Looking for factors predicting outcome of breast carcinoma using tissue microarrays

van der Vegt, Bert

LOOKING FOR FACTORS PREDICTING OUTCOME OF BREAST CARCINOMA USING TISSUE MICROARRAYS

Bert van der Vegt

De voorkant van dit proefschrift is net als een Tissue Microarray opgebouwd uit allemaal kleine fragmenten uit afbeeldingen van mammatumoren. Deze fragmentjes zijn voorzien van een tegenkleur. Het kleurenpatroon is geïnspireerd op het schilderij 'Ancient Sound' van de Duits/Zwitserse kunstschilder Paul Klee (1879-1940).

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Promotores:	Prof. dr. H. Hollema Prof. dr. G.H. de Bock
Copromotor:	Dr. J. Wesseling
Beoordelingscommissie:	Prof. dr. M.J. van de Vijver Prof. dr. G.J. den Heeten Prof. dr. A.L.M. Verbeek

Paranimfen: Drs. T. van der Vegt
Drs. G.J. Dijkstra

Contents

1 GENERAL INTRODUCTION

In the Western world, the lifetime risk for developing breast cancer in women is approximately 10%. Breast cancer is rare before the age of 30 after which the incidence rises until the menopausal age. After this the incidence remains relatively constant. The development of a breast carcinoma is a multifactorial process in which several reproductive and lifestyle factors play a role.[1]

5-7% of all breast carcinoma patients present with distant metastases at the time of diagnosis[2], but approximately 30% of patients with stage I, II, or III breast cancer harbour undetected microscopic distant deposits of metastatic carcinoma.[3] It is still unclear which of these micrometastatic deposits make it to macrometastatic lesions. Selection for adjuvant chemotherapy is currently based on generally accepted prognostic and predictive factors including age, tumour size, histological grade, estrogen and progesteron receptor status, Her2/neu status, menopausal status and lymph node status.[4,5] These factors perform relatively well in group based statistics, but for an individual patient it remains difficult to predict outcome. In the Netherlands adjuvant chemotherapy is given to approximately 90% of breast cancer patients younger than 35 years, to 55% of breast cancer patients 35-50 years of age, and to 20% of breast cancer patients 50-70 years of age (period: 1998 to 2002, n=8437; Netherlands Cancer Registry; http://iknl.nl). If chemotherapy is given, the risk for recurrent disease within 10 years is reduced with approximately 35% among women aged under 50 and approximately 20% among those aged 50-70[6,7], as confirmed by the 15 year follow-up analysis[2], implying that a part of those patients would have survived without this toxic and expensive therapy. This underlines the importance of good prediction strategies to tailor treatment for each individual patient.[8]

In recent years many microarray procedures have been developed which made it possible, from genome to protein, to assess multiple factors (e.g. the expression of many genes or proteins) per patient in one experiment and relate them to clinical endpoints. With these techniques it has become possible to differentiate between clinically relevant breast cancer subtypes and to search for new prognostic indicators in breast cancer.

In this thesis the tissue microarray (TMA) technique is used to assess the added value of novel protein markers that have been related to breast carcinoma to better predict outcome in (in-situ) breast carcinoma. The TMA technique was introduced by Kononen in 1998.[9] Using this technique it has become possible to compile paraffin embedded material from up to 1000 donor paraffin blocks from different test samples (i.c. tumours) into one acceptor paraffin block. This enables highly efficient testing of large cohorts of tumours for the expression of one protein in one staining procedure. This in contrast to the other microarray methods in which the relative quantity of thousands of chromosomal regions, genes, SNPs or mRNA present in one tumour sample are assessed in a single test. In chapter 2 a critical overview of the different microarray techniques, including the TMA technique, is given.

MUC1 (episialin, epithelial membrane antigen (EMA), CA15-3 antigen), a transmembrane glycoprotein expressed on the apical surface of normal glandular epithelial cells, has a controversial role in breast cancer.[10,11] In vitro and some in vivo studies have described cell adhesion inhibition as well as increased metastatic and invasive potential of tumour cells by overexpression of MUC1.[12-14] Other in vivo studies however, found a better outcome for patients overexpressing MUC1[15-17] or no relation between MUC1 expression and outcome at all.[18-21] These divergent results might be explained by the different expression patterns for MUC1 that exist. The tissue microarray array technique is used in a series of in-situ ductal carcinomas (chapter 3) and invasive ductal carcinomas (chapter 4) to investigate a novel scoring system that is based on the location and pattern of MUC 1 expression in in-situ and invasive breast carcinomas.

The introduction of a nation-wide breast cancer screening programme led to an increased incidence of in-situ ductal carcinomas (DCIS).[22-24] Because DCIS does not always progress to invasive breast carcinoma some authors consider screen-detected DCIS to be overdiagnosis.[25,26] Using the tissue microarray technique we studied pathological and biological differences between screen-detected and interval DCIS hypothesizing that screen-detected is biologically more aggressive, because it has been detected in the screening due to linear

branching- and coarse microcalcifications which are frequently associated with high grade DCIS. This is described in chapter 5. Although the introduction of the breast cancer screening programme led to a substantial decrease in advanced breast carcinoma and to a decline of breast cancer related mortality[27], 36% of the tumours found in patients participating in the Dutch screening programme still emerge as interval carcinomas; clinically symptomatic carcinomas detected between two screening moments.[28] There is discussion whether these interval carcinomas differ biologically from screen detected carcinomas and should perhaps be treated differently.[29] In chapter 6 we investigated the biological aggressiveness of these interval carcinomas using the tissue microarray technique.

Her2/neu receptor status is an important factor in the treatment of breast carcinoma as patients with Her2/neu receptor positive breast cancer have reduced mortality and recurrence rates when receiving combined adjuvant treatment with the anti-Her2/neu antibody trastuzumab and chemotherapy. Her2/neu receptor status is commonly assessed using immunohistochemistry.[30,31] Immunohistochemistry for Her2/neu suffers from significant interobserver variation and poor interlaboratory reproducibility.[32-35] In recent years, rabbit monoclonal antibodies have been developed that should lead to more reliable test results than the previously used mouse monoclonal antibodies.[36,37] In chapter 7 the potential of a novel rabbit monoclonal antibody against Her2/neu is tested using the tissue microarray technique.

Finally, chapter 8 contains a general discussion on the most important results of this study.

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2

MICROARRAY METHODS TO IDENTIFY FACTORS DETERMINING BREAST CANCER PROGRESSION

Potentials, limitations, and challenges
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B. van der Vegt[1]
G.H. de Bock[2]
H. Hollema[1]
J. Wesseling[3]

Departments of [1]Pathology and Laboratory Medicine and [2]Epidemiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, and [3]Department of Pathology, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital.

Abstract

65 to 80 percent of the patients with breast cancer might not benefit from the adjuvant therapy they receive based on 'classical' markers used for the selection for adjuvant therapy. Therefore it is necessary to develop new markers that are able to tailor treatment for an individual patient. A number of microarray methods have been developed in recent years to accommodate this search for new factors that determine breast cancer progression. We give an overview of the most commonly used microarray methods to identify tumour progression markers (oligo- or cDNA arrays, CGH arrays, PCR arrays, and tissue microarrays). Their applications will be illustrated using the most influential examples from literature. The potentials, limitations and the related statistical analyses of each method are discussed. We conclude that microarray studies have led to an increase in the understanding of the complexity and diversity of breast carcinoma and have provided clinical relevant subgroups of breast cancer that may benefit from patient tailored treatment. Still, more extensive external validation and long-term follow-up will be necessary before such assays can be implemented into routine clinical practice. Most likely, these novel prognostic indicators will be complementary to the already available classical prognostic factors.

1 Introduction

In the Western world, the life time risk for developing breast cancer in women is approximately 10%. 5-7% of patients present with distant metastases at the time of diagnosis, but 30-40% will develop metastases and die of the disease within 15 years.[1] Currently, selection for adjuvant chemotherapy is based on generally accepted prognostic and predictive factors including age, tumour size, histological grade, hormone receptor status, Her2/neu status, menopausal status and lymph node status.[2; 3] Although these factors perform relatively well in group based statistics, they poorly predict the outcome for the individual patient. Prediction in breast cancer is difficult because it is a very diverse disease comprising many biological subtypes that are all classified as invasive ductal breast carcinoma (breast carcinoma not otherwise specified (NOS)), because they cannot be added to one of the currently recognized specific breast cancer subtypes. Approximately 30% of patients with stage I, II, or III breast cancer harbour undetected microscopic distant deposits of metastatic carcinoma[4], and it is still unclear which of these micrometastatic deposits make it to macrometastatic lesions. In the period 1998 to 2002 adjuvant chemotherapy was given to circa 90% of breast cancer patients younger than 35 years, to 55% of breast cancer patients 35-50 years of age, and to 20% of breast cancer patients 50-70 years of age (n=8437; Netherlands Cancer Registry; http://www.ikcnet.nl/page.php?id=97) to treat micrometastases that go undetected at the time of diagnosis. If chemotherapy is given, the relative risk of reduction of relapse within 10 years is reduced with approximately 35% among women aged under 50 and approximately 20% among those aged 50-70[5], as confirmed by the 15 year follow-up analysis.[1] However these studies also show that substantial numbers of patients considered high-risk who did not receive adjuvant therapy in the old trials did not develop distant metastases, implying that many patients currently treated with adjuvant therapy are actually overtreated. This underlines the importance of good prediction strategies to tailor treatment for each individual patient.[6] In recent years many microarray procedures have been developed which made it possible, from genome to protein, to assess multiple factors (e.g. the expression of many genes or proteins) per patient in one experiment and relate them to clinical endpoints. With these techniques it has become

possible to differentiate between clinically relevant breast cancer subtypes and to search for new prognostic indicators in breast cancer.

In this review we give an overview of the currently used microarray methods and their applications will be illustrated using the most influential examples from literature. The potentials and limitations and the related statistical analyses of each method will be discussed.

2 DNA MICROARRAYS

2.1 Technique

2.1.1 General principle

The DNA microarray technique was first described by Fodor.[7] Using this technique up to 50,000 known single stranded DNA fragments are immobilized at predefined spots on a solid surface.[8] Using the natural quality of DNA to bind complementary DNA, study samples can be tested for gene expression. In this way, thousands of genomic or gene expression features of one tumour sample can be assessed in a single test.

2.1.2 cDNA array

cDNA arrays are the most commonly used microarrays. In this technique a microarray of oligonucleotides, cDNA (copy DNA), or mRNA sequences is compiled. Genes to be included are selected from a gene bank.[9] cDNA from a test sample, usually generated from tumour mRNA, and reference cDNA, usually generated from mRNA isolated from multiple human cell lines, are labelled using two different detectable markers, e.g. a fluorescent dye (fig. 1a). Both cDNAs are than mixed (fig. 1b). Single stranded DNA fragments from known genes of interest are immobilized on a solid surface, a microarray (fig. 1c). The mixture of test- and reference cDNA is then hybridized to this microarray (fig. 1d). After the excess of cDNA has been washed off, the intensity of the markers is read using a laser (fig. 1e). Relative expression of the test sample cDNA (compared to the reference cDNA) is calculated (fig. 1f). This is repeated for all the test samples.[10]

2.1.3 Array Comparative Genomic Hybridization (array-CGH)

In the development of many tumours chromosomal damage leads to gain or loss of genomic material. The increase of number of copies of the Her2/neu gene is the best known example of this. Using CGH technique it is possible to detect the number of copies of DNA sequences for the whole genome. Originally the copy number was mapped to metaphase chromosomes to assess the localisation, but in array-CGH artificial chromosomes from bacteria (BAC), vectors derived from bacteriophages, containing DNA sequences (cosmid or P1), or cDNA clones are immobilized on a glass surface and hybridized with a mixture of fluorescently labelled test and reference DNA. Using the same technique as in cDNA arrays the relative number of DNA sequence copies can then be calculated.[11] Array CGH can also be performed on commercially available oligonucleotide platforms.

2.1.4 Single Nucleotide Polymorphism (SNP) microarray

SNPs are DNA sequence variations consisting of a single nucleotide. Certain SNPs are associated with an increased risk for disease. The technique of SNP microarrays is comparable to cDNA microarrays. Known SNPs are immobilized on a glass surface and a mixture of fluorescently labelled test and reference DNA is hybridized on this surface. Afterwards fluorescence intensities

are measured for each allele of each SNP.[12] Using this technique it is also possible to assess loss of heterozygosity (LOH), a chromosomal mutation in which a complete allele is lost, as is frequently observed in breast cancer.

2.2 Applications

There are several applications for the microarray technique in breast cancer research. In the next section these applications will be discussed.

2.2.1 Class definition

The first studies in breast carcinoma published using expression-arrays performed unsupervised clustering of microarray data. These analyses aimed to reveal whether previously unknown subtypes of breast carcinoma can be recognized based on differences in expression profile. The first and probably best known example is the study by Perou et al, who used a cDNA microarray

representing 8102 genes to subtype 65 breast carcinomas into four distinct subtypes, of which two were previously unknown: an oestrogen receptor (ER) positive group, a normal breast like group that highly expressed genes seen in adipous- and other non-epithelial cell types, an ErbB2 positive group over expressing several genes on the ErbB2 amplicon, and a basal like group overexpressing keratins 5 and 17, laminin and fatty acid binding protein 7.[13] Later, the ER positive group was divided into at least two subgroups: a luminal 'A' subgroup, characterized by a high expression of the ER α gene, GATA binding protein 3, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor α and LIV-1, and luminal 'B and C' subgroups expressing low to moderate levels of these luminal specific genes.[14] It was shown that these 'molecular portraits' are maintained throughout the metastatic process of breast cancer.[14; 15] Using a supervised clustering method (see statistics section 5.2.3) others found gene sets that were able to divide patient groups by ER status[16; 17] and axillary lymph node status[16] suggesting a different biological background for these groups. In a genome-wide association study by genotyping 528,173 SNPs in 1,145 postmenopausal women with invasive breast cancer and 1,142 controls four SNPs in intron 2 of FGFR2, a region encoding a tyrosine kinase receptor commonly associated with breast cancer, were found that were highly associated with breast cancer. This association was verified in the same study using 1,776 affected individuals and 2,072 controls from three additional studies.[18] A whole genome screen of 81 breast carcinoma samples from 19 non-BRCA1/2 breast cancer families using 4720 genome wide SNPs was performed and six regions on chromosomes 2, 3, 4, 7, 11 and 14 were identified as candidates to contain genes involved in breast cancer susceptibility.[19]

Using array-CGH, from a group of 36 patients with a proven BRCA1 mutation and 30 patients with an elevated breast cancer risk (independent bilateral breast carcinomas), a molecular classifier was built that detected 84% of BRCA1 tumours.[20]

2.2.2 Survival prediction

The next logical application of microarrays was to use the retrospectively discovered gene sets to predict outcome. Using the subgroups described

by Perou et al[13], Sorlie was able to predict prognosis in a group of 78 breast carcinoma patients.[14] Mainly the basal like type of breast carcinomas was associated with poor prognosis.

Another important study was performed by Van 't Veer et al who developed a 70 gene set that was able to predict the development of metastasis in follow-up in a group of 98 young patients consisting of 34 patients that had developed metastasis within 5 years, 40 patients that continued to be disease free after 5 years, 18 patients with a BRCA-1 mutation and 2 patients with a BRCA-2 mutation.[21] This 70 gene signature was later validated in a group of 295 early stage breast carcinoma patients, partly consisting of the group used to train the model. Using this signature outcome could be predicted more accurately than using standard parameters.[22] An other gene set that was extensively validated comes from Wang et al. In a group of 286 node negative breast carcinoma patients that had not received adjuvant therapy a gene set containing 76 genes of which 60 genes came from ER-positive patients and 16 genes from ER-negative patients was developed that was able to identify patients who developed distant metastases within 5 years. This gene set was validated in the same study using an independent testing set. In this set the gene set had a high sensitivity (97%) and a mediocre specificity (48%).[23] This gene set was validated externally in two independent groups of 180 and 198 node negative breast carcinoma patients, not receiving adjuvant chemotherapy.[24; 25] In these studies comparable prognostic groups as in the initial studies were found. In a similar manner as the examples given above others developed and validated gene sets that are able to predict survival of specific patient groups.[23; 26-31] The outcomes of those studies were comparable to those described above.

2.2.3 Response Prediction

Microarray studies have been conducted on the role of gene expression profiles in the prediction of response to therapy. All these studies however, were performed in very small study groups which might have confounded their results. From a group of 24 advanced breast carcinoma patients a gene expression profile was derived that predicted the response to neoadjuvant docetaxel treatment.[32] In the same study this gene set was validated using

an independent set of 6 tumours. From a group of 24 advanced breast carcinoma patients, a gene-set predicting pathological complete response after neoadjuvant paclitaxel and fluorouracil, doxorubicin and cyclophosphamide chemotherapy was derived and validated in a group of 18 advanced breast carcinoma patients.[33] From 60 patients treated with adjuvant tamoxifen monotherapy, a gene set was derived predicting the response to therapy. This gene set consisted of only two genes.[34]

3 Polymerase Chain Reaction (PCR)-array

3.1 Technique

'Real time' quantitative reverse transcription (qRT)-PCR techniques which are commonly used for high throughput applications are based on the concept of detecting accumulating PCR products from genetic regions of interest in test samples.[35; 36] RNA from tumour cells is isolated and using reverse transcriptase converted into double stranded DNA (fig. 2a). This DNA is denatured yielding single stranded DNA (fig. 2b). Next primers specific to the 3' and 5' ends of the genetic region of interest are allowed to anneal to the single stranded DNA (fig. 2c). Taq polymerase binds to the primer and extends to the end of the DNA strand synthesizing a new complementary DNA strand (fig. 2d), yielding double stranded DNA. After this the cycle is repeated. SYBR Green, a fluorescent dye that has low fluorescence in the absence of double stranded DNA, and high fluorescence in presence of double stranded DNA is added. As the PCR process proceeds the amount of fluorescence, and thereby indirectly the amount of double stranded DNA, is measured in 'real time' at each amplification cycle. The amplification cycle at which the fluorescent PCR product is first detectable is called the threshold cycle (fig. 2e). This is an extremely accurate measure for the quantity of the target gene in the test sample. This quantity can than be compared with that of other test samples and 'normal' controls.

3.2 Applications

3.2.1 Subtyping

Ahr et al applied a PCR assay containing 15 genes from a prior microarray experiment and 11 genes of which the role in breast cancer had already

been described or which were considered useful as surrogate markers for proliferation (MKi67, PoloLikeKinase), IFN inducible genes (STAT1), stromal cells (DDR2) and vascularization (VEGFR), to a panel of 94 specimens (containing 73 breast carcinomas) and were able to identify a subgroup of breast carcinomas associated with poor clinical characteristics.[37] Using a PCR array containing 47 genes selected from literature on a group of 199 breast carcinomas others identified a subgroup (designated 'subgroup 7') of carcinomas with a low recurrence risk.[38] The results from the DNA microarray study by Perou[13] were confirmed in a group of 123 breast samples using a PCR array containing 53 genes.[39]

3.2.2 Survival prediction

Paik et al developed a 21 gene PCR assay (Oncotype DX™) from a candidate set of 250 genes, selected from literature and the results of cDNA studies which included 16 cancer-related genes and 5 reference genes by using the results from three independent studies compromising a total of 447 patients. Using the results from this assay they calculated a Recurrence Score (RS). This RS represents the risk of developing distant recurrences in follow-up.[40] In the same study this assay was also successfully used to predict outcome in an independent group of 668 node-negative tamoxifen treated breast carcinoma patients. However, when applied in a group of 149 patients with node-negative breast cancer who had not received adjuvant systemic therapy the assay was unable to predict distant disease recurrence.[41]

3.2.3 Response prediction

Like the mRNA/cDNA microarray studies, the PCR-assay introduced by Paik et al[40] was later used successfully to predict the response to chemotherapy in locally advanced breast carcinoma patients.[42]

4 Tissue microarray

4.1 Technique

The tissue microarray (TMA) technique was introduced by Kononen in 1998.[43] Using this technique it is possible to compile paraffin embedded material from up to 1000 donor paraffin blocks from different test samples (i.c. tumours) into one acceptor paraffin block. This enables highly efficient testing of large cohorts of tumours for the expression of one protein in one staining procedure. This in contrast to the microarray methods in which the relative quantity of thousands of chromosomal regions, genes, SNPs, or mRNA present for one tumour sample is assessed in one single test.

