastain Elite ABC kit, Vector, Burlingame, CA), a modification of the labelled-avidin-biotin method. Before antibody was added, sections were first treated with 0.1% trypsin in 0.1% CaCl₂H₂O (anti-CD34) and 0.1% pepsin in 0.1 NHCl (anti-vWF) for 10 min. at room temperature. The antibodies were diluted in

0.5% PBS/BSA solution. Incubation with anti-CD34 was at 4°C overnight; anti-vWF was incubated 45 min. at room temperature.

Only slides of high staining quality (low background staining) were included.

Microvessel density

The highest vascularized areas (hot spots) were selected by scanning the tumour at low power. The hotspots were selected and counted using a 200x magnification (0.60 mm²). The criteria for microvessel recognition were the same as originally described by Weidner². Briefly as microvessels were considered individual or clusters of cells, with or without a lumen and positively stained for factor VIII RAg or CD34. Three hotspots were counted on a 200x field. Both the mean of the three hotspots and the highest single value were recorded for each case. For analysis both values were used. Microvessel density was assessed without knowledge of patients outcome or characteristics.

Twenty samples were analysed independently by a second investigator to establish interobserver variability.

Other immunohistochemical determinations

For other immunohistochemical staining procedures the anti-p53 mouse monoclonal antibody DO7 (DAKO A/S, Denmark; 1:200, two hours incubation at room temperature), the mouse monoclonal anti-bcl-2 antibody (clone 124, DAKO, Denmark; 1:100, two hours incubation at room temperature), the mouse monoclonal antibody MIB-1 (Dianova, Germany; 1:100, one hour incubation at room temperature), rabbit polyclonal antibody NCI-pS2 (Novocastra Laboratories, UK; 1:400, one hour incubation at room temperature), and mouse monoclonal antibody against CD44v6 (R&D Systems, UK; 1:1000, one hour incubation at room temperature) were used. Antigen unmasking by microwave antigen retrieval was done for p53, bcl-2, MIB-1 and CD44v6 staining.

Immunohistochemistry score

For bcl-2 tumours were considered positive if more than 10% of cells showed positive staining, based on prior studies¹⁵ (only for bcl-2 tumours were scored semiquantitatively: negative; 0-10%; 10-25%; 25-50%; 50-75% and >75% of tumour cells staining). The same cut-off point was used for p53¹⁶. For MIB-1 the median value of 7% was used. Tumours were considered pS2-positive in case of any positive staining, while for CD44v6 5% was used as cut-off point.

Steroid receptors

The ER and PR assays were all performed on histologically proven breast cancer tissues using the dextran-coated charcoal method with multiple-point scatchard-plot analysis.

For all the assays the minimum cytosol protein concentration was 2 mg/ml cytosol. PR status was determined only in patients entered from August, 1983 (n=252).

Flow cytometric evaluation of ploidy status and S-phase fraction

Flow cytometric evaluation of ploidy status and S-phase fraction was done as described previously¹⁷.

Statistical Analysis

Disease-free survival was defined as the time from the day of diagnosis till the time of first relapse, death or last follow-up.

Overall survival was defined as the time from the day of diagnosis till the day of death or last follow-up.

Statistical analysis was done using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-plus (Statistical Sciences Europe, Oxford, UK). The association between microvessel density and other possible prognostic factors was analyzed by the chi-square test. Curves for disease-free survival and overall survival were estimated by the Kaplan-Meier method. Differences were analyzed using the logrank test.

Results

Microvessel density

It was not possible to determine the microvessel density in 5 patients for FVIII-RAg and 7 patients for CD34 because of insufficient material or low staining quality (high background staining). With respect to interobserver variability, the results of two investigators showed a very strong interobserver correlation (Pearson correlation $r=0.99$, $p<0.001$), if the same hotspots were counted. If hotspots were chosen independently, the correlation became weaker ($r=0.85$, $p<0.001$). With respect to the intra-observer variability the Pearson correlation coefficient was 0.86 ($p<0.001$). Looking at the highest microvessel count, the median value for FVIII-RAg was 114/mm² (range 15-353) and for CD34 131/mm² (range 13-370). With respect to the median value of the three hotspots the median value for FVIII-RAg was 104/mm² (range 6-272) and 113/mm² for CD34 (range 12-329). As could be expected the correlation between the highest count and the mean of three highest counts was very good (Pearson correlation coefficient 0.96 for FVIII-RAg and 0.98 for CD34, both $p<0.001$). Correlation between microvessel density by FVIII-RAg and CD34 was moderate (Pearson correlation coefficient 0.54 for the highest counts and 0.58 for the mean of three hotspots, both $p<0.001$).

Association of microvessel density with age and other prognostic variables

When tumours were categorized in three equal groups with low, intermediate and high microvessel density, both FVIII-RAg and CD34 showed a significant association with age ($p=0.002$ and $p=0.03$ for highest count respectively, $p=0.001$ and $p=0.02$ for mean of three hotspots respectively). For both antigens the mean microvessel density was lower in patients of 70 years and older, compared to the patients of 50 years and younger and the patients of 51-69 years which was only significant for FVIII-RAg (see table VI.1). Furthermore the microvessel density by FVIII-RAg showed a significant negative association with S-phase fraction ($p=0.02$ both for highest count and mean of three hotspots), and the microvessel density by CD34 showed a significant association with axillary lymph node status, i.e. patients with high microvessel density had more often axillary lymph node metastases ($p<0.001$ both for highest count and mean of three hotspots).

Both FVIII-RAg and CD34 showed no association with steroid receptors, pS2, MIB-1 labelling index, p53, bcl-2, CD44v6, T-stage, histology, and ploidy status. When microvessel density was dichotomized at the median, comparable results were obtained.

Table VI.1 Mean values ($\pm$ SD) of microvessel density in different age categories.

Prognostic variable	Age ≤ 50 years	Age 51-69 years	Age ≥ 70 years	p-value
MVD* (FVIII-RAg-highest value)	147.9 $\pm$ 65.5	117.4 $\pm$ 45.7	107.4 $\pm$ 34.6	<0.0001
MVD* (FVIII-RAg-mean 3 hot spots)	124.5 $\pm$ 51.6	102.2 $\pm$ 41.2	94.5 $\pm$ 31.0	<0.0001
MVD* (CD34-highest value)	148.5 $\pm$ 59.3	139.2 $\pm$ 64.1	129.1 $\pm$ 56.0	0.16
MVD* (CD34-mean 3 hot spots)	127.7 $\pm$ 51.6	118.3 $\pm$ 54.1	110.1 $\pm$ 49.0	0.12

* MVD=microvessel density

Microvessel density and survival

Microvessel density as determined by FVIII-RAg showed no prognostic value for disease-free survival or overall survival, neither for the highest count nor for the mean of the three hot spots. This was true when patients were divided in three categories with low, intermediate or high microvessel density or dichotomized at the median value.

When the microvessel density as determined by CD34 was analysed as a

continuous variable, there was an increasing risk of relapse with a rising number of microvessels (figure VI.1). However, no cut-off point could be found that lead to a significant difference in disease-free survival. When patients were again divided in three equal groups with low, intermediate or high microvessel density, also no significant difference in disease-free survival was found (figure VI.2)($p=0.15$). Only when the group with the highest microvessel density was compared with the two other groups, there was a borderline significant difference in disease-free survival ($p=0.06$). Thereby the differences in disease-free survival were fully accounted for by a different risk for distant metastases, whereas CD34 had no association with the risk for a locoregional relapse. Furthermore, also for overall survival no differences between the three groups could be demonstrated ($p=0.33$). In multivariate analysis for disease-free survival only axillary lymph node status, MIB-1 labelling index and T3 or T4 stage were independent prognostic factors (see table VI.2).

Table VI.2 Multivariate analysis for disease-free survival.

	RHR*	p-value	95% Confidence interval
Age (> 50 vs ≤ 50 years)	1.086	0.68	0.734-1.607
Node status (positive vs negative)	1.791	0.002	1.231-2.606
ER (> 10 vs ≤ 10 fmol/mg protein)	1.516	0.08	0.946-2.430
PR (> 10 vs ≤ 10 fmol/mg protein)	0.912	0.65	0.616-1.350
pS2 (positive vs negative)	1.229	0.25	0.862-1.751
SPF (> 8 vs ≤ 8%)	1.228	0.40	0.762-1.979
Ploidy status (aneuploid vs diploid)	1.410	0.13	0.901-2.207
p53 (> 10 vs ≤ 10%)	1.370	0.09	0.951-1.973
hcl-2 (positive vs negative)	0.904	0.64	0.597-1.371
CD44v6 (positive vs negative)	1.029	0.88	0.717-1.477
MIB-1 (> 7 vs ≤ 7%)	1.567	0.01	1.094-2.246
MVD* (intermediate vs low)	0.987	0.96	0.619-1.575
MVD* (high vs low)	1.417	0.14	0.893-2.248
T-stage (T ₂ vs T ₁)	1.341	0.13	0.920-1.956
T-stage (T ₃ vs T ₁)	1.899	0.04	1.040-3.466

* RHR = relative hazard rate; * MVD = microvessel density as determined by highest count for CD34.

Figure VI.1 Relative risk of relapse as determined by the number of microvessels/mm² (highest value of CD34).