The construction of a TMA starts by selecting the most representative tumour spot (fig. 3a) on the donating paraffin block using a Haematoxylin & Eosin (H&E) stained section for orientation purposes (fig. 3b). Most authors use a manual tissue arraying instrument (Beecher Instruments, Silver Springs, MD, USA) to take a number of core punches from the donating paraffin block (fig. 3c) and arraying them into a predestined spot in the new acceptor paraffin block (fig. 3d). There is discussion on the number of tumour cores that should be included in a TMA to be representative for a tumour, especially because breast tumours tend to be very heterogeneously. It was shown that only two 0.6mm cores need to be included in an acceptor block to be representative for a whole tumour in more than 95% of the cases.[44] Most authors however include three or four tumour cores in an acceptor block.[45] After arraying the tumour cores in the acceptor block most authors note the localisation in an Excel worksheet for later reference. From the recipient block standard 3 um microtome sections can be cut (fig. 3e). On these sections all commonly applied immunohistochemistry and in-situ techniques can be performed.

4.2 Applications

In general, TMA is not suitable for testing on a single patient basis. However, in retrospective studies, TMAs can be applied to select for which limited set of proteins the expression should be determined to be the most informative for tumour classification, survival prediction, and prediction of response to therapy.

4.2.1 Class definition

Using immunohistochemistry for the key markers of the Perou study[46] defining the breast carcinoma subtypes two different studies were able to confirm these subtypes on FFPE material embedded in TMAs containing 107 and 1,076 cases respectively.[47; 48]

Zhang et al performed immunohistochemistry for 7 breast carcinoma related genes (ER, PR, c-ErbB2, p53, Cox-2, VEGF and PDGF) on a TMA containing 97 tumours. Using unsupervised clustering they found an ER+ and an ER- subgroup.[49]

4.2.2 Survival Prediction

Makretsov et al presented a series of 438 breast carcinomas arrayed in a TMA.[50] They performed immunohistochemistry for a panel of 31 markers related to breast cancer. This panel was gathered from the results the microarray studies. By performing hierarchical clustering on the expression data of these 31 genes a gene set of 11 genes was compiled, that gene set was able to predict different prognostic classes in breast carcinoma independent of lymph node metastasis, tumour size, and tumour grade in multivariate analysis.

Using a TMA consisting of over 600 breast tumours van de Rijn et al, showed that a subgroup of breast carcinomas expressing cytokeratins 17 and 5/6 was correlated with poor prognosis.[51]

4.2.3 Response Prediction

Using a TMA, Tovey et al showed in a group of 402 ER positive patients treated with tamoxifen that a group of HER1-3-positive and/or PR-negative patients were significantly more likely to have an early relapse under tamoxifen treatment in univariate and multivariate analysis.[52] In a series of 178 node positive breast carcinoma patients, treated with doxorubicin based chemotherapy, Park et al used a TMA to assess Cox-2 expression in this group of patients. Patients with a tumour expressing Cox-2 had a significantly decreased over overall and disease-free survival when compared to patients with tumours that did not express Cox-2. The authors conclude that Cox-2 expression may represent a doxorubicin resistant phenotype of breast cancer.[53]

5 Statistics

5.1 General

One thing all gene expression profiling experiments have in common is the vast amount of data points generated. Authors use several statistical tools, with a varying degree of complexity, to look for patterns in these data. For tissue microarray technique, multiple samples from one tumour must generate one final result. When a gene expression profile has been discovered it needs to be validated. In this section methods for data reduction and validation of gene expression experiments will be discussed.

5.2 Data reduction

5.2.1 TMA

Because multiple cores from one tumour are often incorporated in a TMA, data from TMA experiments usually features multiple scores from one tumour sample. After assessing that each of those cores consists of representative tumour material, these scores need to be combined. Several rules can be applied to combine multiple scores. One could average the scores, or take the highest or lowest score. Which rule applies best is dependent on the biological properties of the marker stained for. If any degree of loss of a certain marker in a tumour is biologically relevant it is best take the lowest score, but for a marker of proliferation, e.g. Ki-67, it would be best to take the average score. TMA combiner, a computer program available free of charge, is able to combine multiple scores noted in an Excel worksheet by applying one of the combining rules.[54]

5.2.2 Unsupervised clustering

In unsupervised clustering data is divided into clusters. Two approaches are commonly used. In hierarchical clustering gene expression for each of the study samples is calculated and samples are arranged on similarities in the gene expression profile. This a graphically represented in a dendrogram. Afterwards this dendrogram can be divided in two or more clusters. In k-means clustering the number of clusters is predefined. For each of the samples a gene expression is calculated. Each of the samples is than added to one of the clustering ensuring that the centre of each cluster is maximally differentiated

from the others to define specific subgroups in the study population. A good example of unsupervised clustering is the study by Perou where a group of ductal carcinomas was divided and new classes were found.[13]

Unsupervised clustering will always generate several clusters it is therefore important to relate the outcome of a cluster analysis to clinical data and to perform a validation of the model.[55]

5.2.3 Supervised clustering

In supervised clustering two or more groups are predefined before a cluster algorithm is used, e.g. patients that will and will not develop distant metastasis in follow-up. Between these two groups a clustering algorithm will calculate which genes best differentiate both groups. The van 't Veer study is an example of a study where supervised clustering was used.[21]

A problem with supervised clustering is over-fitting. Most studies use a small sample of the total study group to train a cluster algorithm and use the remaining part of the study group to test this algorithm. When the algorithm build on the small training group is perfectly fitted to this small group it will lose predictive power for the remaining part of the group.[55]

5.3 Validation

One of the largest problems of gene expression studies is multiple testing; because the number of samples included in a study is small compared to the enormous number of genes tested, a large number of the significantly tested genes may have been found purely by chance. Because genes in a gene expression profile are not always independent of one another it is difficult to correct for multiple testing.[56] To test if a gene set found in a 'training' group also applies to other groups it is necessary to validate such a gene set. Several methods of internal and external validation are used.

5.3.1 Internal validation

In internal validation (a part of) the study group is used to validate the model.

In split sample validation half of the study population is used to 'train' a gene profile and afterwards this profile is tested independently on the other

half of the group. Because the number of samples in the gene expression study is usually limited, the disadvantage of this method is clear: both the training and the testing group comprise a relatively low number of samples.[57] To avoid this problem several methods of cross-validation have been developed. In cross-validation gene profiles are trained using the study group minus one ("leave-one-out" cross validation) or a few of the samples and afterwards using the samples left out to test the profile (so-called 'boot-strapping). This is repeated until all the samples have been in the training and testing group. For each of the tests a prediction error is calculated. Afterwards these prediction errors are totalled. This total represents the fitting of the model.[58; 59] Because the 'training' and the 'test' set are not independent of one another this method of validation may lead to biases in the model.[56]

5.3.2 External validation

The objective of external validation is to determine whether use of a completely specified diagnostic classifier for therapeutic decision making in a defined clinical context results in patient benefit.[57] To make it possible to easily interpret and to verify or validate microarray data, standards for the presentation of microarray data have been developed, the MIAME (Minimal Information About a Microarray Experiment).[60] To perform an optimal external validation some rules apply to the study group used: (a) the inclusion criteria of the patients should be the same as the group in which the profile was trained, (b) the clinical end point of the study should be the same as the initial study and (c) exactly the same prediction rule as used in the initial study should be used in the validation study. This includes the genes selected, the method used to measure their expression, the equation and the cut-off point for the different classes of the initial study.[61] External validation studies should focus on the 'low-risk' class of the expression profile, as this is the group of patients that will possibly benefit from omitting therapy. Because this group is often small and has to be randomly divided in a treated and a non-treated group, large studies are necessary. Also a long follow-up is necessary to show a difference in survival between both groups with already low recurrence risk.[57] Therefore if possible it would be useful to plan such studies on archived material of patients from previously conducted multicenter clinical

trails.[57] The multigene PCR assay of Paik was validated in this manner.[40]

Problem of such studies are the often out-dated adjuvant therapy regimes used in these patient groups, which confound the results of such studies. In the multicentre phase III trail TAILORx (Trial Assigning IndividuaLized Options for Treatment (Rx)) trial this PCR array will be prospectively tested. Patients with axillary lymph node negative breast cancer and a Oncotype DX™ recurrence score of 11-25 are randomly assigned to receive combined adjuvant chemotherapy and hormal therapy or adjuvant hormal therapy alone (http://www.cancer.gov/clinicaltrials/ECOG-PACCT-1).

The gene set of Perou was validated by Sorlie on independent datasets, but not all the genes assessed in the initial study were available for the independent datasets. Also the clinical end-points for the independent datasets was different.[62] The 70 gene model of van 't Veer was validated by van de Vijver in a study group that partly consisted of patients used in the training model, therefore this validation was not entirely independent and might have been biased.[63] However, this model was retrospectively validated in an independent group, with similar results.[64] The MINDACT (Microarray In Node negative Disease may Avoid ChemoTherapy) trial is a multicentre, prospective, phase III trial in which 6000 node negative patients will be included.[65] For all patients included a risk profile will be calculated using 'classical' clinico-pathological markers and using the 70-gene signature. In cases where both methods are discordant a patient is randomly assigned to a treatment decision based 'classical' clinico-pathological markers or the 70-gene signature. The aim of this study is a reduction of 10-15% of chemotherapy treatment in the low risk group.

6.1 Discussion and future directions

The development of array comparative genomic hybridization, gene expression profiling, and the tissue microarray technique has led to a revolution in how to classify breast tumours and how to classify and predict prognosis and response to therapy. None of the techniques described in this review is likely to be superior to the other, but rather complementary. While DNA microarrays are useful to assess a large number of genes in a small study group, PCR arrays and tissue microarrays are excellent techniques to

validate the 'key'-markers from DNA microarray studies in large patient groups. Nevertheless, most attention has been paid to gene expression profiling for clinical purposes to discriminate between low versus high risk tumours which might guide decision making for administration of adjuvant therapy as is currently being tested in clinical trials.[65] Most strikingly, there is no or hardly any overlap between the gene sets tested in the different available predictive or prognostic signatures.[66] Partially, this can be explained by the use of different gene expression profiling platforms. Even within a single dataset a multitude of signatures can be selected that perform equally well with respect to prognosis prediction. This was illustrated by Ein-Dor et al. who showed that the selection of patients to be included in the training set of the model very much defines the gene list.[67] This was supported by Michiels et al who randomly sampled 500 groups of 78 patients from the Van 't Veer's group of patients and calculated a gene expression profile for each of those groups using the same technique and the same genes van 't Veer et al used to calculate their profile. Only 14 of the genes 'original' gene expression profile appeared in more than 50% of the 500 calculated profiles and 10 genes that were not in the van 't Veer profile also appeared in more than 50% of the 500 profiles.[59] This might be explained by the way test samples are selected, as most of the gene expression profiles were drawn from and optimised for specific patient groups (e.g. young, node negative patients). This might lead to applicability only in these specific groups of patients, although in a recent analysis the Amsterdam profile appeared to perform equally well for patients with 1-3 positive lymph nodes.[68] On the other hand, the selection of test samples comprising a consecutive series of young breast cancer patients with lymph node negative disease comprised a few BRCA-1 gene mutation carriers which did not affect the prognostic power of the test.[21] In addition, Fan et al published a study in which five gene expression profiles were applied to the same dataset.[69] The outcome predictions of the various models showed overlap, suggesting that although different genes are included in the models, the biological subgroups predicted by those genes are equal and that these different signatures are sufficiently robust to predict outcome. In addition, the expression signatures might perform relatively well because the interobserver variation between pathologists in assessing tumour diameter and grade might

result in suboptimal prognosis prediction.

Most predictive models derived from gene expression profiles do not provide an improved prognostic classification when compared to the 'classical' prognostic factors.[70] It has been shown on the 234 patients from van de Vijver et al who were not in the first study that the gene signature added a moderate but not significant improvement in predictive accuracy when added to the prognostic factors: age, nodal involvement, estrogen receptor status and tumour grade.[71] Another study compared five gene expression profiles and found that these profiles showed prognostic value independent of classical prognostic indicators, including grade, but that it is not clear that these models provide more prognostic information than the combination of currently available markers.[72]

A study by Sun et al, who compared the predictive power of clinical prognostic indicators and gene expression profiles alone, with a combination of clinical prognostic indicators and a gene expression profile in predicting the development distant metastases in breast cancer patients. The combination of both methods gained a far higher power.[73] We therefore believe that the use of the novel prognostic indicators as discussed in this review, should rather be complementary to 'classical' prognostic indicators instead of replacing these indicators.

There are some practical issues that should to be resolved before wide-scaled introduction of DNA microarrays can take place, as: the RNA in formalin fixed paraffin embedded material is to fragmented to be used in gene expression profiling. To prevent this, sampling fresh tumour tissue for cryopreservation shortly after surgery is required.

Some therefore argue that PCR arrays have advantages over DNA microarrays in daily clinical practice, because this an easily reproducible method that requires only a limited amount of tumour tissue.[74] Because PCR array can be performed on formalin fixed paraffin embedded (FFPE) material, no tumour tissue is lost for freezing.[34; 40; 75] However, with this type of array the expression of only a small number of genes can be assessed. DNA microarrays and PCR arrays measure the total amount of a certain gene product in a cell at the mRNA level, but this is an indirect method of determining gene expression.

mRNA levels do not always parallel the level of protein expression. Moreover, arrays assessing the level of mRNA expression do not provide any information about the biological activity of the encoded proteins, especially when proteins are involved in signal transduction. The advantage of TMA technique is that it is not only applicable on FFPE material, that it enables subcellular localization of the encoded protein as the histological context is maintained. This makes it possible to determine if the product has any biological relevance. For instance, E-cadherin has a well established function when expressed at the cellular membrane. The function of cytoplasmically expressed E-cadherin is unclear. In a TMA it is possible to differentiate between those two expression patterns. Because of the labour intensive scoring of TMAs the amount of genes scored using a TMA is relatively low. The scoring of IHC-staining by hand causes intra- and inter-observer variability. TMA evaluation is limited to known genes with an antibody available, therefore no new genes can be discovered using this method. In addition, a TMA is of no advantage on a single patient basis during the diagnostic work-up of surgically removed breast tissue.

A technique that is currently under way is ChIP (chromatin immunoprecipitation) on chip technique, which provides an assay for the genome-wide location and functional analysis of DNA-binding proteins.[76] DNA-binding proteins bind to specific sites at the genome to regulate genome expression and maintenance, thereby regulating for example RNA synthesis or allowing cells to move through the cell cycle. Using ChIP on chip technology specific DNA-binding proteins can be assessed across the genome of many samples. Because this is a functional analysis, it is the most precise method of detecting DNA activation. Another emerging technique is the application of protein arrays, where it is possible to assess the expression of proteins on a similar manner as a DNA microarray.[77] Both methods have to our knowledge not been applied in breast cancer research yet.

In conclusion we believe that microarray studies, by discovering patterns in gene expression of breast carcinomas, should be used to gain insight in the biological pathways of breast cancer progression, and that well validated signatures, either at the genomic, mRNA, or protein level, containing 'key'-

markers, may be combined with 'classical' prognostic indicators for use in daily clinical practice. However, the exact clinical value for the gene profiles described in this article remains to be established. Not until these profiles are validated in well designed prospective clinical trials such as the MINDACT[65] and TAILORx, treatment decisions should be made based on gene expression profiling results. In the mean time, the refinement of microarray techniques including more sophisticated data mining and statistical analysis methods to establish biologically and clinically relevant signatures is still ongoing at high speed. This will improve unraveling the factors determining tumour type, prognosis, and response to treatment, including the factors responsible for constitutional or acquired resistance to conventional or targeted adjuvant therapy. Eventually these developments will enable the most optimal medical treatment for each individual breast cancer patient.

FIGURES

Figure 1 (cDNA array)

Fig. 1a: test- and reference cDNA is labelled using two different fluorescent dyes. Fig. 1b: test- and reference cDNA is mixed. Fig. 1c: Selected gene sequences are immobilized on a solid surface, a microarray. Fig. 1d: test- and reference cDNA mixture is hybridized to the microarray. Fig. 1e: the intensity of the markers is read using a laser. Fig. 1f: Relative expression of the test sample cDNA is calculated.

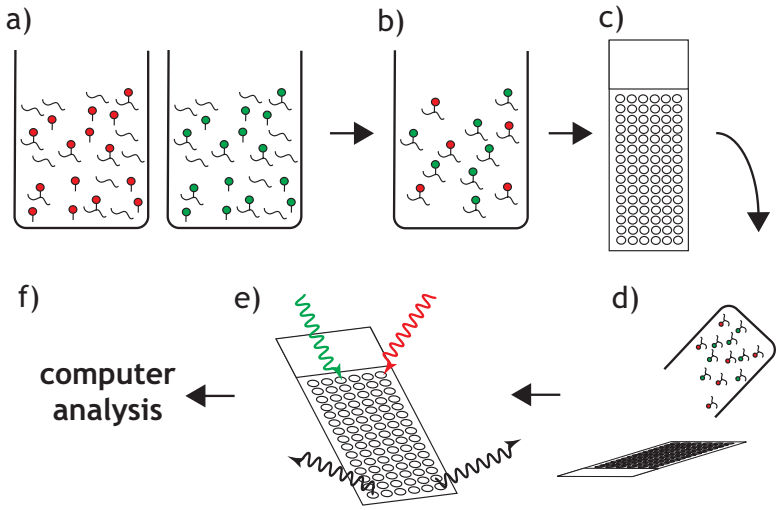


Figure 2 (PCR array)

Fig. 2a: Double stranded DNA . Fig. 2b: DNA denaturing yielding single stranded DNA . Fig. 2c: primers anneal to specific 3' and 5' ends of the genetic region of interest. Fig. 2d: Taq polymerase synthesizes a new complementary DNA strand, SYBR Green binds to double stranded DNA. Fig. 2e: PCR product is detected at the threshold cycle.

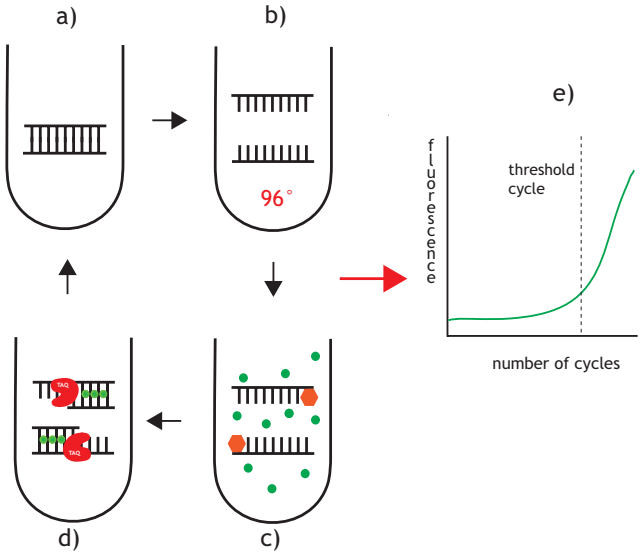
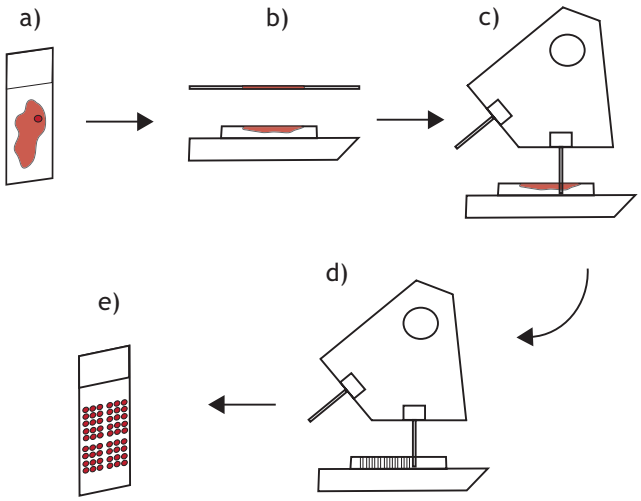


Figure 3 (TMA)

Fig. 3a: the most representative tumour spot is marked on a H&E section. Fig. 3b: the tumour spot is located on the donating tumour paraffin block. Fig. 3c: a manual tissue arraying instrument is used to take a core punch from the donating paraffin block. Fig. 3d: the core punch is arrayed in a predestined spot in the new acceptor paraffin block. Fig. 3e: Standard 3 um microtome sections are cut from TMA blocks.



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3

THE EXPRESSION PATTERN OF MUC1 (EMA) IS RELATED TO TUMOUR CHARACTERISTICS AND CLINICAL OUTCOME IN 'PURE' DUCTAL CARCINOMA IN SITU OF THE BREAST

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de Roos MA[1]
van der Vegt B[2]
Peterse JL[3]
Patriarca C[4]
de Vries J[1]
de Bock GH[5]
Wesseling J[2]

Departments of Surgical Oncology[1], Pathology[2] and Epidemiology[5] University Medical Center Groningen, University of Groningen, Department of Pathology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital[3], and Department of Pathology, Azienda Ospedaliera di Melegnano (Milan)[4]

Abstract

Aims: To evaluate five predefined types of MUC1 expression in a series of cases with pure ductal carcinoma in situ (DCIS), and to investigate the relation between the pattern of MUC1 expression and co-expression of biological markers, clinico-pathological parameters and prognosis.