Figure VI.2 Disease-free survival for patients with low, intermediate and high microvessel density as determined by the highest count for CD34 ($p=0.15$)

Discussion

The last years many studies have been done to investigate the prognostic value of microvessel density in primary breast cancer. As stated in several reviews, most studies have demonstrated a significant association between microvessel density and

disease-free and overall survival in univariate analysis and often also in multivariate analysis¹. In our study, we have demonstrated an increasing risk of relapse with an increasing microvessel density as determined by CD34. However, we were not able to find a cut-off point leading to a statistically significant difference. Negative studies have in the past been criticized mainly because of methodological problems¹⁸, and have lead to some vivid discussions in the literature^{19,23}.

Therefore, recently an international consensus on the methodology and criteria of evaluation with respect to the quantification of angiogenesis in solid tumours has been proposed⁵. Our methodology was quite similar to the proposed standard method, but we used the original method of Weidner² instead of the Chalkley point graticule method to count microvessels. Furthermore, anti-CD34 was used instead of anti-CD31.

Looking at the studies on intratumoural microvessel density and prognosis in operable breast carcinoma as summarized in a recent review¹, it seems that another factor could be of importance. None of the 4 studies with a median follow-up longer than 9 years has demonstrated an association of microvessel density with prognosis either in univariate or in multivariate analysis^{9,19,24,25}. This suggests that microvessel density might be a time-dependent prognostic factor. Although the disease-free survival curves in our own study could suggest such a time-dependent effect, statistically this could not be confirmed. Because in none of the 4 articles mentioned above with a follow-up of more than 9 years survival curves are provided, this cannot be judged from the data presented. These results are, however, contradictory to a more recent published study in which after a follow-up of more than 15 years the microvessel count by the use of monoclonal anti-CD34 was an independent prognostic factor²⁶. The methodology in that study was more different from the proposed standard because of the use of a small microscopic field, i.e. 0.1452 mm² (400 x magnification).

Remarkably, our results demonstrate an association between age older than 69 years and lower microvessel density both for FVIII-RAg and CD34. This is in concordance with the findings of two other studies^{27,28} and suggests a better prognosis for older patients with primary breast cancer.

In our study as in most other studies we have not found an association of microvessel density with other prognostic factors like steroid receptors, p53, ploidy status, proliferative activity and histology. With respect to T- and N-stage data in the literature are conflicting. While the majority of studies has not demonstrated an association with T-stage, about half of the studies does show an association with N-stage. Our findings demonstrate an association between CD34 and axillary lymph node status.

When comparing the different antibodies (anti-FVIII-RAg and anti-CD34), it was easier to count microvessels in slices stained with CD34, especially because of low background staining. Furthermore from a theoretical point of view anti-CD34

should be preferred above anti-FVIII-RAg because of better specificity^{7,8}.

In conclusion, angiogenesis is without doubt important in tumour development and spread. However, determination of angiogenetic activity by microvessel count has given rise to conflicting results mainly because of methodological problems. To our opinion, the proposed standard method can only partly solve these problems because of several subjective aspects. Therefore, at this moment it is unlikely that microvessel density as determined by the current antibodies is a prognostic factor that is useful in routine clinical practice for patients with primary breast cancer. Furthermore, assuming that microvessel density is a prognostic factor despite methodological problems, the correlation of lower microvessel density with age above 69 years suggests a better prognosis in these older patients.

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Chapter VII

**Summary and
general discussion**

Summary and general discussion

Breast cancer is the most frequent malignancy in women in the Netherlands, affecting more than 10.000 new patients each year. Despite a rising incidence the mortality over the last few years has remained stable. This improvement in prognosis is most likely explained by two factors: firstly tumours are diagnosed at an earlier and, therefore, more curable stage, and secondly systemic adjuvant treatment with hormonal therapy, chemotherapy or both has resulted in better disease-free and overall survival. Unfortunately, despite these advances, not every patient benefits from adjuvant therapy. Historically, only patients with axillary lymph-node metastases have been treated with adjuvant therapy, although there is now an increasing tendency to treat unfavourable subsets of patients without axillary lymph node metastases. There remains, however, uncertainty regarding which are the most important prognostic factors. There is an need to define factors which can predict outcome for individual patients such that those who have a high chance of cure with loco-regional therapy alone are spared the side effects of systemic therapy whilst those patients with a high risk of recurrence can receive tailored systemic therapy. Over the last two decades many prognostic factors have been investigated in primary breast cancer. We have investigated the value of several more recently developed prognostic factors by immunohistochemistry in a group of 349 patients with primary breast cancer from one hospital, uniformly treated and with a median follow-up of more than 10 years.

In Chapter I a review is given of the literature on prognostic factors. Hundreds of studies have investigated dozens of prognostic factors, mostly in retrospective studies often with relatively few patients and generally short follow-up. It can be concluded that only more traditional prognostic factors such as axillary lymph-node status, tumour size, proliferative activity and probably tumour grade can be considered as established. With regard to the more recently developed prognostic factors this can only be stated for p53 and probably also microvessel density.

In Chapter II the prognostic value of the immunohistochemical pS2 determination is described. Although in pS2-negative tumours there was a trend for less locoregional relapse, the prognostic value of immunohistochemical pS2 determination could not be demonstrated neither for disease-free survival nor for overall survival. This is in line with the literature where no other study using this immunohistochemical method has found prognostic significance for the pS2 status of the tumour, at least in multivariate analysis. On the contrary several studies using the more laborious cytosolic assay have demonstrated better disease-free and/or overall survival in multivariate analysis. Probably with both methods different epitopes of the protein are measured. Furthermore, in our study pS2 was

the most important factor for post-relapse survival in a multivariate analysis including axillary lymph node status, ER, PR, ploidy and S-phase fraction. This suggests that pS2 is a predictive factor for response to systemic therapy, although we were not able to formally address this question in our study. Because there are indications that pS2 expression reflects the functional status of ER and is also involved in growth regulation, the pS2 status could have predictive value both for hormonal therapy and chemotherapy. In two other studies the predictive value of the pS2 status has been demonstrated for chemotherapy as well as for hormonal therapy. Further prospective studies are needed to investigate if the immunohistochemical pS2 status can be used to predict the response to systemic therapy.

In Chapter III a study on the possible use of the adhesion molecule CD44v6 as a prognostic factor is reported. Although the first study on this subject showed CD44v6 to be an independent prognostic factor, we could not confirm this in a much larger study with longer follow-up. Several other studies have also been unable to demonstrate the value of CD44v6 as a prognostic factor at least in multivariate analysis. It can, therefore, be concluded that the CD44v6 expression has no prognostic value in primary breast cancer. Such prognostic value seems also unlikely on biological grounds because CD44v6 is often expressed in normal tissues including normal breast tissue. More studies are, however, needed to further elucidate the role of CD44 isoforms in the progression of cancer.

In Chapter IV it is demonstrated that the MIB-1 labelling index is an independent prognostic factor for disease-free survival. This is true when a cut-off at the median value of 7% is used and also when MIB-1 is analysed as a continuous variable. Several other studies have shown that MIB-1 or Ki-67 are important prognostic factors in multivariate analysis for patients with primary breast cancer. There are other methods of measuring the proliferative activity of a tumour, such as the assessment of the S-phase fraction, thymidine-labelling index and mitotic index. These methods have been investigated in several studies and all of them have been found to have prognostic value sometimes in multivariate analysis. In our patients S-phase fraction only had an effect with respect to prognosis in univariate analysis. Only a few studies have directly compared different methods of measuring the proliferative activity of a primary breast tumour. We observed a very weak association between MIB-1 labelling index and the S-phase fraction. Several other authors have reported a higher association between S-phase fraction and MIB-1 or Ki-67. Furthermore, in our patients in multivariate analysis MIB-1 labelling index but not S-phase fraction was a prognostic factor. The contrary has, however, also been reported. Most of these differences are probably explained by different methodologies. For routine clinical practice immunohistochemical methods

seem most suitable. Because several studies have demonstrated the independent prognostic significance of Ki-67 and MIB-1, prospective studies with these antibodies are needed in which there should be consensus on the methodology.

In Chapter V the proto-oncogene *bcl-2* and the tumour suppressor gene *p53* were investigated with respect to their prognostic role in primary breast cancer. At the end of the whole follow-up period *bcl-2* expression had no influence on disease-free or overall survival. This seems at variance with most of the literature since in several studies it has been found that *bcl-2* expression, at least in univariate analysis, is associated with better disease-free and/or overall survival. These differences can be explained by the length of the follow-up period, which was shorter in most studies. This suggests that *bcl-2* expression is a time-dependent prognostic factor.

In our patients *p53*-positivity was associated with worse overall survival for the whole group of patients and the node-negative subgroup. In multivariate analysis *p53* expression was also associated with decreased overall survival. With respect to disease-free survival there was a trend for better disease-free survival in *p53* negative patients, but this did not reach statistical significance. These results correspond with those reported in the literature, but as has been stated in a review the prognostic effect of *p53* is probably small. The fact that in our study *p53* expression was correlated with poor post-relapse survival in multivariate analysis suggests that *p53* expression could be predictive of the response to systemic therapy in patients with breast cancer. This is in line with the few data in the literature on the predictive value of *p53* expression.