Methods and Results: With a manual tissue arrayer, 92% (n=80) of the 87 DCIS samples were successfully targeted. Slides were stained for MUC1, estrogen receptor (ER), progesterone receptor (PR), Her2/Neu, p53, and cyclin D1. Entire membrane expression was related with Her2/neu negativity (p=0.042). Apical membrane expression was associated with low grade (p=0.027), Her2/neu negativity (p=0.014), and PR positivity (p=0.005). Focal cytoplasmic expression was related with high grade (p=0.006). Diffuse cytoplasmic expression was associated with high grade (p=0.004), large tumour size (p=0.046), Her2/neu positivity (p=0.042), and cyclin D1 positivity (p=0.002). On the basis of former analyses the four patterns were classified as membrane- or cytoplasmic expression. In multivariate analysis MUC1 cytoplasmic expression (Hazard Ratio 8.5, 95% Confidence Interval 1.0-73.0, p=0.04) was the only independent predictor of local recurrence.

Conclusions: Four patterns of MUC1 expression are recognized in DCIS that suggest a relation with functional differentiation and can be divided into two types that are clinically relevant and could therefore be helpful in the discrimination between different subgroups of DCIS.

INTRODUCTION

MUC1, also known as epithelial membrane antigen (EMA) or episialin, is a mucin-like transmembrane glycoprotein encoded by a gene on 1q21 and is expressed at the luminal surface of the epithelial cell membrane of normal breast ducts[1]. In vitro and in vivo studies have indicated that increased MUC1 expression can facilitate the process of metastasis by modification of intercellular adhesion.[2-5]

Although MUC1 expression patterns have been extensively studied in invasive ductal carcinoma, there are only a few analyses of the MUC1 expression patterns in ductal carcinoma in situ (DCIS). Diaz et al[6] described luminal, membranous, and cytoplasmic staining of MUC1 in DCIS. In that study cytoplasmic staining was associated with a higher grade. In a study by Mommers et al[7] three different patterns of MUC1 expression in DCIS are described: staining of the apical (luminal) membrane, staining of the entire cell membrane and cytoplasmic staining. In that study membrane expression of MUC1 was related with high grade, although cytoplasmic expression was not evaluated being too difficult to distinguish from background staining.

Detection of MUC1 by immunohistochemistry is strongly influenced by the degree of glycosylation and the primary antibody used, as most MUC1 antibodies bind the extracellular mucin-like domain and are sensitive to the degree and make-up of glycosylation of their antigenic site.[8] For that reason, we used an antibody that is robust in binding MUC1 because it is almost insensitive to the degree of glycosylation of MUC1.[9]

Recently, we identified five patterns of MUC1 expression in invasive breast carcinomas in a parallel study and it is likely that these patterns have clinico-pathological relevance (unpublished observations). In this study the classification of MUC1 was applied to a set of pure ductal carcinoma in situ tumours arranged in a tissue microarray (TMA) in order to determine the relation of these MUC1 expression patterns with the expression of established biological markers, clinico-pathological characteristics and with local recurrence.

Materials and methods

Patients and tumors

Patients were selected on the availability of the original pathological slides and sufficient paraffin embedded tissue. Eighty-seven patients were included in the study and all patients had been treated for pure DCIS between July 1992 and October 2001. Clinical and pathological data of patients were reviewed from pathology reports, radiology reports and medical charts and follow-up was evaluated by the reports of outpatient clinic visits. Radiological and histo-pathological characteristics have been reported previously.[10] The patients characteristics are depicted in Table 1. Median age was 57.7 (range 36.8-77.5 years). Fifty-two cases (59%) were detected by the National Screening Programme for Breast Cancer. Forty-eight patients had been treated with mastectomy and 39 patients with breast conserving surgery (BCS), including post-operative radiotherapy in 20; none received adjuvant endocrine therapy. The median follow-up time for all patients was 39 months and ipsilateral recurrence was recorded as DCIS or invasive carcinoma.

Tissue microarray construction

Slides from all blocks were evaluated for representative areas with DCIS and tissue microarrays were prepared as described earlier.[11] In brief, the most representative area of DCIS was marked on the original hematoxylin- and eosin (H&E) stained section. With this marked section as an orientation, three 0.6 mm punches were taken from the selected area in the donor blocks and mounted in a recipient block containing approximately 110 biopsies, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD, USA). Out of 87 cases, representative tissue cores were obtained in 80 cases (92%) and acceptable immunohistochemistry (at least one of the three cores was stained sufficiently) was achieved in 69 (79%) cases for p53, 70 (80%) cases for cyclin D1- and PR- staining, 73 cases (84%) for ER- and MUC1 staining and 80 cases (92%) for Her2/neu staining.

Immunohistochemistry

For immunohistochemistry, 3 µm sections of the tissue microarrays were deparaffinised in 2 changes of xylene for 10 minutes each and gradually

rehydrated through changes of graded ethanol from 100% to distilled water. Antibodies and antigen retrieval methods are summarised in Table 2. The endogenous peroxidase reaction was blocked by incubating the sections in 3% perhydrol for 30 minutes. Primary antibodies were diluted in PBS containing 1% Bovine Serum Albumin and incubated at room temperature for 1 hour. Samples were then washed in PBS and incubated with secondary and tertiary antibodies. For visualization of the antibody-antigen complex, the diaminobenzidine tetrahydrochloride/ peroxidase reaction was used. After a final wash with distilled water, counterstaining was performed with hematoxylin. Sections were dehydrated through rising concentrations of ethanol and mounted.

Evaluation of immunohistochemical staining

All slides stained for molecular markers were read by one author (MdR) and checked by another (JW). ER, PR, p53, and cyclin D1 were graded based on the percentage of cells showing positive nuclear staining in the ducts with DCIS. ER, PR and cyclin D1 were considered positive if nuclear staining was present in >10% of the cells, and p53 was considered positive in case of more than 30% positively stained nuclei. Her2/neu expression was graded as recommended by the manufacturer's scoring guidelines: 0: no staining at all or membrane staining in <10% of the tumour cells; 1+: a faint/barely perceptible partial membrane staining in >10% of the tumour cells; 2+: weak to moderate complete membrane staining in >10% of the tumour cells; 3+: strong complete membrane staining in >10%. Her2/neu was considered positive if the score was 3+. For the classification of the MUC1 expression, 5 expression patterns were used as illustrated in figure 1. MUC1 expression was considered positive if there was staining in >10% of the tumour cells.

Statistical analysis

Chi-square tests were performed in order to assess the relation between the types of MUC1 expression and biological markers and clinico-pathological parameters. After performing the chi-square tests the four types of MUC1 expression could be reduced to two pathologically and biologically different types of expression. The relation of these two types of MUC1 expression with

local recurrence was investigated in univariate and multivariate analysis. Univariate analysis and multivariate analysis were performed by Cox regression. The elimination of variables in a stepwise manner identified the statistically significant predictors in multivariate analysis. A p-value of <0.05 was considered as significant. All calculations were performed with SPSS 12.01 (SPSS inc., Chicago, IL, USA).

Results

Immunohistochemical detection of MUC1

MUC1 expression patterns are exemplified in Figure 1. MUC1 was expressed in all cases (100%, n=73). Predominant entire membrane expression was present in 20 cases (27%). Apical membrane staining was seen in 23 cases (32%). Focal cytoplasmic staining was described in 39 cases (53%). In most cases (n=58, 84%) diffuse cytoplasmic staining was present. Inside-out staining was not found. The majority of the DCIS (n=57, 78%) displayed more than one type of MUC1 expression. The most frequently pure pattern of staining was diffuse cytoplasmic immunoreactivity (n=12, 16%). The most frequent promiscuous pattern of staining was the association between focal cytoplasmic and diffuse cytoplasmic expression (n=30, 41%).

MUC1 expression and clinico-pathological characteristics

The association between MUC1 expression and clinico-pathological parameters is outlined in Table 3. The only relation of clinical parameters with MUC1 expression was the relation of a negative family history of breast cancer with MUC1 focal cytoplasmic expression (p=0.042). Screen-detected lesions and menopausal status were not associated with any type of MUC1 expression. MUC1 apical membrane expression was associated with low grade DCIS (European Pathologists Working Group [EPWG] classification [p=0.027] and Van Nuys classification [p=0.032]). MUC1 focal cytoplasmic expression was related with high grade according to the Van Nuys classification (p=0.006). MUC1 diffuse cytoplasmic expression was associated with high grade according to the Van Nuys classification (p=0.004) and a large tumour size (p=0.046).

MUC1 expression and biological markers

The relation of well known biological markers with MUC1 expression is displayed in Table 4. MUC1 entire membrane expression was related with Her2/neu negativity (p=0.042). MUC1 apical membrane staining was associated with Her2/neu negativity (p=0.014) and PR positivity (p=0.005). MUC1 focal cytoplasmic expression was associated with cyclin D1 positivity (p=0.042). Diffuse cytoplasmic staining was related with Her2/neu positivity (p=0.043) and with cyclin D1 positivity (p=0.002).

MUC1 expression and local recurrence

After performing the above mentioned analyses it was noticed that the four types of MUC1 expression could be broadly divided into two different types of expression. Entire- and apical membrane expression, that both had a pathological and biological less aggressive signature, were combined and classified as membane expression. Focal- and diffuse cytoplasmic expression, that both had a pathological and biological more aggressive signature, were also combined and classified as cytoplasmic expression. In order to evaluate the relation between these two types of MUC1 expression with local recurrence the dominant type of MUC1 expression in each tumor was classified as membrane or cytoplasmic expression. In the case of multiple expression patterns in one lesion, the dominant type of expression was defined by the largest percentage of cells that displayed this type of expression.

Thirty-nine patients were treated with BCS. Due to small numbers patients that had been treated with and without radiotherapy were evaluated for local recurrence. The overall local recurrence rate in the study group was 7 cases (2 invasive carcinoma and 5 DCIS). In univariate analysis low grade (Hazard Ratio [HR] 0.1, 95% Confidence Interval [CI] 0.02-0.9, p=0.04) was related with local recurrence-free survival and MUC1 cytoplasmic expression (HR 8.1, 95%CI 1.0-68.0, p=0.04) was associated with local recurrence (Log Rank 5.2, p=0.02; Figure 2). In multivariate analysis cytoplasmic MUC1 expression was the only independent predictor of local recurrence (HR 8.5, 95% CI 1.0-73.0, p=0.04).

Discussion

Five predefined different types of MUC1 expression were studied in a series of pure DCIS cases arranged in a tissue microarray. MUC1 expression was observed in all DCIS lesions (n=73, 100%) and most cases displayed a variable expression pattern (78%). Four out of five different types of MUC1 expression previously noticed in invasive breast cancer were present in DCIS. Entire membrane expression was related with Her2/neu negativity (p=0.042). Apical membrane expression was associated with low grade (p=0.027 and p=0.032), Her2/Neu negativity (p=0.014) and PR positivity (p=0.005). Focal cytoplasmic expression was related with high grade (p=0.006) and diffuse cytoplasmic expression was associated with high grade (p=0.004), a large tumour size (p=0.046), cyclin D1 positivity (p=0.002) and Her2/Neu positivity (p=0.043). Inside-out staining was not observed in DCIS. These four types of MUC1 expression can be divided into two types of expression in which the expression patterns with a pathological and biological similar signature are combined. MUC1 cytoplasmic expression is an independent predictor of local recurrence (HR 8.5, 95% CI 1.0-73.0, p=0.04).

Construction of TMA in invasive breast carcinoma is more successful than in DCIS because DCIS lesions are more difficult to target manually. The scattered distribution and the small size of the ducts with tumour make it more difficult to obtain representative tissue punches. In this study, the most representative H&E section of every DCIS lesion was selected and after delineation of the largest lesions on the section by a permanent marker, three punches per lesion were taken out of the corresponding paraffin block. This strategy proved to be successful in 80 (92%) out of 87 lesions. In another study by Jirström et al, the success rate of targeting DCIS lesions for TMA was much lower (52%) and the authors claim that part of this low success rate was due to extensive sectioning of all paraffin blocks prior to the TMA procedure.[12]

The MUC1 gene encodes a sialylated transmembrane glycoprotein with a large mucin-like domain consisting of 20-amino acid repeats which are rich in serines, threonines and prolines.[13,14] After synthesis in the rough endoplasmatic reticulum, it is packaged in the Golgi apparatus and carried to the luminal domain of the cell membrane, where it exerts anti-adhesive and lubricant functions.[15] Therefore, in normal breast tissue, MUC1 is expressed at the apical

membrane. During malignant transformation of breast carcinoma cells, MUC1 expression may undergo changes that have morphological consequences._ The evaluation of immunohistochemical staining of MUC1 in breast malignancies has been described in several reports. In invasive carcinoma cytoplasmic- and membrane staining have been reported[16-19], whereas other studies report apical staining[20-23] and staining of intracytoplasmic canaliculi[24] in addition to the former two types of staining. In the study by Hayes et al.[22] cytoplasmic staining is divided in cytoplasmic-vacuolar and cytoplasmic-nonvacuolar expression. Many investigators also use the percentage of stained tumour cells[18-20,25] and the intensity of staining as a quantification of MUC1 expression.[18,26] The expression of MUC1 in DCIS is less extensively evaluated than in invasive carcinoma, but apical- , membrane- and cytoplasmic staining have been reported.[6,7]

In a review by Rahn et al.[23] the overall percentage of MUC1 expression in invasive cancer was found to be increased in lower grade and estrogen receptor positive tumors. In that review high perentage of MUC1 expression was associated with a better prognosis, and cytoplasmic and circumferential membrane staining were associated with a worse prognosis. Subgroup analysis of MUC1 diffuse cytoplasmic expressing tumours in our invasive series also shows a relation with a worse prognosis whereas entire membrane staining, in that study, has no prognostic power (unpublished observations). A recent report by Rakha et al.[20] investigated a large series of invasive breast carcinomas (n=1447) and found a positive relation of MUC1 expression with lower histological grade, smaller tumour size, ER expression and absence of both regional recurrence and distant metastasis. In that study, cytoplasmic and membranous expression of MUC1 were associated with poor outcome.[20] Cytoplasmic expression of MUC1 is positively correlated with Her2/neu overexpression and grade and cytoplasmic distribution is also inversely correlated with estrogen receptor status.[15,26] In DCIS, cytoplasmic expression of MUC1 is associated with a high grade but it has also been reported that membrane staining is related with high grade if cytoplasmic expression is excluded.[6,7]

In the evaluation of these reports, one should be aware of the fact that in almost all studies antibodies are used that bind to the extracellular

repeat domain of MUC1 which is glycosylated to various degrees with different compositions of the carbohydrate side chains. As a consequence, immunohistochemical detection of MUC1 depends to a great extent on the sensitivity of a particular antibody to the degree and make-up of the glycosylation.[8] For that reason we used an antibody which is almost insensitive to these factors.[9]

The patterns of MUC1 expression that have been predefined in our previous studies in a set of invasive ductal carcinomas of the breast were applied to DCIS in the present study.[27] Because most DCIS lesions displayed a high overall percentage of MUC1 expression, we did not further evaluate the overall expression as a prognostic parameter. Entire membrane expression was related with Her2/neu negativity (p=0.042). Apical membrane expression was associated with low grade (p=0.027 and p-0.032), Her2/Neu negativity (0.014) and positive staining for PR (0.005). In our observations in invasive ductal carcinoma apical membrane expression was also related with lower pathological grade and PR expression but also with ER positivity (unpublished data). Focal cytoplasmic expression was related with high grade (p=0.004). Diffuse cytoplasmic expression was positively associated with grade (p=0.004) and Her2/neu overexpression (p=0.043) as is observed in invasive cancer.[18,26] It can be concluded from these data that the classification for MUC1 expression is strongly related with tumour differentiation as shown by pathological characteristics and biological markers.

From the former observations two types of expression were identified; membrane expression has a pathological and biological less aggressive signature, whereas cytoplasmic expression has a more aggressive signature which is also demonstrated by the relation with local recurrence (HR 8.5, 95% CI 1.0-73.0, p=0.04). One might think that the fact that only half of the patients was treated with adjuvant radiotherapy has confounded the results of this outcome analysis. Due to small numbers patients treated with BCS with and without adjuvant radiotharpy were analysed together. However, we think that, in this study, the effect of radiotherapy can be disregarded because in the univariate analysis adjuvant radiotherapy was not significantly related to local recurrence (HR 1.5, 95% 0.3-8.4, p=0.63).

It has been extensively described that aberrant expression and/or

overexpression of MUC1 is involved in modulating cell adhesion, in inducing invasive potential, and in affecting various signal transduction pathways.[28] Based on the first two of these features of MUC1 and on the relationship between clinico-pathological parameters and pattern of MUC1 expression, it is likely that MUC1 can play a decisive role in the transition from in situ to invasive ductal carcinoma. The absence of the inside-out staining pattern in DCIS as described here supports this notion, also because MUC1 is expressed at the leading edge of tumor cells localized at the invasive front of adenocarcinomas.[29] In contrast to DCIS which are almost exclusively MUC1 positive, a minority of invasive ductal carcinomas are MUC1 negative and appear to be a subgroup with a poor prognosis (unpublished data). This indicates that both aberrant MUC1 expression and absent MUC1 expression can favour more aggressive tumour progression, probably via different mechanisms comprising different factors involved in e.g. cell adhesion and invasion.

In summary, patterns of MUC1 expression were evaluated in invasive breast cancer and tested in a series of DCIS lesions. The expression on TMA shows that the different types of MUC1 staining, that are expressed in invasive breast cancer, are also present in DCIS, except for inside-out staining. Inside-out expression is present in invasive breast carcinoma, but not all invasive carcinomas, and it was absent in DCIS lesions. This indicates that inside-out expression is a marker for invasive disease, possibly with a worse prognosis. Entire- and apical membrane staining and focal- and diffuse cytoplasmic staining were associated with pathological grade and biological markers, which suggests that the present classification of MUC1 expression patterns is an indicator of functional differentiation. A division of these four types of expression in membrane- and cytoplasmic expression is of clinical relevance and could therefore be helpful in the discrimination between different subgroups of DCIS.

Table 1. patient- and tumour characteristics.

Characteristics	n	%
Age		
	57.7 (36.8-77.5)	-
Screen-detected		
Yes	52	59.8
No	35	40.2
Menopausal status		
Pre-menopausal	78	90
Post-menopausal	9	10
Family history of breast cancer		
Yes	15	17.2
No	72	82.8
Therapy		
BCS	19	21.8
BCS+XRT	20	23
Mastectomy	48	55.2
Tumour size		
15mm	27	31
16-40mm	30	34.5
41mm	30	34.5
EPWG (grade)		
I	12	13.8
II	44	50.6
III	31	35.6
Van Nuys (grade)		
I	12	13.8
II	37	42.5
III	38	43.7

n: number of cases; %: percentage; BCS: breast conserving surgery; XRT: radiotherapy; mm: millimeter; EPWG: Classification according to European Pathologists Working Group; Van Nuys: Classification according Van Nuys.

TABLE 2. ANTIGEN RETRIEVAL METHODS AND ANTIBODIES.

ANTIBODY	CLONE	SUPPLIER	DILUTION	ANTIGEN RETRIEVAL	SECONDARY ANTIBODY	SUPPLIER	TERTIARY ANTIBODY	SUPPLIER
MUC1	214D4	Dr. J. Hilkens*	1:100	-	RAMP0	Dako	GARP0	Dako
ER	6F11	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 980C microwave	RAMBI0	Dako	SARBI0	Dako
PR	1A6	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 980C microwave	RAMBI0	Dako	SARBI0	Dako
Her2/Neu	CB11	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 980C microwave	RAMBI0	Dako	SARBI0	Dako
p53	BP-53-12-1	Biogenix	1:800	Tris/HCL 0.1M (pH 9.5) 30' 980C microwave	RAMBI0	Dako	SARBI0	Dako
Cyclin D1	SP4	Neomarkers	1:50	Tris/HCL 0.1M (pH 9.5) 30' 980C microwave	RAMBI0	Dako	SARBI0	Dako

*ER, Estrogen Receptor; PR, Progesteron Receptor; * , prediluted by supplier; RAMBI0, Rabbit anti mouse biotin; SARBI0; Swine anti rabbit biotin; RAMP0; Rabbit anti mouse polyclonal; GARP0; Goat anti rabbit polyclonal -, no antigen retrieval necessary. * See acknowledgements;[27].*

TABLE 3. CLINICO-PATHOLOGICAL PARAMETERS AND MUC1 EXPRESSION.