Of interest are the findings in *bcl-2/p53* subgroups. In the *bcl-2* positive subgroup there was a large difference between *p53* negative and *p53* positive patients with respect to disease-free survival (77 vs 53% 5-year survival) and overall survival (86 vs 59% 5-year survival). In the *bcl-2*-negative subgroup, however, *p53* status did not essentially influence disease-free or overall survival. Generally comparable tendencies were found in node-negative and node-positive patients. For post-relapse survival *bcl-2* positive/*p53* negative patients clearly had a better prognosis than the other 3 subgroups. More studies on these subgroups are needed and an explanation for this phenomenon, if it is confirmed, is not easy to give. Because *bcl-2* expression in breast cancer is probably related to slower growth, it could be speculated that in *bcl-2* positive breast tumours *p53* status is important for the prognosis of the patient but not necessarily related to apoptosis. If on the other hand loss of *bcl-2* expression is correlated with faster growth of breast cancer, it is conceivable that *p53* no longer plays a role.

In Chapter VI the results of counting microvessel density with anti-CD34 and anti-fVIII-RAg in the same patients are described. We were not able to confirm the results as mentioned in most of the literature, stating that microvessel density is an independent prognostic factor in primary breast cancer. Although in our patients a higher microvessel density as determined by anti-CD34 was associated with an increasing risk of relapse when analysed as a continuous variable, no single cut-off could be found that lead to a significant difference in disease-free survival. Microvessel density as determined by anti-fVIII-RAg showed no influence on both overall and disease-free survival. Several other investigators were also unable to demonstrate an (independent) prognostic value for microvessel density. This has resulted in active discussions in the literature. From these discussions it can be concluded that determining the microvessel density is technically difficult with several subjective aspects. The present method of microvessel counting, therefore, seems unsuitable for use in routine practice for patients with cancer.

Furthermore, in our patients age above 69 years was correlated with a lower microvessel density. This confirms the findings in two other studies. Assuming that microvessel density despite methodological problems is a prognostic factor, this suggests that older age is associated with a better prognosis in primary breast cancer.

In conclusion, with respect to all investigated prognostic variables, MIB-1 labelling index proved to be an independent prognostic factor for disease-free survival and p53 expression for overall survival. This supports much of what is found in the literature, however with a substantially longer follow-up than what has mostly been reported in other studies. In line with the literature none of the more recently developed factors studied in this thesis can be used to make treatment decisions in individual patients. Moreover about 20 years of research on these newer prognostic factors in primary breast cancer has not lead to much progress in this subject. Improvement on this subject will only be possible when more uniform methods are used in prospective studies. It seems, however, unlikely at this moment that any single prognostic factor can be used in the future to decide which individual patient can benefit from (adjuvant) systemic therapy.

It could be more fruitful to use these newer factors to tailor adjuvant therapy for an individual patient. The fact that in our patients both pS2 and p53 had independent prognostic value for post-relapse survival in multivariate analyses, suggests that these factors can be used to predict response to systemic therapy. There is not much literature on this subject, but several studies now lend support to the idea that these newer factors better can be used as predictive than as prognostic factors. More studies on this subject, preferably prospective in character and with uniform methodologies are needed.

Further it can be speculated that molecular-biological methods investigating specific

genetic alterations give better prognostic information. Although at present most techniques used to screen or detect genetic alterations are laborious and costly, it might be anticipated, that for example with the development of DNA chip technology, genetic screening will become more generally available and can be used for prognostication.

Chapter VIII

**Samenvatting en algemene
discussie**

Samenvatting en algemene discussie

Borstkanker is de meest voorkomende maligniteit bij vrouwen in Nederland. De diagnose wordt per jaar bij meer dan 10.000 nieuwe patiënten gesteld. Ondanks een stijgende incidentie is de mortaliteit de laatste jaren ongeveer stabiel gebleven. Deze verbetering in de prognose is het meest waarschijnlijk te verklaren door twee factoren: ten eerste worden tumoren gediagnostiseerd in een gunstiger stadium, en ten tweede heeft adjuvante systemische behandeling met hormonen, chemotherapie, of een combinatie van beide, geresulteerd in betere ziektevrije en totale overleving. Echter, zeker niet alle patiënten met een primair mammacarcinoom hebben profijt van adjuvante systemische therapie. Historisch gezien werden alleen patiënten met okselkliermetastasen behandeld met adjuvante chemotherapie. Recent is er een toenemende tendens om patiënten zonder okselkliermetastasen met ongunstige prognostische factoren ook te behandelen. Er blijft onzekerheid bestaan welke de meest belangrijke prognostische factoren zijn. Het is noodzakelijk factoren te definiëren die de prognose van individuele patiënten bepalen. Op die manier kunnen patiënten met een hoge kans op curatie na loco-regionale therapie alléén de bijwerkingen van systemische therapie bespaard worden, terwijl patiënten met een hoge kans op recidief of metastasen een individueel aangepaste therapie kunnen krijgen. Vooral de laatste 20 jaar zijn vele prognostische factoren onderzocht bij het primaire mammacarcinoom. Wij hebben de waarde onderzocht van diverse, meer recent ontwikkelde prognostische factoren, met behulp van immuunhistochemische methoden in een groep van 349 patiënten met primair mammacarcinoom uit één ziekenhuis, die zoveel mogelijk uniform behandeld zijn en met een mediane follow-up van meer dan 10 jaar.

In Hoofdstuk I wordt een overzicht gegeven van de literatuur over prognostische factoren. In honderden studies zijn tientallen prognostische factoren onderzocht, voornamelijk retrospectief met relatief weinig patiënten en in het algemeen een korte follow-up.

Uit de literatuur hebben wij geconcludeerd dat slechts meer traditionele prognostische factoren, zoals de okselklierstatus, de grootte van de primaire tumor, proliferatieve activiteit, en waarschijnlijk de tumorgraad, beschouwd kunnen worden als gevestigde prognostische factoren.

M.b.t. de meer recent ontwikkelde prognostische factoren kan dit slechts gesteld worden voor p53 en waarschijnlijk ook voor microvaatdichtheid.

In Hoofdstuk II wordt de prognostische waarde van de immuunhistochemische pS2 bepaling beschreven. Hoewel in pS2-negatieve tumoren er een trend was voor minder locoregionale relapse, kon de prognostische waarde van immuunhistochemische pS2 bepaling niet worden aangetoond, noch voor ziektevrije overleving,

noch voor totale overleving. Dit komt overeen met wat in de literatuur wordt gesteld, waarbij geen enkele andere studie, die de immunohistochemische methode heeft gebruikt, een prognostische waarde heeft aangetoond voor de pS2 status van de tumor, althans in multivariate analyse. Daarentegen hebben diverse studies, die de meer arbeidsintensieve cytosol-assay hebben gebruikt, een betere ziekte-vrije en totale overleving aangetoond in multivariate analyse. Het meest waarschijnlijk worden met beide methoden verschillende epitopen van het eiwit gemeten.

Verder was in onze studie pS2 de meest belangrijke factor voor de overleving na relapse in een multivariate analyse met axillaire lymfklierstatus, oestrogeenreceptor, progesteronreceptor, ploïdie-status en S-fase fractie. Dit suggereert dat pS2 meer een predictieve factor is voor de response op systemische therapie, alhoewel wij niet in staat waren deze vraag in onze studie formeel te beantwoorden. Omdat er aanwijzingen zijn dat de pS2 expressie de functionele status van de oestrogeenreceptor weerspiegelt, maar ook dat pS2 betrokken is bij de groeiregulatie, zou de pS2-status predictieve waarde kunnen hebben, zowel met betrekking tot hormonale therapie als chemotherapie.

In twee andere studies werd de predictieve waarde van de pS2-status aangetoond voor chemotherapie en hormonale therapie. Verdere prospectieve studies zijn nodig om te onderzoeken of de immunohistochemische pS2-status kan worden gebruikt om de response op systemische therapie te voorspellen.

In Hoofdstuk III wordt een studie beschreven betreffende het mogelijk gebruik van het adhesiemolecuul CD44v6 als een prognostische factor.

Hoewel de eerste studie betreffende dit adhesiemolecuul aantoonde dat CD44v6 een onafhankelijk prognostische factor is, konden wij dit in een veel grotere studie, met langere follow-up, niet bevestigen. Diverse andere kleinere studies konden ook de waarde van CD44v6 als een prognostische factor niet aantonen, althans in multivariate analyse. Daarom kan worden geconcludeerd dat de CD44v6-expressie geen prognostische waarde heeft bij het primaire mammacarcinoom. Een dergelijke prognostische waarde lijkt op biologische gronden ook minder waarschijnlijk, omdat CD44v6 vaak tot expressie komt in normaal weefsel, inclusief normaal mammaweefsel. Meer onderzoek is echter nodig om de rol van CD44 isovormen bij de progressie van maligniteiten te verhelderen.

In Hoofdstuk IV wordt aangetoond dat de MIB-1 labeling index als een maat voor de proliferatie een onafhankelijke prognostische factor is met betrekking tot ziekte-vrije overleving. Dit is zowel waar bij een cut-off punt bij de mediane waarde van 7%, als wanneer MIB-1 wordt geanalyseerd als een continue variabele. Diverse andere studies hebben aangetoond dat MIB-1 of Ki67 belangrijke prognostische factoren zijn in multivariate analyse voor patiënten met een primair mammacarcinoom. Er zijn echter ook andere methoden om de proliferatieve

activiteit van de tumor te meten, zoals het meten van de S-fase fractie, de thymidine labeling index en de mitotische index. Deze methoden zijn ook in diverse studies onderzocht en van alle is prognostische waarde aangetoond, soms in multivariate analyse. Bij onze patiënten had S-fase fractie alleen waarde met betrekking tot prognose in univariate analyse.