MUC1 staining	MUC1 entire membrane expression		MUC1 apical membrane expression		MUC1 focal cytoplasmic expression		MUC1 diffuse cytoplasmic expression	
Clinico-pathological parameters	positive	negative	positive	negative	positive	negative	positive	negative
Screen detected								
Yes	15(75)	28(52.8)	12(52.2)	31(62)	23(57.5)	20(60.6)	32(54.2)	3(21.4)
No	5(25)	25(47.2)	11(47.8)	19(38)	17(42.5)	13(39.4)	27(45.8)	11(78.6)
N=73								
		p=0.086		p=0.428		p=0.788		p=0.096
Menopausal status								
Postmenopausal	19(95)	47(88.7)	21(91.3)	45(90)	38(95)	28(84.8)	53(89.8)	13(92.9)
Premenopausal	1(5)	6(11.3)	2(8.7)	5(10)	2(5)	5(15.2)	6(10.2)	1(7.1)
N=73		p=0.413		p=0.860		p=0.143		p=0.730
Family history								
Positive	1(5)	12(22.6)	5(21.7)	8(16)	4(9.8)	9(28.1)	12(20.3)	1(7.1)
Negative	19(95)	41(77.4)	18(78.3)	41(84)	37(90.2)	23(71.9)	47(79.7)	13(92.9)
N=73								
		p=0.079		p=0.552		p=0.042		p=0.246
Tumour size								
< 15mm	7(36.8)	15(28.8)	7(31.8)	15(30.6)	12(31.6)	10(30.3)	14(24.6)	8(57.1)
16-40mm	5(26.3)	19(36.5)	9(40.9)	15(30.6)	14(36.8)	10(30.3)	20(35.1)	4(28.6)
> 41mm	7(36.8)	18(34.6)	6(27.3)	19(38.8)	12(31.6)	13(39.4)	23(40.4)	2(14.3)
N=71								
		p=0.692		p=0.590		p=0.764		p=0.046
EPWG (grade)								
I	2(10)	10(18.9)	7(30.4)	5(10)	8(20)	4(12.1)	8(13.6)	4(28.6)
II	12(60)	23(43.4)	12(52.2)	23(46)	19(47.5)	16(48.5)	27(45.8)	8(57.1)
III	6(30)	20(37.7)	4(17.4)	22(44)	13(32.5)	13(39.4)	24(40.7)	2(14.3)
N=73								
		p=0.412		p=0.027		p=0.629		p=0.131
Van Nuys (grade)								
I	2(10)	8(15.1)	5(21.7)	5(10)	10(24.4)	0(0)	4(6.9)	6(40)
II	10(50)	21(39.6)	13(56.5)	18(36)	13(31.7)	18(56.3)	26(44.8)]	5(33.3)
III	8(40)	24(45.3)	5(21.7)	27(54)	18(43.9)	14(43.8)	28(48.3)	4(26.7)
N=73								
		p=0.692		p=0.032		p=0.006		p=0.004

Values between parenthese are percentages. Chi-square analysis. EPWG: European Pathologists Working Group.

TABLE 4. BIOLOGICAL MARKERS AND MUC1 EXPRESSION.

MUC1 STAINING	MUC1 ENTIRE MEMBRANE EXPRESSION		MUC1 APICAL MEMBRANE EXPRESSION		MUC1 FOCAL CYTOPLASMIC EXPRESSION		MUC1 DIFFUSE CYTOPLASMIC EXPRESSION	
Biological Markers	positive	negative	positive	negative	positive	negative	positive	negative
Her2/neu								
Positive	9(47.4)	12(22.6)	3(12)	18(38.3)	10(25)	11(34.4)	20(34.5)	1(7.1)
Negative	10(52.6)	41(77.4)	22(88)	29(61.7)	30(75)	21(65.6)	38(65.5)	13(92.9)
N=72								
		p=0.042		p=0.014		p=0.384		p=0.043
ER								
Positive	13(68.4)	39(75)	20(87)	32(66.7)	24(75)	28(71.8)	41(71.9)	11(78.6)
Negative	6(31.6)	13(25)	3(13)	16(33.3)	8(25)	11(28.2)	16(28.1)	2(21.4)
N=71								
		p=0.579		p=0.071		p=0.761		p=0.615
PR								
Positive	9(47.4)	26(51)	17(73.9)	18(38.3)	20(51.3)	15(48.4)	26(46.4)	9(64.3)
Negative	10(52.6)	25(49)	6(26.1)	29(61.7)	19(48.7)	16(51.6)	30(53.6)	5(35.7)
N=70								
		p=0.788		p=0.005		p=0.810		p=0.232
P53								
Positive	13(72.2)	36(73.5)	16(72.7)	33(73.3)	9(23.1)	9(32.1)	13(24.5)	5(35.7)
Negative	5(27.8)	13(26.5)	6(27.3)	12(26.7)	30(76.9)	19(67.9)	40(75.5)	9(64.3)
N=67								
		p=0.919		p=0.958		p=0.409		p=0.401
Cyclin D1								
Positive	14(73.7)	34(68)	15(65.2)	33(71.7)	23(59)	25(83.3)	43(78.2)	5(35.7)
Negative	5(26.3)	16(32)	8(34.8)	13(28.3)	16(41)	5(16.7)	12(21.8)	9(64.3)
N=69								
		p=0.647		p=0.579		p=0.029		p=0.002

Values between parentheses are percentages; ER: estrogen receptor; PR: progesteron receptor.

Table 5. Univariate analysis investigating the relation of pathological and biological characteristics with local recurrence in patients treated with breast conservation.

Pathological and biological features	n (%)	HR	95%CI	p-value
Margins				
Positive	7(18)	4.0	0.9-18.1	0.07
Free	32(82)	1		
(n=39)				
Tumour size				
‹16mm	19(48.7)	0.4	0.06-3.1	0.40
16-40mm	13(33.3)	0.7	0.1-4.1	0.67
›40 mm	7(18)	1		
(n=39)				
Grade (EPWG)				
1	5(12.8)	0.4	0.04-3.6	0.40
2	21(53.9)	0.9	0.09-8.9	0.93
3	13(33.3)	1		
(n=39)				
Grade (Van Nuys)				
1	5(12.8)	0.1	0.02-0.9	0.04
2	18(46.2)	0.3	0.04-1.7	0.16
3	16(41.0)	1		
(n=39)				
Radiotherapy				
Yes	19(48.7)	1.5	0.3-8.4	0.63
No	20(51.3)	1		
(n=39)				
MUC1 expression				
Cytoplasmic	19(51.4)	8.1	1.0-68.0	0.04
Membrane	18(48.6)	1		
(n=37)				
Her2/neu				
Positive	9(23.1)	3.9	0.8-20.1	0.10
Negative	30(76.9)	1		
(n=39)				
ER				
Positive	29(76.6)	0.4	0.1-2.4	0.34
Negative	9(23.4)	1		
(n=38)				
PR				
Positive	19(51.4)	0.9	0.2-4.1	0.89
Negative	18(48.6)	1		
(n=37)				
p53				
Positive	11(30.5)	4.0	0.9-18.1	0.07
Negative	25(69.5)	1		
(n=36)				
Cyclin D1				
Positive	22(59.5)	0.9	0.2-4.2	0.93
Negative	15(40.5)	1		

n: number of cases; %: percentage; HR: Hazard Ratio; 95% CI: 95% Confidence Interval; ER: estrogen receptor; PR: progesterone receptor.

Table 6. Multivariate analysis investigating the relation of pathological and biological characteristics with local recurrence in patients treated with breast conservation.

Characteristics	HR	95% CI	p-value
MUC1 Cytoplasmic Membrane	 8.5 1	 1.0-73.0	 0.04

Elimination of variables in a stepwise manner; HR, Hazard Ratio; 95%CI, 95% Confidence Interval.

Figures

Figure 1.

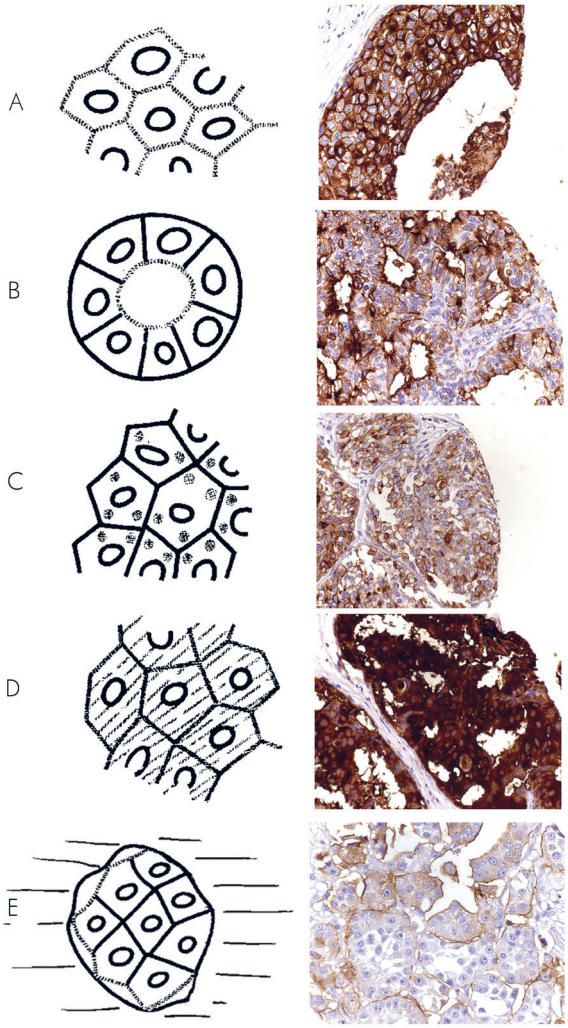
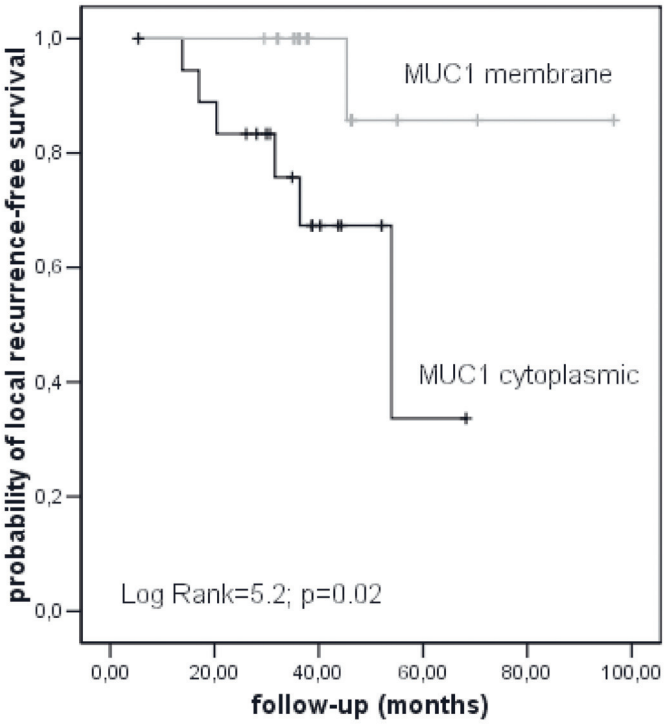


Figure 2.


probability of local recurrence-free survival
1,0
0,8
0,6
0,4
0,2
0,0
MUC1 membrane
MUC1 cytoplasmic
Log Rank=5.2; p=0.02
0,00
20,00
40,00
60,00
80,00
100,00
follow-up (months)

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4

THE EXPRESSION PATTERN OF MUC1 (EMA) IS RELATED TO TUMOUR CHARACTERISTICS AND CLINICAL OUTCOME OF INVASIVE DUCTAL BREAST CARCINOMA

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van der Vegt B[1]
de Roos MA[2]
Peterse JL[3]
Patriarca C[4]
Hilkens J[5]
de Bock GH[6]
Wesseling J[1]

Departments of Pathology[1], Surgical Oncology[2] and Epidemiology[6] University Medical Center Groningen, University of Groningen, The Department of Pathology[3] and Tumour biology[5], The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, and Department of Pathology, Azienda Ospedaliera di Melegnano (Milan)[5]

Abstract

Aims: MUC1 expression was classified according to five predefined expression patterns in invasive ductal breast carcinoma and related to clinico-pathological parameters, co-expression of other biological markers and prognosis.

Methods and Results: Samples from 243 consecutive patients with primary ductal carcinomas were incorporated in tissue microarrays (TMAs). Slides were stained for MUC1, ER, PR, Her2/neu, p53, Cyclin D1. MUC1 apical membrane expression was associated with smaller tumours ($p=0.001$), lower tumour grades ($p<0.001$), ER positivity ($p=0.049$), PR positivity ($p=0.003$) and increased overall survival (OS, p=0,030). MUC1 diffuse cytoplasmic expression was associated with PR and Cyclin D1 positivity ($p=0.018$ and $p=0.009$), and increased relapse free survival (RFS, $p=0.034$). MUC1 inside-out expression was associated with ER positivity ($p=0.026$). Negativity for MUC1 was associated with ER negativity ($p=0.004$), PR negativity ($p=0.001$), Cyclin D1 negativity ($p=0.009$). In stepwise multivariate analysis MUC1 negativity was an independent predictor of both RFS (HR 3.5, 95% CI 1.5-8.5, $p=0.005$) and OS (HR 14.7, 95% CI 4.9-44.1, $p<0.001$).

Conclusions: The expression pattern of MUC1 in invasive ductal breast carcinoma is related to tumour characteristics and clinical outcome. In addition, a MUC1 negative expression pattern is an independent risk factor for poor RFS and OS, besides 'classical' prognostic indicators.

Introduction

MUC1 (episialin, epithelial membrane antigen (EMA), CA15-3 antigen) is a highly O-glycosylated mucin-like transmembrane glycoprotein encoded by a gene on chromosome 1q21.[1] This protein has a very large extracellular domain mainly consisting of 20 amino acid tandem repeats (TRs), a transmembrane domain, and a cytoplasmic tail.[2-4]

In most normal glandular epithelial cells, MUC1 is expressed on the apical surface[5]. In vitro and in vivo studies have described cell adhesion inhibition as well as increased metastatic and invasive potential of tumour cells by overexpression of MUC1.[6-8] In MUC1 deficient mice primary breast tumours had a significantly lower growth rate.[9] Overexpression of an underglycosylated form of MUC1 occurs in nearly all breast carcinomas.[10-12]

Using numerous different antibodies and scoring methods, many authors described correlations between MUC1 expression and ER status, grade of differentiation and prognosis.[13-15] In contrast with the in vitro work, most of these studies show a better outcome for patients overexpressing MUC1. Four studies, however, found no relation between MUC1 expression and outcome.[16-19] These differences may be explained by the complex scoring system used, the different affinity of the applied antibodies for the glycosylated isoforms of MUC1, and the wide range of histopathological phenotypes of breast carcinomas with different clinical and prognostic implications.[20]

Therefore, we used a monoclonal antibody directed at the protein backbone of MUC1 (mAb 214D4), which is relatively insensitive to the degree and make-up of glycosylation of the molecule[21], to study five patterns of MUC1 expression in primary ductal carcinomas which were predefined by two of the authors (CP and JP). To test the potential of this scoring method we applied it to a set of primary invasive ductal breast carcinomas not otherwise specified arranged in a tissue microarray (TMA) and related the MUC1 expression patterns to clinico-pathological parameters, a series of well established biological markers, and prognosis. This scoring method was also applied to a set of ductal carcinomas in situ.

Materials and methods

Patients

243 consecutive patients treated for a primary operable invasive ductal carcinoma of the breast not otherwise specified at the University Medical Center Groningen between January 1996 and December 2001 were included in this study. Patient and tumour characteristics and data on follow-up were obtained retrospectively from hospital records and are summarized in table 1. The median follow-up was 60.5 months (range 0.4 – 108.2). Follow-up was performed according the regional follow-up guidelines (http://www.ikcnet.nl/page.php?id=97). During follow-up 12 patients developed a local recurrence after a median follow-up of 26.7 months. 33 patients developed distant metastasis after a median follow-up of 36.7 months. In total 41 patients presented with a relapse with a median relapse free survival (RFS) of 27.3 months. 20 patients died due to breast cancer with a median overall survival (OS) of 34.1 months.

Tissue microarray construction

From the patient's tumour paraffin block, three 0.6 mm core samples of the most representative tumour area were included in a tissue microarray. The technique of tissue microarray production has been described and validated for breast carcinoma by others.[22, 23] In brief, the most representative tumour area was marked on the original hematoxylin- and eosin (H&E) stained section. Using this section as an orientation, three 0.6 mm core punches were taken from the selected area in the donor blocks and mounted in a recipient block, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD, USA).

Immunohistochemistry

Immunohistochemistry for MUC1, ER, PR, Her2/neu, Cyclin D1 and p53 was performed on these sections. The antibodies and antigen retrieval methods used are summarized in table 2. The immunostaining protocol was as follows: sections were deparafinized in pure xylene, rehydrated in decreasing concentrations of ethanol and washed in distilled water. Antigen retrieval was performed. The endogenous peroxidase reaction was blocked by

incubating in 3% perhydrol for 30 minutes. The primary antibody diluted in PBS containing 1% Bovine Serum Albumin was incubated for one hour, after which the secondary (1:100 diluted in PBS containing 1% BSA and 1% AB-serum) and tertiary (1:100 diluted in PBS containing 1% BSA and 1% AB-serum) were incubated for 30 minutes each. Visualisation was performed using the diaminobenzidine tetrahydrochloride / peroxidase reaction. Counterstaining was performed using haematoxylin. Sections were dehydrated using rising concentrations of alcohol and were mounted.

Evaluation of immunohistochemistry

Scoring of the stainings was performed by a well trained resident (BvdV). The scoring was randomly verified by an experienced pathologist (JW). ER, PR , p53 and Cyclin D1 were graded based on the percentage of tumour cells showing positive nuclear staining. ER, PR and Cyclin D1 were considered positive if nuclear staining was present in .10% of the cells, and p53 was considered positive in case of a substantial percentage of positively stained nuclei (>30%). Her-2/neu expression was graded as recommended by the manufacturerer's scoring guidelines: 0: no staining at all or membrane staining in <10% of the tumour cells; 1+: a faint/barely perceptible partial membrane staining in >10% of the tumour cells; 2+: weak to moderate complete membrane staining in >10% of the tumour cells; 3+: strong complete membrane staining in >10%. Her-2/neu was considered positive if the score was 3+. MUC1 was graded according the five expression patterns as depicted in figure 1. MUC1 expression was considered positive if there was staining in >10% of the tumour cells.

Data analysis

Data analysis was performed using the SPSS 12.0.1 statistical package (SPSS inc., Chicago, IL, USA). Chi-square tests were used to evaluate the associations of MUC1 expression with clinico-pathological parameters and biological markers. If applicable the Fisher Exact Test was used. Kaplan Meier (KM) curves were plotted and log-rank scores were calculated. P values of <0.05 were considered significant. After performing the analysis mentioned above the six expression patterns were divided into three subgroups according to expression

location: entire membrane, apical membrane and inside-out expression were classified as membrane expression; diffuse cytoplasmic and focal cytoplasmic were classified as cytoplasmic expression; tumours negative for MUC1 were classified as negative. These groups and other well established prognostic indicators were entered in an univariate Cox regression analysis to analyse the relationship with RFS and OS. Variables from the univariate analysis with a p value of ‹0.05 were then entered in a stepwise multivariate Cox regression analysis to investigate the relationship with RFS and OS.

Results

Tissue microarray and immunohistochemistry

Of the 243 cases included, the tissue cores of 237 cases were adequately represented in the TMA. Immunohistochemistry could be evaluated in all cases (100%, n=237) for MUC1, p53 and Cyclin D1, in 235 cases (99.2%) for Her2/neu, in 232 cases (97.9%) for ER and in 230 cases (97.0%) for PR.

In the assessable cases, MUC1 was expressed in 221 cases (93.2%) showing either a single or a combination of expression patterns. 16 cases (6.8%) did not show any expression of MUC1. Entire membrane expression was seen in 48 cases (20.3%). 64 cases (27.0%) showed apical expression. In 21 cases (8.9%) focal cytoplasmic expression was seen. The most common expression was diffuse cytoplasmic (73.0%, n=173). Inside-out expression was seen in 23 cases (9.7%). 117 cases (49.4%) showed a single expression pattern. The most common single expression pattern was diffuse cytoplasmic expression (70.1%, n=82). 100 cases (42.2%) showed a combination of two patterns and 4 cases (1.7%) showed a combination of three expression patterns. The most common combination of expression patterns was apical and diffuse cytoplasmic expression (40.3%, n=42).