Slechts weinig studies hebben verschillende methoden om de proliferatieve activiteit van de primaire mammatumor te meten direct vergeleken. Wij konden slechts een zeer zwakke associatie tussen MIB-1 labeling index en S-fase fractie aantonen. Diverse andere studies hebben een hogere associatie tussen S-fase fractie en MIB-1 of Ki67 gevonden.

Verder was bij onze patiënten met behulp van multivariate analyse wel de MIB-1 labelingsindex, doch niet de S-fase fractie, een prognostische factor. Het tegenovergestelde is echter ook beschreven. De meeste van deze verschillen kunnen waarschijnlijk worden verklaard door verschil in de methodologie. In de normale routinepraktijk lijken immuunhistochemische methoden het meest geschikt. Omdat diverse studies de onafhankelijke prognostische waarde van Ki67 en MIB-1 hebben aangetoond, zijn prospectieve studies met deze antilichamen noodzakelijk, waarbij er consensus dient te zijn betreffende de methodologie.

In Hoofdstuk V werden het proto-oncogen bcl-2 en het tumorsuppressorgen p53 onderzocht met betrekking tot de prognostische rol bij het primair mammacarcinoom. Na de gehele follow-up periode had bcl-2 expressie geen invloed op de ziektevrije totale overleving. Dit lijkt afwijkend van het merendeel van de literatuur, omdat in diverse studies is gevonden dat de bcl-2 expressie op z'n minst in univariate analyse is geassocieerd met een betere ziektevrije en/of totale overleving. Deze verschillen kunnen waarschijnlijk worden verklaard door de lengte van de follow-up periode, die in de meeste studies beduidend korter is. Dit suggereert dat de bcl-2 expressie een tijdsafhankelijke prognostische factor is.

In onze patiënten was p53-positiviteit geassocieerd met een slechtere totale overleving voor de hele groep patiënten en de klier-negatieve subgroep. Ook in multivariate analyse was p53-expressie geassocieerd met een verminderde totale overleving. Wat betreft de ziektevrije overleving was er een trend voor een betere ziektevrije overleving bij p53-negatieve patiënten, doch dit verschil was niet statistisch significant. Deze resultaten komen overeen met wat in de literatuur wordt gemeld, maar zoals in een review werd gesteld is het prognostisch effect van p53 waarschijnlijk klein.

Het feit dat in onze studie p53 expressie was gecorreleerd met een slechte overleving na relapse, ook in multivariate analyse, suggereert dat p53-expressie de response op systemische therapie in patiënten met mammacarcinoom kan voorspellen. De overigens beperkte gegevens in de literatuur betreffende de predictieve waarde van p53-expressie ondersteunen dit.

Belangrijk zijn verder de bevindingen in de bcl-2/p53 subgroepen. In de bcl-2-positieve subgroep was er een groot verschil tussen p53-negatieve en p53-positieve patiënten wat betreft de ziektevrije overleving (77 vs 53% 5-jaars overleving) en totale overleving (86 vs 59% 5-jaars overleving). In de bcl-2-negatieve subgroep echter had de p53-status geen noemenswaardige invloed op de ziektevrije of totale overleving. In het algemeen werden in de subgroepen van klier-positieve en klier-negatieve patiënten vergelijkbare tendensen gevonden. Ook wat betreft de overleving na relapse hadden bcl-2-positieve/p53-negatieve patiënten duidelijk een betere prognose dan de drie andere subgroepen.

Meer studies betreffende deze subgroepen zijn nodig en een verklaring voor dit fenomeen, indien het wordt bevestigd, is niet gemakkelijk te geven. Omdat bcl-2-expressie bij het mammacarcinoom waarschijnlijk gekoppeld is aan een langzamere groei, kan worden gespeculeerd dat bij bcl-2-positieve mammacarcinomen de p53-status belangrijk is voor de prognose van de patiënt, hoewel dit niet noodzakelijkerwijze gerelateerd hoeft te zijn aan apoptose. Indien anderzijds verlies van bcl-2-expressie is gerelateerd met snellere groei van het mammacarcinoom, is het denkbaar dat de p53-status geen rol meer speelt.

In Hoofdstuk VI worden de resultaten beschreven van het bepalen van de microvaatdichtheid met anti-CD34 en anti-factor VIIIIRAg in dezelfde groep patiënten. Wij waren niet in staat de resultaten, zoals vermeld in het merendeel van de literatuur, te bevestigen, waarin gesteld wordt dat microvaatdichtheid een onafhankelijke prognostische factor is bij het primair mammacarcinoom.