MUC1 expression and clinico-pathological parameters

The relationship between MUC1 expression pattern and clinico-pathological parameters is shown in table 3. MUC1 apical expression is associated with smaller tumours (p=0.001) and with lower tumour grades (p‹0.001).

MUC1 expression and biological markers

Table 4 shows the relationship between MUC1 expression and other biological markers.

For MUC1 apical expression significant associations with ER (p=0,049) and PR (p=0,003) expression were found. Diffuse cytoplasmic MUC1 expression showed associations with PR (p=0.018) and Cyclin D1 (p=0.009). For MUC1 inside-out expession there was a signifcant association with ER (p=0.026). Negativity for MUC1 expression was associated with ER (p=0.004), PR (p=0.001) and Cyclin D1 (p=0.009).

MUC1 expression and clinical outcome

Kaplan Meier survival curves showed no significant correlation between MUC1 expression at the entire membrane and OS or RFS. Patients with tumours that had apical MUC-1 expression displayed a better OS (p=0.030; fig. 2b). No relationship between focal cytoplasmic MUC1 expression and survival was found. Patients with tumours that showed diffuse cytoplasmic MUC1 expression had a better RFS than patients with tumours that did not show diffuse cytoplasmic MUC1 expression (p=0.034; fig. 2a). For MUC1 inside-out expression, no correlation with survival was found. MUC1 negativity was significantly associated with worse RFS (p= <0.001) and OS (p= <0.001; fig. 2c, fig. 2d).

Analysis of combinations of MUC1 expression patterns, clinico-pathological parameters, biological markers, and clinical outcome

In order to increase the power of an outcome analysis the expression patterns were broadly divided into three patterns on the basis of location of MUC1 expression. Apical membrane expression and inside-out expression, that both had a biological less aggressive signature, were combined with entire membrane expression and classified as membrane expression. Diffuse cytoplasmic expression was combined with focal cytoplasmic expression and classified as cytoplasmic expression. Tumours that did not show MUC1 expression were classified as MUC1 negative. In order to evaluate the relation between these three types of MUC1 expression with RFS and OS

the dominant type of MUC1 expression in each tumour was classified as membrane or cytoplasmic expression, or as MUC1 negative. In the case of multiple expression patterns in one lesion, the dominant type of expression was defined by the largest percentage of cells that displayed this type of expression.

The results from univariate Cox regression analysis for RFS are shown in table 5. Significant relations were found for tumour size (HR 2.2, 95% CI 1.1-4.5, p=0.03 for tumours between 2.0 and 5.0 cm; HR 3.8, 95% CI 1.4-10.2, p=0.009 for tumours >5 cm), tumour grade (HR 2.3, 95% CI 1.2-4.2, p=0.009), MUC1 expression (HR 3.4, 95% CI 1.5-8.1, p=0.005 for MUC1 negativity), Her2/neu expression (HR 2.8, 95% CI 1.1-7.1, p=0.03), ER expression (HR 0.5, 95% CI 0.3-1.0, p=0.05), PR expression (HR 0.4, 95% CI 0.2-0.7, p<0.01) and relapse free survival.

In table 6 the results from univariate Cox regression analysis for OS are shown. Significant results were found for tumour size (HR 6.6, 95% CI 1.6-26.4, p=0.008 for tumours >5 cm), tumour grade (HR 3.6, 95% CI 1.5-8.7, p=0.005), axillary lymph node status (HR 3.0, 95% CI 1.1-7.8, p=0.03), reception of adjuvant chemotherapy (HR 3.2, 95% CI 1.1-8.8, p=0.02), MUC1 expression (HR 6.0, 95% CI 2.2-16.7, p=0.001), Her2/neu expression (HR 6.3, 95% CI 2.2-17.5, p<0.001), ER expression (HR 0.3, 95% CI 0.1-0.8, p=0.02), PR expression (HR 0.4, 95% CI 0.2-1.0, p=0.05) and overall survival.

The results from the stepwise multivariate analysis for RFS are shown in table 7. MUC1 expression (HR 4.6, 95% CI 1.5-8.5, p=0.005 for MUC1 negativity) and PR expression (HR 0.4, 95% CI 0.2-0.8, p=0.09) were significant independent predictors of relapse free survival.

In table 8 the results from multivariate analysis for OS are shown. Axillary lymph node status (HR 4.7, 95% CI 1.7-13.0, p=0.003), MUC1 expression (HR 14.7, 95% CI 4.9-44.1, p<0.001 for MUC1 negativity) and Her2/neu expression (HR 3.7, 95% CI 1.4-9.5, p=0.006) were significant independent predictors for overall survival.

Discussion

This study investigated the relationship between MUC1 expression patterns in invasive ductal carcinomas of the breast not otherwise specified, tumour characteristics, expression of a series of well established tumour markers, and clinical outcome. To avoid ambiguous results due to the heterogeneity of breast cancer, we focused on this by far most common type of breast cancer.

We found expression in 93.2% percent of the cases. MUC1 apical expression was significantly associated with smaller tumours, lower tumour grades, ER positivity and PR positivity. MUC1 diffuse cytoplasmic expression showed a significant association with PR and Cyclin D1 positivity. MUC1 inside-out expression was associated with ER positivity. Negativity for MUC1 was significantly associated with ER negativity, PR negativity and Cyclin D1 negativity. Patients with apical MUC1 expressing tumours and patients with diffuse cytoplasmic MUC1 expressing tumours displayed a significantly increased RFS. Patients with tumours negative for MUC1 showed a significantly decreased RFS and OS in both univariate and multivariate analysis.

Before discussing the associations found for the different expression patterns of MUC1 in more detail, it is important to discuss the antibodies used to detect MUC1 in various studies. Almost all anti-MUC1 antibodies used are directed against the O-glycosylated extracellular MUC1 tandem repeat domain.[13] However, the degree and make-up of glycosylation may vary extensively among MUC1-positive adenocarcinomas[24, 25] and the affinity for MUC1 of the vast majority of these antibodies depends on the extent and composition of glycosylation[26, 27]. As a consequence, the variety of anti-MUC1 antibodies used to determine MUC1 expression in breast carcinomas may explain at least part of the discrepancies between various studies as discussed below.

Detecting almost all glycosylated MUC1 isoforms is important to study its significance for tumour progression, relationship to other tumour progression markers and to clinical outcome. Some well established functions of MUC1, e.g. the inhibition of cell-cell and cell-extracellular matrix adhesion, are only to a minor extent dependent of the MUC1 glycosylation status.[7, 28] For that reason we used mAb 214D4, a monoclonal antibody which is also directed to the protein backbone of the MUC1 repeat domain, but for which the affinity is

almost independent of the glycosylation status.[21]

In normal glandular epithelium, MUC1 is expressed at the apical surface.[5] For that reason apical expression in breast carcinomas (designated 'pattern B'; fig.1) indicates normal routing of MUC1 molecules and as a consequence relatively intact glandular differentiation. Indeed, in our series, apical MUC1 expression is associated with many indicators of good prognosis and a better OS. The association with lower tumour grades[11, 13, 15, 29], ER[15, 29] and PR[29] positivity and the absence of distant metastasis[15] has been described. Some authors found an increased rate of axillary lymph node negativity[15] and longer RFS for patients with tumours expressing MUC1 apically.[15, 30] In our data these findings could not be confirmed. Study size, follow-up and patients included in these studies might account for this difference, e.g. the study by Hayes et al only included node positive patients. In accordance with our series an increase in OS of patients with tumours expressing MUC1 apically has been reported elsewhere.[15, 31] One relatively small study did not find an association between apical expression and clinico-pathological variables.[32]

Entire membrane MUC1 expression (designated 'pattern A'; fig. 1) is more often seen in mucinous carcinomas than in ductal carcinomas of no special type.[12] Although this expression pattern appears to be the effect of misrouting in the MUC1 pathway, no unambiguous results on the role of MUC1 expression on the entire membrane in breast cancer have been described. Where Parham et al showed that high entire membrane expression of MUC1 associates with low tumour grades[17], Rahn et al showed the contrary.[13] The former study also found an association with positive lymph node status. In this study no significant associations between MUC1 entire membrane expression and clinico-pathological parameters were found. Entire membrane MUC1 expression did not associate with clinico-pathological characteristics and outcome in these series. Two other studies that looked at a relation between expression of MUC1 on the entire membrane and outcome also did not find such a relation.[11, 17] By combining entire membrane and cytoplasmic MUC1 expression Rakha et al were able to show a significant decrease in OS and RFS in this group.[15] We did not perform such a subgroup analysis.

Inside-out expression (designated 'pattern E'; fig. 1) for MUC1 was present in a small percentage of the tumours and has been described by two of the

authors (CP, JP) before.[33] This pattern is specific for invasive micropapillary carcinoma, a subtype of ductal breast carcinoma with a high potential to metastasize to the axillary lymph nodes.[34] We did not find such a relation however, nor did we find an association between 'inside out' expression and outcome. The small number of cases in these series might account for this.

Diffuse cytoplasmic expression of MUC1 was associated with good prognosis in these series. Previous reports have linked cytoplasmic expression of MUC1 to ER negativity[15], high Her2/neu expression[35], decreased RFS[11, 15, 31] and decreased OS.[15, 31] The study by Lundy et al, found that MUC1 cytoplasmic expression was related to ER positivity and lower tumour grades.[32] In this study a positive relation of MUC1 diffuse cytoplasmic expression with PR and Cyclin D1 positivity was found which might be explained by the common combination of apical and diffuse cytoplasmic expression in these series. Results from the subgroup analysis of combined apical and diffuse cytoplasmic MUC1 expression versus strictly cytoplasmic MUC1 expression show that tumours with diffuse cytoplasmic MUC1 expression have a clinico-pathological profile that is usually associated with worse outcome, but that when there is a combination with apical MUC1 expression (so a part of the MUC1 is routed correctly) this negative effect disappears.

Focal cytoplasmic expression of MUC1 has been described in lobular carcinomas.[12, 36] To our knowledge it has not been described in ductal carcinomas before. We did not find any relationship between focal cytoplasmic expression (designated 'pattern C') and any of the investigated variables.

We observed that tumours negative for MUC1 had a very poor outcome with respect to RFS and OS (figure 2 C and 2 D). In addition, absence of MUC1 expression was associated with absence of ER, PR and Cyclin D1. These findings support the observation by Luna-More et al, that tumours negative for MUC1 have high tumour grades, are ER and PR negative, and are more frequently associated with positive axillary lymph nodes.[29] Other studies have related low or negative MUC1 expression to higher tumour grades[13] and poor prognosis.[37] In inflammatory breast carcinoma patients with MUC1 negative tumours had a significantly shorter OS.[38] Remarkably, our MUC1-negative group of breast carcinomas appears to be a subgroup with poor prognosis that can not be identified with the common prognostic indicators; for both RFS and OS

survival MUC1 negativity was the strongest independent predictor (see tables 7 and 8).

We also performed a study with MUC1 expression in ductal carcinoma in situ (unpublished results). When comparing the results of that study with the current study some interesting differences can be noted. The inside-out expression pattern is exclusively seen in invasive ductal carcinomas and not in ductal carcinoma in situ (DCIS). Also in DCIS no MUC1 negative tumours were found. As mentioned before the inside-out expression pattern is specific for invasive micropapillary carcinoma. MUC1 negative tumours are a subgroup of tumours that is non-luminal, non mucin producing. These tumours are probably fast growing and aggressive and may not have a stage of non-invasive growth that can be diagnosed because of early invasion. Loss of MUC1 might play a role in this process of early invasiveness. Remarkably, this seems to be in contrast with in vitro and in vivo data which show that membranous MUC1 overexpression favours adhesion modulation, invasive potential, and metastatic capacity of tumour cells.[6-9] These effects are very likely due to steric hindrance of adhesion molecules by the high density of large and elongated extracellular MUC1 domains at the cell surface.[7] Without doubt, there are more mechanisms available for acquiring invasive potential, e.g. inactivation of the E-cadherin/β-catenin complex as in invasive lobular breast carcinoma. To investigate a potential relationship between MUC1 and E-cadherin expression, we performed immunohistochemistry for E-cadherin and β-catenin. However, both stainings were too heterogeneous and irreproducible for a reliable semiquantitative analysis (data not shown).

We realize that in this outcome study patients have been treated in a very heterogeneous manner (radiotherapy, chemotherapy and hormonal therapy), and that this may have confounded the results somewhat. However, in univariate analysis radiotherapy is not an predictor for both OS and RFS. In multivariate analysis MUC1 negativity remains an independent predictor of RFS and OS suggesting an effect independent from adjuvant chemotherapy. Also one could argue that the great amount of analysis undertaken in this study has led to significant results. However, in this study we focused on the comparison of MUC-1 expression with clinico-pathological parameters and

biomarkers selected based on their established role in the tumour biology of carcinomas and in breast carcinoma in particular. Therefore the authors think the conclusions of this study are still valid.

In conclusion, this study has shown that determination of the MUC1 expression pattern may play a role in the classification and prognosis prediction of breast cancer. Normal apical expression of MUC1 was associated with a good prognosis whereas cytoplasmic MUC1 expression was associated with worse prognostic tumour characteristics. A MUC1 negative expression pattern is an independent risk factor for poor RFS and OS, besides 'classical' prognostic indicators.

Table 1. Patient and tumour characteristics.

	N	%
Age at diagnosis		
median (range)	58 (27-89)	
Menopausal status		
premenopausal	75	30.9
postmenopausal	168	69.1
Family history		
positive	34	14.0
negative	157	64.6
unknown	52	21.4
Therapy		
BCT	145	59.6
mastectomy	98	44.9
Axillary nodal status		
negative	131	53,9
positive	107	44,0
not assessed	5	2,1
Pathological tumour size (mm)		
median (range)	20 (2-140)	
Pathological tumour stage		
T1	109	44.9
T2	109	44.9
T3	18	7.4
unknown	7	2.9
Grade of differentiation		
well	57	23,5
moderate	110	45,3
poor	75	30,9
missing	1	0,4
Adjuvant chemotherapy		
yes	61	25,1
no	182	74,9
Adjuvant hormonal therapy		
yes	87	35,8
no	156	64,2

n: number of cases; %: percentage; BCT: breast conserving therapy; mm: millimeter; T1: tumour diameter < 20 mm; T2: tumour diameter ≥ 20 mm but < 50 mm in diameter; T3: tumour diameter ≥ 50 mm.

TABLE 2. ANTIBODIES AND ANTIGEN RETRIEVAL METHODS.

ANTIBODY	CLONE	SUPPLIER	DILUTION	ANTIGEN RETRIEVAL	SECONDARY ANTIBODY	SUPPLIER	TERTAIRY ANTIBODY	SUPPLIER
MUC1	214D4	Dr. J. Hilkens[1]	1:100	-	RAMPO	Dako	GARPO	Dako
ER	6F11	Ventana	[2]	Tris/HCL 0.1 M (pH 9,5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
PR	1A6	Ventana	[2]	Tris/HCL 0.1 M (pH 9,5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
p53	BP-53-12-1	Biogenix	1:800	Tris/HCL 0.1 M (pH 9,5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
Cyclin D1	SP4	Neomarkers	1:50	Tris/HCL 0.1 M (pH 9,5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
Her2 Neu	CB11	Ventana	[2]	Tris/HCL 0.1 M (pH 9,5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako

ER: Estrogen receptor; PR: Progesteron Receptor; RAMPO: Rabbit anti mouse polyclonal; RAMBIO: Rabbit anti mouse biotin; GARPO: Goat anti rabbit polyclonal; SARBIO: Swine anti rabbit biotin; -: No antigen retrieval neccesary.1Gift from Dr. J. Hilkens; 2 Prediluted by supplier.

TABLE 3. MUC1 EXPRESSION AND CLINICO-PATHOLOGICAL PARAMETERS.

MUC1 staining	MUC1 entire membrane expression		MUC1 apical expression		MUC1 focal cytoplasmic expression		MUC1 diffuse cytoplasmic expression		MUC1 Inside-out expression		MUC1 All staining patterns	
Clinico-pathological parameters	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative
Menopausal status												
Premenopausal	16 (33.3)	59 (31.2)	19 (29.7)	56 (32.4)	3 (14.3)	72 (33.3)	54 (31.2)	21 (32.8)	7 (30.4)	68 (31.8)	67 (30.3)	8 (50.0)
Postmenopausal	32 (66.7)	130 (68.8)	45 (70.3)	117 (67.6)	18 (85.7)	144 (66.7)	119 (68.8)	43 (67.2)	16 (69.6)	146 (68.2)	154 (69.7)	8 (50.0)
(n=237)												
Family history												
Positive	4 (11.8)	30 (19.7)	9 (18.4)	25 (18.2)	3 (18.8)	31 (18.2)	27 (20.3)	7 (13.2)	3 (15.8)	31 (18.6)	31 (18.1)	3 (20.0)
Negative	30 (88.2)	122 (80.3)	40 (81.6)	112 (81.8)	13 (81.3)	139 (81.8)	106 (79.7)	46 (86.8)	16 (84.2)	136 (81.4)	140 (81.9)	12 (80.0)
(n=186)												
Pathological tumour stage												
T1	17 (36.2)	89 (48.6)	39 (65.0)	67 (39.4)	11 (55.0)	95 (45.2)	77 (45.3)	29 (48.3)	9 (39.1)	97 (46.9)	98 (45.8)	8 (50.0)
T2	25 (53.2)	82 (44.8)	21 (35.0)	86 (50.6)	7 (35.0)	100 (47.6)	83 (48.8)	24 (40.0)	10 (43.5)	97 (46.9)	100 (46.7)	7 (43.8)
T3	5 (10.6)	12 (6.6)	0 (0.0)	17 (10.0)	2 (10.0)	15 (7.1)	10 (5.9)	7(11.7)	4 (17.4)	13 (6.3)	16 (7.5)	1 (6.3)
(n=230)				*								
Tumour grade			≠≠									
I	8 (16.7)	48 (25.5)	25 (39.1)	31 (18.0)	6 (30.0)	50 (23.1)	39 (22.7)	17 (26.6)	6 (26.1)	50 (23.5)	55 (25.0)	1 (6.3)
II	25 (52.1)	83 (44.1)	30 (46.9)	78 (45.3)	9 (45.0)	99 (45.8)	80 (46.5)	28 (43.8)	9 (39.1)	99 (46.5)	101 (45.9)	7 (43.8)
III	15 (31.3)	57 (30.3)	9 (14.1)	63 (36.6)	5 (25.0)	67 (31.0)	53 (30.8)	19 (29.7)	8 (34.8)	64 (30.0)	64 (29.1)	8 (50.0)
(n=236)				*								
Lymph node status												
negative	20 (42.6)	107 (57.8)	37 (59.7)	90 (52.9)	13 (65.0)	114 (53.8)	94 (56.0)	33 (51.6)	10 (43.5)	117 (56.0)	117 (54.2)	10 (62.5)
positive	27 (57.4)	78 (42.2)	25 (40.3)	80 (47.1)	7 (35.0)	98 (46.2)	74 (44.0)	31 (48.4)	13 (56.5)	92 (44.0)	99 (45.8)	6 (37.5)
(n=232)												
Adjuvant radiotherapy												
Yes	29 (60.4)	113 (59.8)	39 (60.9)	103 (59.5)	13 (61.9)	129 (59.7)	103 (59.5)	39 (60.9)	16 (69.6)	126 (58.9)	131 (59.3)	11 (68.8)
No	19 (39.6)	76 (40.2)	25 (39.1)	70 (40.5)	8 (38.1)	87 (40.3)	70 (40.5)	25 (39.1)	7 (30.4)	88 (41.1)	90 (40.7)	5 (31.3)
(n=237)												
Adjuvant chemotherapy												
Yes	29 (60.4)	89 (47.1)	29 (45.3)	89 (51.4)	10 (47.6)	108 (50.0)	86 (49.7)	32 (50.0)	14 (60.9)	104 (48.6)	112 (50.7)	6 (37.5)
No	19 (39.6)	100 (52.9)	35 (54.7)	84 (48.6)	11 (52.4)	108 (50.0)	87 (50.3)	32 (50.0)	9 (39.1)	110 (51.4)	109 (49.3)	10 (62.5)
(n=237)												

*T1: tumour diameter ‹ 20 mm; T2: tumour diameter › 20 mm but ‹ 50 mm in diameter; T3: tumour diameter › 50 mm. * significant.*

TABLE 4. MUC1 EXPRESSION RELATED TO BIOLOGICAL MARKERS.

MUC1 staining	MUC1 entire membrane expression		MUC1 apical expression		MUC1 focal cytoplasmic expression		MUC1 diffuse cytoplasmic expression		MUC1 Inside-out expression		MUC1 All staining patterns	
Biological Markers	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative
Her-2/neu												
Negative	42 (87.5)	162 (86.6)	58 (92.1)	146 (84.9)	20 (95.2)	184 (86.0)	147 (85.5)	57 (90.5)	20 (87.0)	184 (86.8)	191 (86.8)	13 (86.7)
Positive	6 (12.5)	25 (13.4)	5 (7.9)	26 (15.1)	1 (4.8)	30 (14.0)	25 (14.5)	6 (9.5)	3 (13.0)	28 (13.2)	29 (13.2)	2 (13.3)
(n=235)												
ER												
Negative	7 (14.9)	46 (24.9)	9 (14.1)	44 (26.2)	3 (14.3)	50 (23.7)	36 (21.2)	17 (27.4)	1 (4.3)	52 (24.9)	45 (20.6)	8 (57.1)
Positive	40 (85.1)	139 (75.1)	55 (85.9)	124 (73.8)	18 (85.7)	161 (76.3)	134 (78.8)	45 (72.6)	22 (95.7)	157 (75.1)	173 (79.4)	6 (42.9)
(n=232)												
PR												
Negative	14 (29.2)	71 (39.0)	13 (21.3)	72 (42.6)	5 (23.8)	80 (38.3)	54 (32.3)	31 (49.2)	8 (36.4)	77 (37.0)	73 (34.1)	12 (75.0)
Positive	34 (70.8)	111 (61.0)	48 (78.7)	97 (57.4)	16 (76.2)	129 (61.7)	113 (67.7)	32 (50.8)	14 (63.6)	131 (63.0)	141 (65.9)	4 (25.0)
(n=230)				*								*
P53												
Negative	46 (95.8)	176 (93.1)	62 (96.9)	160 (92.5)	19 (90.5)	203 (94.0)	165 (95.4)	57 (89.1)	21 (91.3)	201 (93.9)	209 (94.6)	13 (81.3)
Positive	2(4.2)	13 (6.9)	2(3.1)	13 (7.5)	2 (9.5)	13 (6.0)	8 (4.6)	7 (10.9)	2 (8.7)	13 (6.1)	12 (5.4)	3 (18.8)
(n=237)												
Cyclin D1												
Negative	12 (25.0)	61 (32.3)	17 (26.6)	56 (32.4)	8 (38.1)	65 (30.1)	45 (26.0)	28 (43.8)	4 (17.4)	69 (32.2)	63 (28.5)	10 (62.5)
Positive	36 (75.0)	128 (67.7)	47 (73.4)	117 (67.6)	13 (61.9)	151 (69.9)	128 (74.0)	36 (56.3)	19 (82.6)	145 (67.8)	158 (71.5)	6 (37.5)
(n=237)								*				*

*T1: tumour diameter ‹ 20 mm; T2: tumour diameter › 20 mm but ‹ 50 mm in diameter; T3: tumour diameter › 50 mm. * significant.*

Table 5. Univariate analysis investigating the relation of pathological and biological characteristics with relapse free survival.

Pathological and biological features	n (%)	HR	95% CI	p-value
Tumour size				
<20mm	109 (46.2)	1		
20-50mm	109 (46.2)	2.2	1.1-4.5	0.03
>50 mm	18 (7.6)	3.8	1.4-10.2	0.009
(n=236)				
Grade				
1 & 2	167 (69.0)	1		
3	75 (31.0)	2.3	1.2-4.2	0.009
(n=242)				
MUC1 expression				
Cytoplasmic	144 (60.8)			
Membrane	77 (32.5)	1	0.5-2.0	1.0
Negative	16 (6.7)	3.4	1.5-8.1	0.005
(n=237)				
Her2/neu				
Negative	225 (94.5)	1		
Positive	13 (5.5)	2.8	1.1-7.1	0.03
(n=238)				
ER				
Negative	54 (22.9)	1		
Positive	182 (77.1)	0.5	0.3-1.0	0.05
(n=236)				
PR				
Negative	86 (36.9)	1		
Positive	147 (63.1)	0.4	0.2-0.7	0.003
(n=233)				
p53				
Negative	226 (93.8)	1		
Positive	15 (6.2)	1.5	0.5-4.3	0.5
(n=241)				
Cyclin D1				
Negative	75 (30.9)	1		
Positive	168 (69.1)	0.6	0.3-1.2	0.2
(n=243)				

n: number of cases; %: percentage; Hazard Ratio ; ER: estrogen receptor; PR: progesterone receptor.

Table 6. Univariate analysis investigating the relation of pathological and biological characteristics with overall survival.

Pathological and biological features	n (%)	HR	95% CI	p-value
Tumour size				
<20mm	109 (46.2)	1		
20-50mm	109 (46.2)	3.0	1.0-9.2	0.06
>50 mm	18 (7.6)	6.6	1.6-26.4	0.008
(n=236)				
Grade				
1 & 2	167 (69.0)	1		
3	75 (31.0)	3.6	1.5-8.7	0.005
(n=242)				
Adjuvant chemotherapy				
No	123 (50.7)	1		
Yes	120 (49.3)	3.2	1.1-8.8	0.02
(n=243)				
MUC1 expression				
Cytoplasmic	144 (60.8)	1		
Membrane	77 (32.5)	0.7	0.2-2.3	0.6
Negative	16 (6.7)	6.0	2.2-16.7	0.001
(n=237)				
Her2/neu				
Negative	225 (94.5)	1		
Positive	13 (5.5)	6.3	2.2-17.5	<0.001
(n=238)				
ER				
Negative	54 (22.9)	1		
Positive	182 (77.1)	0.3	0.1-0.8	0.02
(n=236)				
PR				
Negative	86 (36.9)	1		
Positive	147 (63.1)	0.4	0.2-1.0	0.05
(n=233)				
p53				
Negative	226 (93.8)	1		
Positive	15 (6.2)	2.4	0.7-8.1	0.2
(n=241)				
Cyclin D1				
Negative	75 (30.9)	1		
Positive	168 (69.1)	0.6	0.2-1.5	0.3
(n=243)				

n: number of cases; %: percentage; Hazard Ratio ; ER: estrogen receptor; PR: progesterone receptor.

Table 7. Stepwise multivariate analysis investigating the relation of pathological and biological characteristics with relapse free survival.

Characteristics	HR	95% CI	p-value
MUC1 expression			
Cytoplasmic	1		
Membrane	1.1	0.5-2.2	0.8
Negative	3.5	1.5-8.5	0.005
PR			
Negative	1		
Positive	0.4	0.2-0.8	0.09

HR, Hazard Ratio; 95%CI, 95% Confidence Interval.

Table 8. Stepwise multivariate analysis investigating the relation of pathological and biological characteristics with overall survival.

Characteristics	HR	95% CI	p-value
Axillary lymph node status			
Negative	1		
Positive	4.7	1.7-13.0	0.003
MUC1 expression			
Cytoplasmic	1		
Membrane	0.6	0.2-2.0	0.4
Negative	14.7	4.9-44.1	<0.001
Her2/neu			
Negative	1		
Positive	3.7	1.4-9.5	0.006

HR, Hazard Ratio; 95%CI, 95% Confidence Interval.

Figures

Figure 1. MUC1 staining patterns as classified in this study.

A. Entire membrane staining. B. Apical staining. C. Focal cytoplasmic staining. D. Diffuse cytoplasmic staining. E. Inside-out staining.

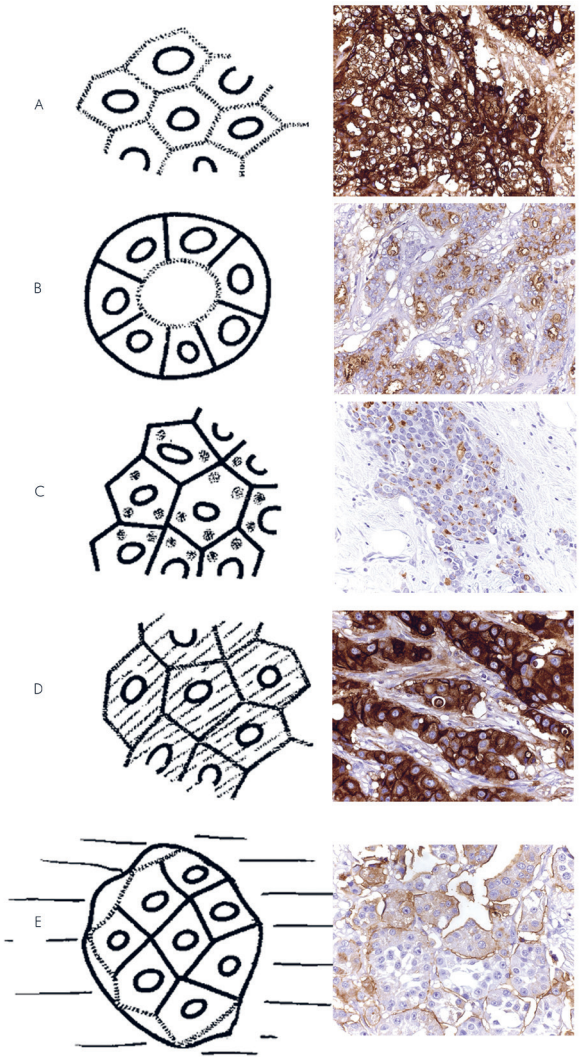
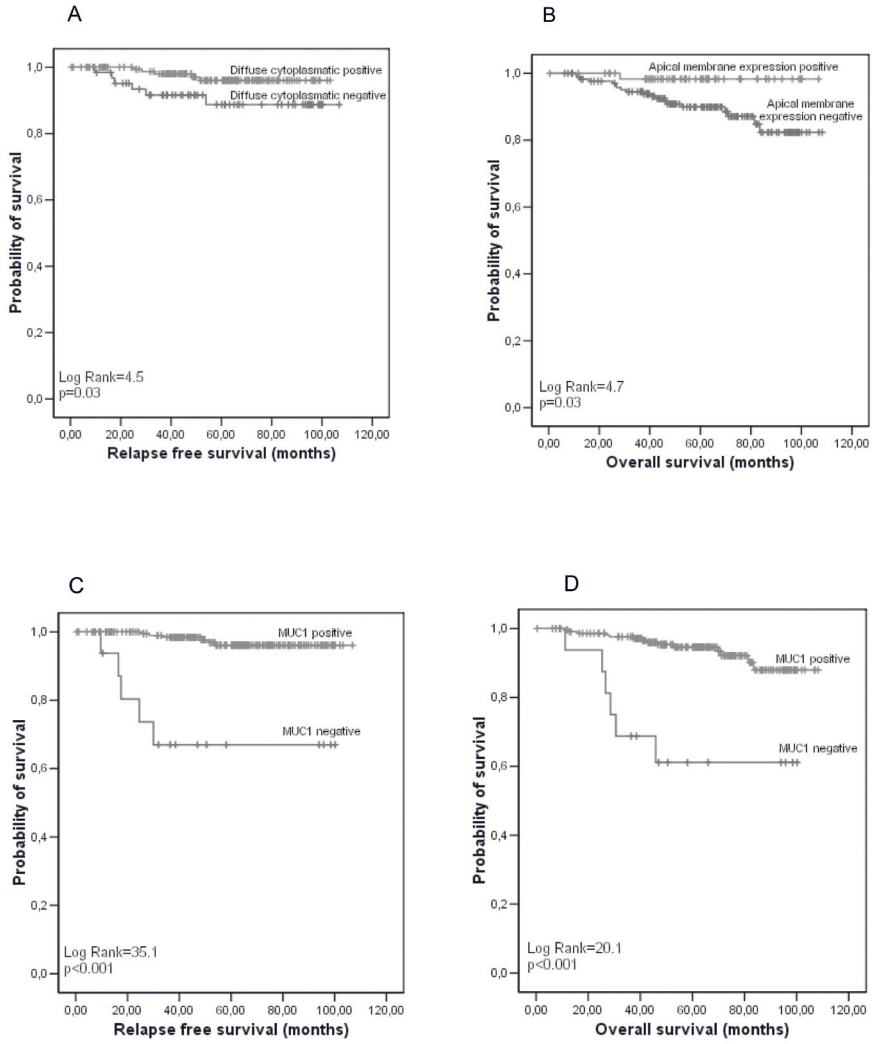


Figure 2. Correlation between the MUC1 expression pattern and relapse free (A & C) and overall (B & D) survival (Kaplan Meier method and log-rank test).



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5

PATHOLOGICAL AND BIOLOGICAL DIFFERENCES BETWEEN SCREEN-DETECTED AND INTERVAL DUCTAL CARCINOMA IN SITU OF THE BREAST

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de Roos MA[1]
van der Vegt B[2]
de Vries J[1]
Wesseling J[2]
de Bock GH[3]

Departments of Surgical Oncology[1], Pathology[2] and Epidemiology[3] University Medical Center Groningen, University of Groningen

Abstract

Background: The incidence of ductal carcinoma in situ (DCIS) has risen dramatically with the introduction of screening mammography. The aim was to evaluate differences in pathological and biological characteristics between patients with screen-detected and interval DCIS.

Methods: From January 1992 to December 2001, 128 consecutive patients had been treated for pure DCIS at our institute. From these 128,102 had been attending the Dutch breast cancer screening programme. Sufficient paraffin embedded tissue was available in 74 out of the 102 cases to evaluate biological marker expression (Her2/neu, ER, PR, p53 and cyclin D1) on tissue microarrays (TMA-group). Differences in clinico-pathological characteristics and marker expression between screen-detected and interval patients were evaluated. Screen-detected DCIS was classified as DCIS detected by screening mammography, when the two-year earlier examination failed to reveal an abnormality. Interval patients were classified as patients with DCIS detected within the two-year interval between two subsequent screening rounds.

Results: Screen-detected DCIS was related with linear branching and coarse granular microcalcifications on mammography ($p<0.001$) and with high grade according to the Van Nuys classification ($p=0.025$). In univariate analysis screen-detected DCIS was related with Her2/neu overexpression (Odds Ratio [OR]=6.5; 95%CI 1.3-31.0; $p=0.020$) and interval DCIS was associated with low grade (Van Nuys, OR=7.3; 95% CI 1.6-33.3; $p=0.010$) and PR positivity (OR=0.3; 95%CI 0.1-1.0; $p=0.042$). The multivariate analysis displayed an independent relation of Her2/neu overexpression with screen-detected DCIS (OR=12.8; 95%CI 1.6-104.0; $p=0.018$).

Conclusion: These findings suggest that screen-detected DCIS is biologically more aggressive than interval DCIS and should not be regarded as overdiagnosis.

Introduction

With the introduction of widespread screening mammography, the incidence rates of ductal carcinoma in situ (DCIS) have risen dramatically in Western Europe and North America.[1-3] DCIS now accounts for nearly 20% of all screen-detected breast malignancies.[4]

As a consequence, treating physicians are confronted with a cumulative caseload because it is not known how many women with screen-detected DCIS will develop an invasive carcinoma in their lifetimes. The proportion of untreated cases of DCIS that would progress to invasive malignancy has been difficult to evaluate, because DCIS is usually excised when detected. Because DCIS is a non-obligatory precursor to invasive carcinoma, and, therefore, has a relatively benign nature, screen-detected DCIS has been argued to represent an overdiagnosis.[5-,6] This argument is supported by autopsy studies in which the median prevalence of DCIS was 8.9% suggesting some cases do not progress to clinically significant lesions in a patient's lifetime.[7] On the contrary, patients with DCIS treated with biopsy alone in the premammography era had a higher rate of subsequent occurrences (14-50%) of invasive breast cancer than expected.[8,9] Large clinical trials, in which patients had been treated with lumpectomy alone, have also indicated that DCIS can recur as invasive ductal carcinoma.[10,11]

Screen-detected DCIS is more often presented as linear branching microcalcifications on mammography than symptomatic DCIS.[12] The screen-detected group in the previously mentioned study had a larger proportion of patients with comedocarcinoma. Therefore, it was suggested that linear branching microcalcifications were related with a more aggressive type of DCIS.[12] This is confirmed in other reports which have indicated that linear branching microcalcifications on mammography are associated with high grade.[13,14]

We believe that screen-detected DCIS is more often associated with suspicious microcalcifications representing high grade DCIS which has been detected before it has had the chance to progress to invasive cancer. Therefore, it is hypothesized that screen-detected DCIS is biologically more aggressive than interval DCIS. In order to compare screen-detected DCIS with interval DCIS in such retrospective study, the clinico-pathological and biological

characteristics of both groups were evaluated for differences. Screen-detected DCIS was classified as DCIS detected by screening mammography, when the two-year earlier examination failed to reveal an abnormality. Interval DCIS was classified as DCIS detected within the two-year interval between two subsequent screening rounds, when the earlier examination failed to reveal an abnormality. Age, tumour size, and pathological grade were studied for their known relation with local recurrence. Finally, the expression of established prognostic biomarkers in breast cancer was studied by immunohistochemistry for oestrogen receptor (ER), progesterone receptor (PR), Her2/neu, p53 and cyclin D1.

Patients and methods

Patients and tumours

The Dutch screening programme for breast cancer has been gradually implemented in the North Netherlands since 1991. It offered biennial mammography to women aged 50-69 years and since 1999 women aged 70-74 have also been included. Women received mammography in the cranio-caudal and medio-latero-oblique direction for each breast. Two radiologists evaluated the mammograms by a double, independent reading.

In the period from January 1992 to December 2001 128 consecutive patients had been treated for pure DCIS at our institution. To identify patients for inclusion in the study all women who had actually attended the screening programme at least two subsequent rounds with a two-year interval at the time of diagnosis were considered as attenders. Patients that had skipped one or more screening rounds previous to the diagnosis and patients who had not been attending the programme at all were considered non-attenders. In order to obtain this information patients records were checked and if there was no information regarding the participation of the screening programme at the time of diagnosis the general practioner was consulted. Out of the 128 consecutive patients 102 attenders and 26 non-attenders could be identified. For immunohistochemistry patients were selected on the availability of sufficient paraffin embedded tissue. Thirteen out of the 26 non-attenders and

74 out of the 102 attenders remained, respectively, for evaluation of Her2/neu overexpression, oestrogen receptor (ER) expression, progesterone receptor (PR) expression, p53 expression and cyclin D1 expression using tissue microarray analysis as part of a project protocol that had been approved by the medical ethics committee. The patients in the study-group (n=74) were divided into two groups. Patients with DCIS that had been detected by screening mammography were classified as screen-detected patients, when the two-year earlier examination failed to reveal an abnormality (n=54). Patients with DCIS that had been detected within the two-year interval between two subsequent screening rounds were classified as interval patients, when the earlier examination failed to reveal an abnormality (n=20).

Mammography and pathological assessment

Mammographic and pathological characteristics were derived from mammography and pathological reports, respectively. Data were delivered and evaluated anonymously. If data were missing, mammography and pathological slides were re-evaluated. Mammographic appearances were scored as microcalcifications, a mass, a combination of the two, or as occult. Microcalcifications were scored as fine granular, coarse granular or as linear branching. Pathological size had been estimated and the grade had been scored according to the European Pathologists Working Group (EPWG[15]) and according to the Van Nuys classification.[16]

Tissue microarray construction

Slides from all blocks were evaluated for representative areas with DCIS and tissue microarrays were prepared as described earlier[17]. In brief, the most representative area of DCIS was marked on the original haematoxylin and eosin (H&E) stained section. With this marked section as an orientation, three 0.6 mm punches were taken from the selected area in the donor blocks and mounted in a recipient block containing approximately 110 biopsies, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD, USA). The presence of DCIS in the arrayed samples was verified on haematoxylin eosin stained sections.

Immunohistochemistry

For immunohistochemistry, 3 µm sections of the paraffin embedded tissue arrays were deparaffinised in 2 changes of xylene for 5 minutes each and gradually rehydrated through changes of graded ethanol from 100% to distilled water. Antigen retrieval methods and antibodies are summarized in Table 1. The endogenous peroxidase reaction was blocked by incubating the sections in 3% perhydrol for 30 minutes. Primary antibodies were diluted in PBS contaning 1% Bovine Serum Albumin and incubated at room temperature for 1 hour. Samples were then washed in PBS and incubated with secondary and tertiary antibodies. For visualization of the antibody-antigen complex, the diaminobenzidine tetrahydrochloride/ peroxidase reaction was used. After a final wash with distilled water, sections were counterstained with hematoxylin. Sections were dehydrated through rising concentrations of ethanol and mounted. Immunohistochemistry was successful in 81/87 cases for Her2/neu staining, 73/87 cases for ER staining, 71/87 for PR and cyclin D1 staining and 70/87 for p53 staining.