Hoewel bij onze patiënten een hogere microvaatdichtheid, zoals vastgesteld met anti-CD34, geassocieerd was met een toenemend risico op een relapse bij analyse als een continue variabele, kon er geen cut-off waarde worden vastgesteld, die aanleiding gaf tot een significant verschil in ziektevrije overleving. Microvaatdichtheid, zoals bepaald door anti-factor VIIIIRAg, had geen invloed op zowel de ziektevrije als de totale overleving. Diverse andere onderzoekers waren ook niet in staat om de (onafhankelijke) prognostische waarde van microvaatdichtheid aan te tonen. Dit heeft geleid tot levendige discussies in de literatuur. Uit deze discussies kan worden geconcludeerd dat het meten van de microvaatdichtheid een technisch moeilijke methode is met diverse subjectieve aspecten. Daarom lijkt de huidige methode van het bepalen van de microvaatdichtheid nauwelijks bruikbaar voor gebruik in de routinepraktijk voor patiënten met een maligniteit.

Verder was bij onze patiënten leeftijd boven de 69 jaar gecorreleerd met een lagere microvaatdichtheid. Dit bevestigt de bevindingen in twee andere studies. Indien men aanneemt dat microvaatdichtheid ondanks methodologische problemen toch een prognostische factor is, suggereert dit dat oudere leeftijd geassocieerd is met een betere prognose bij het primaire mammacarcinoom.

Concluderend: kijkend naar alle onderzochte prognostische variabelen bleek de MIB-1 labeling index een onafhankelijke prognostische factor te zijn voor ziektevrije overleving en de p53-expressie voor totale overleving. Dit steunt veel van wat is gevonden in de literatuur, echter met een duidelijk langere follow-up dan in de meeste andere studies wordt gemeld. Echter in overeenstemming met de literatuur kan geen enkele van de meer recent ontwikkelde factoren, zoals in dit proefschrift bestudeerd, gebruikt worden voor beslissingen bij de behandeling van een individuele patiënt. Meer dan 20 jaar research met deze nieuwere prognostische factoren bij het primaire mammacarcinoom, heeft zelfs niet geleid tot veel vooruitgang op dit gebied. Een verbetering dienaangaande is alleen mogelijk wanneer meer uniforme methoden worden gebruikt in prospectieve studies. Het lijkt echter op dit moment onwaarschijnlijk dat één enkele prognostische factor in de toekomst gebruikt kan worden om te beslissen welke individuele patiënt profijt kan hebben van (adjuvante) systemische therapie.

Het is waarschijnlijk nuttiger deze nieuwere factoren te gebruiken om systemische therapie aan een individuele patiënt "op maat" te geven. Het feit dat bij onze patiënten zowel pS2 als p53 onafhankelijke prognostische waarde had in multivariate analyse voor de overleving na relapse, suggereert dat deze factoren kunnen worden gebruikt voor het voorspellen van een response op systemische therapie.

Er is niet veel literatuur betreffende dit onderwerp, maar diverse studies zijn op dit moment niet in tegenspraak met de stelling, dat nieuwere factoren beter kunnen worden gebruikt als predictieve dan als prognostische factoren. Meer studies dienaangaande zijn nodig, bij voorkeur prospectief en met uniforme methodologie. Verder kan worden gespeculeerd dat moleculair-biologische methoden, die specifieke genetische veranderingen onderzoeken, betere prognostische informatie geven. Hoewel op dit moment de meeste technieken om genetische veranderingen te ontdekken kostbaar en arbeidsintensief zijn, is het denkbaar dat bv. met de ontwikkeling van DNA-chip technologie, genetische screening meer algemeen toepasbaar wordt en gebruikt kan worden als prognostische factor.

Dankwoord

Dankwoord

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Curriculum Vitae

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De auteur van dit proefschrift werd geboren op 30 mei 1954 te Breda. Na het behalen van het gymnasium- β diploma aan het Bisschoppelijk College te Sittard, studeerde hij van 1972 tot 1979 geneeskunde aan de Katholieke Universiteit Nijmegen. Na het vervullen van de militaire dienstplicht volgde hij in de periode 1981 tot 1986 de opleiding interne geneeskunde in het Gemeenteziekenhuis (nu: Merwedeziekenhuis) in Dordrecht (opleider: dr BA de Planque) en het de Weverziekenhuis (nu: Atrium Medisch Centrum) in Heerlen (opleider: dr FTh Lustermans). Vanaf begin 1986 was hij als internist werkzaam in de Daniël den Hoed Kliniek in Rotterdam bij dr G Stoter. In 1988 en 1989 was hij KWF-fellow, waarbij ook 1 jaar werd gewerkt op een laboratorium van het National Cancer Institute in Frederick, Maryland, Verenigde Staten. Sinds 1 februari 1992 is hij als internist verbonden aan de Werkgroep Haematologie-Oncologie van de Vakgroep Interne Geneeskunde (hoofd: Prof dr HFP Hillen) van het Academisch Ziekenhuis Maastricht.