Evaluation of immunohistochemical staining

All slides stained for molecular markers were read by two authors (MdR and BvdV). The slides were randomly reviewed by a third author (JW) and in case of disagreement between the other two authors. ER, PR and p53 were graded based on the percentage of cells showing positive nuclear staining in the ducts with DCIS. ER and PR were considered positive if nuclear staining was present in .10% of the cases, and p53 was considered positive in case of a substantial percentage of positively stained nuclei (>30%). Her2/neu expression was graded as recommended by the HercepTest™ scoring guidelines: 0: no staining at all or membrane staining in <10% of tumour cells; 1+: a faint/barely perceptible partial membrane staining in >10% of the tumour cells; 2+: weak to moderate complete membrane staining in >10% of tumour cells; 3+: strong complete membrane staining in >10%. Her-2/neu was considered to be overexpressed if the score was 3+. Cyclin D1 expression was scored using a semiquantitative system as described by Vos et al.[18] This system was based on the staining intensity scored as 0 (none), 1 (weak), 2 (moderate), and 3 (strong), and the percentage of positive tumour cell nuclei scored as 0 (0%), 1 (1-25%), 2 (25-

50%), 3 (50-75%), and 4 (>75%). The cyclin D1 staining score was calculated as the sum of the intensity and the percentage of positive tumour cells.

Statistical analysis

Differences in clinico-pathological characteristics between screen-detected and interval patients with DCIS in the study-group, and between the study-group and the exluded group of patients were analysed by chi-square analysis. Differences in clinico-pathological and biological characteristics between the study-group and the non-attenders were also analysed by chi-square analysis. Differences in age were tested by using the Mann-Whitney U test. Univariate analyses, investigating differences in pathological and biological features, was performed by logistic regression, using screen-detected as a dependent variable. Multivariate analyses were performed with a logistic-regression model. The elimination of variables in a stepwise manner identified the statistically significant pathological and biological parameters. A p value of , 0.050 was considered as significant. All calculations were performed with SPSS 12.01 (SPSS inc., Chicago, IL, USA).

Results

Table 2 shows the clinico-pathological characteristics of the patients in the study group (n=74). Screen-detected DCIS was less often symptomatic than interval DCIS (p<0.001). Five patients (25%) in the interval group had no objective signs on presentation; all patients had felt a lump in the breast that could not be verified on clinical examination. On mammography microcalcifications were more often seen in screen-detected DCIS (p=0.002). Screen-detected DCIS was more often presented as linear branching- (44.9%) and coarse granular (55.9%) microcalcifications than interval DCIS (p<0.001). High grade (Van Nuys, 53.1%, p=0.025) was also more often observed in screen-detected DCIS.

Differences in clinico-pathological characteristics between the study-group

(n=74) and the group of patients that had been exluded (n=28) because of insufficient paraffin embedded tissue are outlined in table 3. There was no marked difference in age, microcalcifications, tumour size and pathological grade according to the EPWG classification between both groups. The excluded group of patients represented a relatively large proportion of low grade according to the Van Nuys classification (p<0.001) in comparison with the TMA-group.

Differences in clinico-pathological and biological characteristics between the study-group and the non-attenders (n=13) are summarized in table 4. Non-attenders were younger than the patients in the study group (55.9 years versus 59.6 years; p=0.042).

In Table 5 the relation between pathological characteristics, biological marker expression and mode of detection in the study-group is displayed. Univariate logistic regression analysis indicated that in screen-detected DCIS Her2/neu is more often overexpressed (Odds Ratio [OR]=6.5; 95% Confidence Interval [CI] 1.3-31.0; p=0.020). Interval DCIS is more frequently positive for PR staining (OR=0.3; 95% CI 0.1-1.0; p=0.042) and is related to low pathological grade according to the Van Nuys classification (OR=7.3; 95% CI 1.6-33.3; p=0.010). In multivariate logistic regression, including pathological grade according to the EPWG and Van Nuys classification, Her2/neu overexpression and PR expression in the model, Her2/neu overexpression was the only independent indicator for screen-detected DCIS (OR=12.8; 95% CI 1.6-104.0; p=0.018).

Discussion

Approximately 1 in every 1300 screening mammography examinations leads to a diagnosis of DCIS.[4] Data from a large trial and service screening programmes in the UK, the Netherlands, Australia and the USA have demonstrated that a woman attending prevalence screen has a 19 times greater chance of having a progressive DCIS or an invasive tumour diagnosed than of having a non-progressive DCIS diagnosed.[19] It is questioned what to do with the high detection rate of screen-detected DCIS. It was hypothesized that screen-detected DCIS is biologically more aggressive than interval DCIS

because suspicious microcalcifications, detected by the screening programme, will probably more frequently represent high grade DCIS. Therefore screen-detected DCIS was characterized pathologically and biologically in order to determine whether screen-detected DCIS differed from interval DCIS. The results of this study indicate that screen-detected DCIS is pathologically (OR=7.3; 95% CI 1.6-33.3; p=0.010) and biologically (OR=12.8; 95%CI 1.6-104.0; p=0.018) more aggressive than interval DCIS. Indeed screen-detected DCIS was related with more suspicious microcalcifications (p<0.001). DCIS detected by a prevalence screen was pathologically and biologically comparable to DCIS detected in later rounds (data not shown) suggesting prevalence and incident cases to be both of clinical relevance.

The relative incidence of high grade DCIS in our series of screen-detected patients was 53% which is comparable to the incidence of high grade in a screening population from the Netherlands Cancer Institute (47%).[21] Much data point out that poorly differentiated or high grade DCIS lesions have a greater potential to progress to invasive disease than low grade DCIS.[20] High grade is also an independent risk factor of local recurrence after lumpectomy for DCIS and approximately 50% of these recurrences are invasive cancers.[22,23] There are no studies available comparing screen-detected with interval DCIS in a group of patients that had all been attending the screening programme. Reports on screen-detected DCIS regarding histo-pathological grade vary markedly describing a higher incidence of low grade[24], no difference[25], or a higher incidence of high grade[12,26,27] in screen-detected DCIS. In these reports screen-detected DCIS is compared to symptomatic DCIS or to DCIS detected in a period before the screening programme was introduced. In the present study a higher incidence of high grade lesions, which were classified according Van Nuys (OR=7.3; 95% CI 1.6-33.3; p=0.010) classification, was found in screen-detected DCIS, indicating a higher malignant potential in screen-detected DCIS. These results are consistent with the results from the studies of Evans et al.[26] and Kessar et al.[27]

DCIS lesions from patients in the study group were compared to DCIS lesions from patients who had not attended the screening programme (table 4). Out of the 26 non-attenders there were only 13 patients from which sufficient paraffin embedded tissue was available. Using chi-square analysis there

were no differences in pathological and biological characteristics between the two groups. The difference in age could be explained by the fact that the non-attenders-group also contained patients under 50-years of age. From these analyses it seems that DCIS in non-attenders is not pathologically and biologically more aggressive than DCIS in attenders of the screening programme. However, because of the very small number of patients in the non-attenders group no hard conclusions can be drawn.

Her2/neu overexpression has been found to correlate with various pathologic and biological factors believed to be associated with more aggressive behaviour; high grade, presence of necrosis, ER- and PR-negativity and overexpression of Ki-67 (indicating an increased proliferation rate) are features that are strongly related with Her-2/neu overexpression.[28-30] The report of Walker et al.[24] displayed a Her-2/neu expression of 59% in symptomatic DCIS and of 42% in mammographically detected DCIS. Another study by Idvall et al.[25] reported no difference in Her-2/neu expression between DCIS before and after introduction of mammographic screening. In this present study Her2/neu overexpression was the only independent feature to be related with screen-detected DCIS in multivariate analysis (OR=12.8; 95%CI 1.6-104.0; p=0.018) which indicates a more aggressive profile of screen-detected DCIS when compared to interval DCIS.

Although the statistical methods used were univariate and multivariate analysis, the numbers in both groups are small, which explains the broad 95% CI. The small numbers are due to the selection of patients in this study for study period, attendance of screening rounds and availability of sufficient paraffin embedded tissue. Clearly further studies with larger populations are needed to elucidate the relative significance of the Her2/neu overexpression in women with screen-detected DCIS. Although 28 out of 102 patients were excluded because of lack of sufficient paraffin embedded tissue, there is no reason to assume that this exclusion results in a significant selection bias. Apart from pathological grade according to Van Nuys, there were no differences in clinico-pathological characteristics between the study-group and the group of excluded patients. The group of excluded patients displayed a relatively large amount of low grade DCIS according to Van Nuys (53.6%, p<0.001), which is mainly derived from the interval group (n=10, data not shown).

If all patients would have been included the relation of pathological grade and probably Her2/neu expression with screen-detected patients would be even more significant. The inclusion of patients that actually took part in the screening programme was necessary to analyze differences between screen-detected- and interval DCIS, which, to our knowledge, has not been performed previously.

Expression of ER, p53 and cyclin D1 was not related to screen-detected or interval DCIS. There was, however, a relation between screen-detected DCIS and PR negativity in univariate analysis (OR=0.288; 95%CI 0.087-0.957; p=0.042). Other studies could not demonstrate a relation of screen-detected DCIS with the expression of the above mentioned markers.[12,24,25] In a review by Boland et al.[31] ER and PR positivity are related to low grade DCIS, whereas p53 and cyclin D1 expression are associated with high grade. The fact that screen-detected DCIS is associated with PR-negativity provides indirect evidence for the presence of a more aggressive tumour biology. Obviously PR expression is related to Her2/neu expression, because PR expression was not significant in multivariate analysis.

Ultimately, the question is how we should interpret these findings. The authors think the results from this study represent no evidence to alter patient management and screening recommendations. Instead, they should rather be regarded as support of current clinical practice in DCIS of the breast. They confirm that every DCIS should be treated until we are able to identify DCIS which will progress to invasive cancer if left untreated is.

In conclusion, since the advent of screening, the increased incidence of DCIS has raised concerns about the possibility of overdiagnosis of DCIS. This study has shown that screen-detected DCIS has a more aggressive tumour profile than interval DCIS. Therefore, screen-detected DCIS should not be regarded as an overdiagnosis per se and every woman diagnosed with DCIS by mammographic screening should be treated properly according to existing guidelines or standards of care.

TABLE 1. ANTIGEN RETRIEVAL METHODS AND ANTIBODIES.

ANTIBODY	CLONE	SUPPLIER	DILUTION	ANTIGEN RETRIEVAL	SECONDARY ANTIBODY	SUPPLIER	TERTIARY ANTIBODY	SUPPLIER
ER	6F11	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
PR	1A6	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
Her-2/Neu	CB11	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
p53	BP-53-12-1	Biogenix	1:800	Tris/HCL 0.1M (pH 9.5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako
Cyclin D1	SP4	Neomarkers	1:50	Tris/HCL 0.1M (pH 9.5) 30' 98°C microwave	RAMBIO	Dako	SARBIO	Dako

*ER, Estrogen Receptor; PR, Progesteron Receptor; * , prediluted by supplier; RAMBIO, Rabbit anti mouse biotin; SARBIO; Swine anti rabbit biotin.*

Table 2. Clinico-pathological characteristics of the patients in the study-group and differences between screen-detected and interval patients.

Clinico-pathological characteristics	Screen-detected n=54	Interval n=20	p-value[§]
Age (mean)	58.9	60.7	0.187[¶]
Family history of breast cancer			0.055
Yes	8(14.8)	7(35)	
No	46(85.2)	13(65)	
Signs			<0.001
Palpable mass	5(9.3)	6(30)	
Nipple discharge	2(3.7)	9(45)	
Mastodynia	1(1.9)	0(0)	
No objective signs	46(85.1)	5(25)	
Mammography			0.002
Microcalcifications	46(85.2)	10(50)	
Mass	2(3.7)	6(30)	
Combination mc's and mass	6(11.1)	4(20)	
Microcalcifications			<0.001
Linear branching	22(44.9)	4(28.6)	
Coarse granular	27(55.1)	4(28.6)	
Fine granular	0(0)	6(42.9)	
Mammographic size			0.183
<2 cm	21(42.6)	12(60)	
>2 cm	33(57.4)	8(40)	
BCS	23(42.6)	8(40)	0.841
Mastectomy	31(57.4)	12(60)	
Tumour size			0.787
<16mm	19(35.2)	6(30)	
16-40mm	17(31.5)	8(40)	
>40 mm	18(33.3)	6(30)	
Grade (EPWG)			0.229
1	4(7.5)	4(20)	
2	29(53.7)	11(55)	
3	21(38.9)	5(25)	
Grade (Van Nuys)			0.025
1	4(7.4)	6(30)	
2	24(44.4)	9(45)	
3	26(53.1)	5(25)	

[¶]Mann-Whitney U test. [§]Chi-square analyses. Values between parentheses are percentages.BCS, breast conserving surgery EPWG, European Pathologist Working Group.

Table 3. Comparison of clinico-pathological characteristics between the study-group and the group of patients that were excluded because of insufficient paraffin embedded tissue.

Clinico-pathological characteristics	Study-group n=74	Excluded n=28	p-value[§]
Age (mean)	59.6	61.5	0.381[¶]
Microcalcifications (n=86)			0.063
Linear branching	26(41.3)	5(25)	
Coarse granular	31(49.2)	9(45)	
Fine granular	6(9.5)	6(30)	
Tumour size			0.458
<16mm	25(33.8)	7(25)	
16-40mm	25(33.8)	9(32.1)	
>40 mm	24(32.6)	12(42.9)	
Grade (EPWG)			0.184
1	8(10.8)	6(21.4)	
2	40(54.1)	10(35.7)	
3	26(35.1)	12(42.9)	
Grade (Van Nuys)			<0.001
1	10(13.5)	15(53.6)	
2	33(44.6)	6(21.4)	
3	31(41.9)	7(25)	

¶Mann-Whitney U test. §Chi-square analyses. Values between parentheses are percentages. EPWG, European Pathologist Working Group.

Table 4. Differences in clinico-pathological and biological characteristics between the study-group, and the non-attenders.

Clinico-pathological characteristics	Study-group n=74	Non-attenders n=13	p-value[§]
Age (mean)	59.6	55.9	0.042[¶]
Microcalcifications (n=71)			0.280
Linear branching	26(41.3)	1(12.5)	
Coarse granular	31(49.2)	6(75)	
Fine granular	6(9.5)	1(12.5)	
Tumour size			0.193
<16mm	25(33.8)	3(23.1)	
16-40mm	25(33.8)	3(23.1)	
>40 mm	24(32.6)	7(53.8)	
Grade (EPWG)			0.294
1	8(10.8)	4(30.7)	
2	40(54.1)	3(23.1)	
3	26(35.1)	6(46.2)	
Grade (Van Nuys)			0.659
1	10(13.5)	1(7.7)	
2	33(44.6)	5(38.5)	
3	31(41.9)	7(53.8)	
Her2/neu			0.535
Positive	21(30.9)	3(23.1)	
Negative (n=81)	47(69.1)	10(76.9)	
ER			0.155
Positive	49(79)	7(63.6)	
Negative (n=73)	13(21)	4(36.4)	
PR			0.351
Positive	31(51.7)	4(36.4)	
Negative (n=71)	29(48.3)	7(63.6)	
p53			0.264
Positive	14(23.3)	4(40)	
Negative (n=70)	46(76.7)	6(60)	
Cyclin D1			0.675
Positive	42(70)	7(63.6)	
Negative (n=71)	18(30)	4(36.4)	

[¶]Mann-Whitney U test. [§]Chi-square analyses. Values between parentheses are percentages. EPWG, European Pathologist Working Group.

Table 5. Univariate analysis of pathological and biological characteristics in screen-detected versus interval DCIS in the study-group.

Pathological and biological features	Screen-detected n=54	Interval n=20	OR by screen-detected	95% CI	p-value
Tumour size					
<16mm	19(35.2)	6(30)	0.8	0.2-2.9	0.744
16-40mm	17(31.5)	8(40)	1.1	0.2-2.3	0.827
>40 mm	18(33.3)	6(30)	1		
Grade (EPWG)					
1	4(7.5)	4(20)	3.800	0.7-20.8	0.124
2	29(53.7)	11(55)	1.703	0.5-5.6	0.377
3	21(38.9)	5(25)	1		
Grade (Van Nuys)					
1	4(7.4)	6(30)	7.3	1.6-33.3	0.010
2	24(44.4)	9(45)	1.6	0.5-5.3	0.416
3	26(53.1)	5(25)	1		
Her2/neu					
Positive	19(39.6)	2(10)	6.5	1.3-31.0	0.020
Negative (n=68)	29(60.4)	18(90)	1		
ER					
Positive	33(75)	16(88.9)	0.4	0.1-1.9	0.236
Negative (n=62)	11(25)	2(11.1)	1		
PR					
Positive	18(42.9)	13(65)	0.3	0.1-1.0	0.042
Negative (n=60)	24(57.1)	5(35)	1		
p53					
Positive	10(24.4)	4(21.1)	0.8	0.2-3.1	0.776
Negative (n=60)	31(75.6)	15(78.9)	1		
Cyclin D1					
Positive	29(69.1)	13(72.2)	0.9	0.3-2.9	0.806
Negative (n=60)	13(30.9)	5(27.8)	1		

Univariate analysis using logistic regression. Figures in parentheses are percentages. OR, Odds Ratio. EPWG, European Pathologist Working Group. ER, Oestrogen receptor. PR, Progesteron receptor. 95% CI, 95% Confidence Interval.

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6

AGGRESSIVENESS OF 'TRUE' INTERVAL INVASIVE DUCTAL CARCINOMAS OF THE BREAST IN POSTMENOPAUSAL WOMEN

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van der Vegt B[1]
Wesseling J[2]
Pijnappel RM[3]
Dorrius MD[4]
den Heeten GJ[5]
de Roos MA[6]
de Bock GH[7]

Departments of Pathology[2], Radiology[3] and Epidemiology[7] University Medical Center Groningen, University of Groningen; Department of Pathology The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam[2]; Department of Radiology, Martini Hospital, Groningen[3]; LRCB, University Medical Center Nijmegen, Nijmegen[5], and Department of Surgery, Rivierenland Hospital, Tiel[6], The Netherlands

Abstract

There is debate whether interval carcinomas differ from screen detected tumours biologically. In this study clinico-pathological parameters and the expression of well validated biological markers were compared between 'true' interval carcinomas and screen detected- / missed carcinomas hypothesizing that 'true' interval carcinomas show a more aggressive biological behaviour. The study group consisted of 92 consecutive postmenopausal women attending the breast screening programme and presenting with an invasive ductal carcinoma. All screening mammograms were re-reviewed. 16 patients had a 'true' interval carcinoma. 7 carcinomas were missed at screening, but detected upon re-reviewing of the screening mammogram. Radiological characteristics were assessed from diagnostic mammograms. Data on patient- and tumour characteristics and follow-up data were recorded from hospital records. Median follow-up was 61 months. Immunohistochemistry for ER, PR, Her2/neu and p53 was performed on TMA sections. Univariate and multivariate logistic regression analyses were performed. In univariate analysis 'true' interval carcinomas were significantly larger (OR 7.2, 95% CI 1.8-28.1) and less frequently ER (OR 0.3, 95% CI 0.1-0.9) and PR (OR 0.3, 95% CI 0.1-1.0) positive. In multivariate analysis 'true' interval carcinoma was independently associated with larger tumours (OR 7.0, 95% CI 1.4-36.2). A trend towards ER negativity was found (OR 0.3, 95% CI 0.1-1.1). 'True' interval carcinomas showed a trend towards a decreased relapse free survival (HR 1.7 95% CI 0.9-3.1). Although 'true' interval carcinomas were significantly larger than screen detected- / missed interval carcinomas it remains challenging to find parameters that determine this difference between 'true' interval carcinomas and screen detected lesions.

Introduction

From 1989 till 1997 a nation-wide breast cancer screening programme has gradually been implemented in The Netherlands.[1] Starting as a biennial screening mammography for women aged 50-69, in 1999 the programme was also offered to women aged 70-75 years. The attendance rate is over 80% in the northern part of The Netherlands. The introduction of the screening programme has led to a substantial decrease in the rate of advanced breast carcinoma and to a breast cancer mortality decline of almost 30% in the screened and non-screened population.[2] Despite the participation in the screening programme, a number of women still present with a clinically symptomatic carcinoma between two screening moments, a so-called interval carcinoma. In participants in the Dutch Breast Cancer Screening Programme, 36% of the tumours emerge as interval carcinomas[3] and there is discussion if interval carcinomas differ from screen-detected tumours biologically and should therefore deserve a different, perhaps more aggressive treatment.[4] Over the years many studies have been conducted on the differences between interval- and screen detected carcinomas.[5-16] Comparison of these studies is difficult, because of the great heterogeneity in screening group, screening interval and study design. Therefore in this analysis a very homogeneous group of postmenopausal women is studied. All women presented with an invasive ductal carcinoma and participated in the breast screening programme (as confirmed by the Northern Netherlands Comprehensive Cancer Centre). The patients' screening mammograms were re-reviewed in order to differentiate between 'true' interval carcinomas and false negative mammograms (missed carcinomas). We studied the expression of conventional tumour progression related biological markers (estrogen receptor (ER), progesterone receptor (PR), HER2/neu and p53), radiological characteristics (breast density, tumour outlining and calcifications) and follow-up data hypothesizing that if 'true' interval carcinomas are indeed a more aggressive subgroup of carcinomas, these variables differ between both groups.

Material and methods

Definitions

Screen detected carcinoma: a carcinoma detected in the screening programme.

'Missed' carcinoma: a clinically detected carcinoma that occurred between two screening moments with a visible lesion on re-reviewing of the screening mammogram.

Interval carcinoma: a clinically detected carcinoma that occurred between two screening moments after a 'true' negative screening mammogram.

Patients

99 consecutive post menopausal women from who participation in the biannual breast screening programme could be confirmed by the Northern Netherlands Comprehensive Cancer Centre and who were treated between 01-01-1996 and 31-12-2001 at the University Medical Centre Groningen for a primary operable invasive ductal carcinoma of the breast as defined by the WHO classification[17] were retrospectively included in this study. Seven patients were excluded; one patient because she was already in clinical follow-up for an earlier in-situ lesion and six patients because their screening mammograms were not available for re-reviewing. Therefore the Patient and tumour characteristics and data on follow-up were obtained retrospectively from hospital records and are summarized in table 1. Histology was reviewed on the original hematoxylin- and eosin stained section. The median follow-up was 61 months (range 6.3 – 106.4). Follow-up was performed according the follow-up guidelines of the Northern Netherlands Comprehensive Cancer Centre and consisted of a yearly mammogram in the first five years of follow-up and clinical examination (quarterly in the first year of follow-up, biannually in the second year and annually the third to fifth year. After 5 years patients are referred back to the screening programme.[18] During follow-up 4 patients developed a local recurrence after a median follow-up of 26.7 months. 11 patients developed distant metastasis after a median follow-up of 27.3 months. In total 14 patients presented with a relapse with a median relapse free survival of 26.2 months. 5 patients died related to breast cancer with a median overall survival of 28.6 months.

Re-reviewing of mammograms

The original screening mammogram of all patients was re-reviewed by two of the authors (GJdH and RP), who are both experienced screening radiologists, in order to differentiate between 'true' interval carcinomas and interval carcinomas as a result of a false negative screening mammogram (missed carcinomas). A consensus reading was performed. The following criteria were used: type 1, nothing to be seen; type 2, minimal signs only in retrospect; type 3, significant abnormality. Type 3 tumours were considered missed carcinomas. There was a maximum bias because both radiologists knew the inclusion criteria and question of the study. Breast density was scored on clinical mammograms by one of the authors (RP) using the Breast Imaging Reporting and Data Systems (BIRADS) classification for breast density.[19]

Tissue Microarray Construction

From the patient's tumour paraffin block, three 0.6 mm core samples of the most representative tumour area were included in a tissue microarray. The technique of tissue microarray production has been described and validated for breast carcinoma by others.[20; 21] In brief, the most representative tumour area was marked on the original hematoxylin- and eosin stained section. Using this section as an orientation, three 0.6 mm core punches were taken from the selected area in the donor blocks and mounted in a recipient block, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD, USA).

Immunohistochemistry

Immunohistochemistry for ER, PR, Her2/neu and p53 was performed on sections from the tissue array. The antibodies and antigen retrieval methods used are summarized in table 2. The immunostaining protocol was as follows: sections were deparafinized in pure xylene, rehydrated in decreasing concentrations of ethanol and washed in distilled water. Antigen retrieval was performed. The endogenous peroxidase reaction was blocked by incubating in 3% perhydrol for 30 minutes. The primary antibody diluted in PBS containing 1% Bovine Serum Albumin (BSA) was incubated for one hour,

after which the secondary (biotinylated rabbit anti mouse, DAKO, 1:100 diluted in PBS containing 1% BSA and 1% AB-serum) and tertiary (biotinylated swine anti rabbit, DAKO, 1:100 diluted in PBS containing 1% BSA and 1% AB-serum) antibodies were incubated for 30 minutes each. Visualisation was performed using the diaminobenzidine tetrahydrochloride / peroxidase reaction. Counterstaining was performed using haematoxylin. Sections were dehydrated using rising concentrations of alcohol and were mounted.

Evaluation of immunohistochemistry

Antibody staining was scored by one investigator (BvdV), under supervision of an experienced breast pathologist (JW), who randomly verified the scoring. ER, PR and p53 were graded based on the percentage of tumour cells showing positive nuclear staining. ER and PR were considered positive if nuclear staining was present in >10% of the cells, and p53 was considered positive in case of a substantial percentage of positively stained nuclei (>30%). Her-2/neu expression was graded as recommended by the HercepTest™ scoring guidelines: 0: no staining at all or membrane staining in <10% of the tumour cells; 1+: a faint/barely perceptible partial membrane staining in >10% of the tumour cells; 2+: weak to moderate complete membrane staining in >10% of the tumour cells; 3+: strong complete membrane staining in >10%. Her-2/neu was considered to be overexpressed if the score was 3+.

Data analysis

Data analysis was performed using the SPSS 14.0.2 statistical package (SPSS inc., Chicago, IL, USA). An univariate logistic regression analysis was performed to assess the odd ratios (OR) of clinico-pathological variables and biomarkers in 'true' interval carcinoma versus screen detected- / missed carcinoma. All parameters with an OR of 3.0 or higher in the univariate logistic regression analysis were then entered into a stepwise multivariate logistic regression analysis. A Cox regression analysis was performed to assess relapse free- and overall survival in 'true' interval carcinoma versus screen detected- / missed carcinoma.

Results

Tissue cores from all cases were successfully included in the TMA. Immunohistochemistry was assessable in 90 cases (98,0%) for p53, in 88 (96,0%) for Her2/neu and ER, and in 87 cases (95%) for PR.

The results from the re-reviewing of the mammograms are shown in Table 1. 16 of the 23 cases marked as interval carcinomas, were retrospectively 'true' interval carcinomas. 7 cases showed a retrospectively visible lesion on the screening mammogram. Most of those lesions (5/7) were now classified as 'uncertain benign' where they had earlier been classified as 'benign'. Two cases were now classified as 'malignancy suspected'. One of those cases had originated in very dense breast tissue which might have caused the judgement error. A clinical mammogram was available in 88 cases (96%). Breast density was evenly distributed between 'true' interval carcinomas and screen detected- / missed carcinomas.

Univariate analysis

The results of univariate logistic regression analyses of clinico-pathological parameters and biomarkers in 'true' interval- versus screen detected- / missed carcinomas are shown in table 3. Most parameters did not differ between both groups. 'True' interval carcinomas were significantly larger (OR 7.2, 95% CI 1.8-28.1, p=0.005) and were less often ER positive (OR 0.3, 95% CI 0.08-0.9, p=0.034). A trend towards PR negativity (OR 0.3, 95% CI 0.1-1.0, p=0.06) was found in 'true' interval carcinomas.

Multivariate analysis

Table 4 shows the results of a multivariate analysis. Tumour size was significantly associated with 'true' interval carcinoma (HR 5.1, 95% CI 1.2-21.0, p=0.02). A trend towards ER negativity was also found (HR 0.3, 95% CI 0.08-1.2, p=0.08). PR was eliminated from the equation.

Clinical outcome

The Cox regression analysis showed a trend towards decreased relapse free survival in 'true' interval carcinomas (table 5). No difference in overall survival was found.

Discussion

This study compared clinical, pathological and radiological variables, the expression of conventional biomarkers and follow-up data of 'true' interval carcinomas versus screen detected- and missed carcinomas, hypothesizing that 'true' interval carcinomas express parameters of aggressive behaviour more abundantly. 'True' interval carcinomas were larger and showed a trends towards ER negativity and decreased relapse free survival.

In table 6 the results from a literature search on studies assessing differences between interval- and screen detected breast carcinomas conducted in postmenopausal women are shown. When comparing the results from the studies that defined 'true' interval carcinoma with the current study our finding of increased tumour size and decreased ER expression in 'true' interval carcinoma confirms some of the results of those studies. Some studies also found differences in the number of positive axillary lymph nodes and tumour grade, findings that we could not confirm. Those findings were never confirmed in multivariate analysis however.

Several restrictions apply when comparing studies conducted on the differences between interval- and screen detected carcinomas. First, there is large heterogeneity in study groups, screening interval, type of breast cancer studied and study design. Second, most studies, including the current one, comprise a small study group. One might argue that these study groups are too small and heterogeneous to gain sufficient statistical power to find differences between screen detected and interval carcinomas. To avoid heterogeneity in type of breast carcinomas and the patient population studied, we focused on postmenopausal women in the screening programme, presenting with an invasive ductal carcinoma as defined by the WHO classification[17], as this is by far the most common type of breast cancer. Third, most studies use univariate logistic regression analysis to study differences, making their findings more susceptible to biases.[22] Therefore we performed a stepwise multivariate logistic regression analysis to correct for confounding factors. Fourth, the definition of an interval carcinoma differs between studies. Some authors define all carcinomas detected clinically between two screening

moments as an interval carcinoma. This is a correct definition of interval carcinoma when looking at the sensitivity of the screening programme as a whole. A portion of those interval carcinomas, however, are in fact significant lesions that should have been referred. These tumours are not detected in the interval between two screening moments because of their biological behaviour, but due to restrictions of the screening programme. Therefore, in the current study screening mammograms were re-reviewed in order to differentiate between 'true' interval carcinomas and interval carcinomas as a result of a false negative screening mammogram. We defined an interval carcinoma as a clinically detected carcinoma that occurred between two screening moments after a 'true' negative screening mammogram. Using this definition of interval carcinoma the sensitivity of the screening mammogram as a test for detecting breast carcinoma can be assessed. The programme sensitivity of the Dutch screening programme was 65% in the nationwide evaluation of the programme (meaning that for every two carcinomas discovered in the screening programme in the two years between screening moments another carcinoma is discovered clinically). In our series the programme sensitivity was 75% (using the first definition described above). It is plausible that this difference is caused by the selection of the study group (only invasive ductal carcinomas were included). The Dutch National Evaluation Team Breast Cancer Screening (LETB) has estimated that in general (ductal carcinoma in situ and lobular carcinoma included) from all the interval carcinomas approximately 50% is a 'true' interval carcinoma. 25% of the interval carcinomas show a clear lesion, for which a patient should have been referred for additional diagnostics, on re-reviewing of the screening mammography, and a further 25% show 'minimal signs', that are only suspicious with the knowledge of a clinically discovered interval carcinoma. The percentage of 'true' interval carcinomas in this series was 30.4%, which is comparable to those estimations.

'True' interval carcinomas were five times more often larger sized (>2 cm) tumours in our series. Several explanations for the increased size of interval carcinomas have been suggested in literature. First, interval carcinomas have been associated with dense breast tissue, with poor outlining, and with absence of calcifications on mammography.[12; 23; 24] When tumours from women

with dense breasts become clinically apparent after a negative screening mammogram, they are more ahead in their natural history compared to screen detected carcinomas and are therefore larger, a phenomenon called lead time bias.[25] In our series breast density was evenly distributed between screen detected- / missed carcinomas and 'true' interval carcinomas. Second, several studies suggest that interval carcinomas are rapidly proliferating tumours. Several different parameters for proliferation are used in these studies. Two studies found increased mitotic count and Ki-67 antigen expression in interval carcinomas.[7; 10] One of those studies however was performed a heterogeneous group of breast carcinomas, including lobular carcinomas, which may have confounded the results of this study somewhat. Ki-67 immunohistochemistry was not performed in this series, because this expression is very heterogeneous in ductal breast carcinoma and therefore difficult to interpret on TMA. Others used fraction of tumour cells in the S-phase fraction of the cell-cycle as a marker for proliferation and found an increase of this fraction in interval carcinomas.[11; 13] In this study we used the Bloom-Richardson scoring system, of which mitotic count is a part, to assess grade of differentiation as a marker for proliferation.[26] We did not find a difference between both groups of this study. There are other biological factors outside proliferation rate that may play a role in the development of breast carcinoma and may explain the difference in size between 'true' interval- and screen detected- /missed carcinoma. For example, tumour cells from 'true' interval carcinomas may be less susceptible to apoptosis. One study assessing the number of apoptotic cells in interval- and screen detected carcinomas did not find a difference between both groups.[10] In addition, tumours that have a higher angiogenic potential may grow faster due to reduced tumour cell death. Another key role player is the amount of tumour stroma induced which will affect significantly the size of tumours, especially of invasive ductal carcinomas which are known for their highly variable desmoplasia inducing potential[27]. It remains to be established if and to which extent these explanations contribute to the difference in growth rate between screen detected carcinomas / missed carcinomas and 'true' interval carcinomas.

It is assumed that 50 to 75% of the tumours discovered in the first screening round are small, biologically indolent tumours.[28-30] These tumours are more

often ER and PR positive. We found that screen detected- / missed carcinomas were significantly more often ER positive in univariate analysis, a result that is confirmed by other studies.[7] This association only showed a trend towards significance in multivariate analysis, probably due to the relatively small sample size of this study. These relatively less aggressive tumours might also explain the trend towards increased relapse free survival we found for screen detected carcinomas.

In conclusion, in this small consecutive and homogeneous study group of postmenopausal women with invasive ductal breast carcinoma we found a significant difference in tumour size between 'true' interval- versus screen detected- / missed carcinomas in multivariate analysis. ER expression differed significantly between both groups in univariate analysis. It remains challenging however to find parameters that determine this difference between the 'true' interval carcinomas and screen detected lesions.

Tables

Table 1. patient and tumour characteristics.

	n	%
Age at diagnosis		
median (range)	60.2	50.2 – 74.8
Detection		
screen detected	69	75
interval ('true')	16	17.4
missed	7	7.6
Mammographic breast density on diagnostic mammography (BIRADS)		
I	30	32.6
II	19	20.7
III	23	25.0
IV	16	17.4
Calcifications on screening mammography		
none	63	68.5
cluster	6	6.5
linear	1	1.1
granular	18	19.6
linear + granular	3	3.3
branching	1	1.1
Outlining on screening mammography		
not visible	22	24.2
sharp	5	5.5
unsharp	39	42.9
spiculae	22	24.2
unsharp + spiculae	3	3.3
Therapy		
BCT	54	58.7
mastectomy	38	41.3
Axillary nodal status		
negative	61	66.3
positive	30	32.6
not assessed	1	1.1
Pathological tumour size		
<2 cm	52	56.5
>2-<5 cm	34	37.0
>5 cm	6	6.5
Grade of differentiation		
well	26	28.3
moderate	43	46.7
poor	22	23.9
missing	1	1.1

	N	%
Adjuvant chemotherapy		
yes	30	32.6
no	62	67.4
Adjuvant radiotherapy		
yes	63	68.5
no	29	31.5

n: number of cases; %: percentage; BCT: breast conserving therapy; cm: centimetre.

Table 2. Antibodies and antigen retrieval methods.

ANTIBODY	CLONE	SUPPLIER	DILUTION	ANTIGEN RETRIEVAL
ER	6F11	Ventana	*	Tris/HCL 0.1 M (pH 9.5) 30' 98°C microwave
PR	1A6	Ventana	*	Tris/HCL 0.1 M (pH 9.5) 30' 98°C microwave
Her2/neu	CB11	Ventana	*	Tris/HCL 0.1 M (pH 9.5) 30' 98°C microwave
p53	BP-53-12-1	Biogenix	1:800	Tris/HCL 0.1 M (pH 9.5) 30' 98°C microwave

**prediluted by supplier.*

Table 3. Univariate logistic regression analysis of clinico pathological variables and biological markers in 'true' interval carcinoma vs screen detected- / missed carcinoma.

	'True' interval carcinoma OR (95% CI)	P
Age at diagnosis (n=92)	1.0 (0.9-1.1)	0.808
Mammographic density (BIRADS) (n=88)		
I	1	
II	0.5 (0.1-2.6)	0.389
III	0.6 (0.1-2.7)	0.507
IV	1.3 (0.3-5.6)	0.696
Mammographic calcifications (n=92)		
yes	1	
no	1.5 (0.4-5.0)	0.538
Treatment (n=92)		
BCT	1	
mastectomy	1.5 (0.5-4.5)	0.439
Axillary nodal status (n=91)		
negative	1	
positive	2.4 (0.8-7.2)	0.117
Tumour size (n=88		
<2 cm	1	
>2 cm	7.2 (1.8-28.1)	0.005
Grade of differentiation (n=91)		
well	1	
moderate	0.4 (0.1-1.8)	0.244
poor	2.0 (0.5-7.4)	0.320
Estrogen receptor (n=88)		
negative	1	
positive	0.3 (0.1-0.9)	0.034
Progesterone receptor (n=87)		
negative	1	
positive	0.3 (0.1-1.0)	0.06
Her2/neu (n=88)		
0/1/2	1	
3	1.6 (0.3-8.6)	0.603
P53 ((n=90)		
negative	1	
positive	1.6 (0.2-16.2)	0.701

HR: Hazard Ratio; 95% CI: 95% Confidence Interval; SD: standard deviation; BCT: breast conserving therapy; cm: centimetre; p: significance.

Table 4. Independent predictors of 'true' interval carcinoma.(n=82).

Parameters	OR	95% CI	p-value
Tumour size			
<2 cm	1		
> 2cm	5.1	1.2-21.0	0.02
Estrogen receptor			
negative	1		
positive	0.3	0.1-1.2	0.08

Regression analysis by elimination of variables in a stepwise manner.

OR: odds ratio; 95% CI: 95% confidence interval; cm: centimetre.

Table 5. Relapse free and overall survival for 'true' interval carcinoma vs screen detected- / missed carcinoma.

	HR	95% CI	p-value
Relapse Free Survival(n=91)			
SD/M carcinoma	1		
'true' interval carcinoma	1.7	0.9-3.1	0.08
Overall Survival(n=88)			
SD/M carcinoma	1		
'true' interval carcinoma	1.3	0.1-11.6	0.82

Cox regression analysis.

HR: hazard ratio; 95% CI: 95% confidence interval; SD/M: screen detected- and missed carcinomas.

Table 6. Significant differences between interval- and screen detected tumours from literature.

AUTHOR (YEAR)	NUMBER OF SCREEN DETECTED CARCINOMAS	NUMBER OF INTERVAL CARCINOMAS	AGEGROUPS	SCREENING INTERVAL (YEARS)	'TRUE' INTERVAL CARCINOMA?	ANALYSIS (UNIVARIATE / MULTIVARIATE)	SIGNIFICANT DIFFERENCES
DeGroote[8] (1983)	99	21	30-80	1	yes	univariate	number of positive axillary lymph nodes overall mortality 6 year survival
Heuser[12] (1984)	32	28	-	1	no	univariate	calcifications on mammography age survival
Frisell[9] (1987)	222	60	40-64	2	yes	univariate	tumour stage ER expression
Hatschek[11] (1989)	212	98	40-74	2	no	univariate	S-phase fraction
Bahnsen[5] (1994)	163	22	36-75	2	no	univariate	number of positive axillary lymph nodes
Burrell[6] (1996)	267	82	50-64	varying	yes	univariate	tumour size tumour grade number of positive axillary lymph nodes
Klemi[13] (1997)	385	100	40-74	varying	no	univariate[1]	S-phase fraction age stage
Crosier[7] (1999)	84	51	50-64	3	yes	univariate	tumour size tumour grade ER expression Her2/neu expression p53 expression ki-67 expression
						multivariate	ki-67 expression Her2/neu expression

AUTHOR (YEAR)	NUMBER OF SCREEN DETECTED CARCINOMAS	NUMBER OF INTERVAL CARCINOMAS	AGEGROUPS	SCREENING INTERVAL (YEARS)	'TRUE' INTERVAL CARCINOMA?	ANALYSIS (UNIVARIATE / MULTIVARIATE)	SIGNIFICANT DIFFERENCES
Porter[14] (1999)	279	150	40->80	varying	no	univariate[2]	mitotic count tumour grade ki-67 expression ER expression
Gilliland[10] (2000)	64	63	40-80	varying	no	univariate multivariate	proportion of proliferating cells p53 expression number of apoptotic cells p53 expression ki-67 expression
Raja[15] (2001)	625	230	50-64	3	yes	univariate	tumour grade tumour size number of positive axillary lymph nodes
Shen[16] (2005)	712	280	40-64	1	no	multivariate	breast cancer related death
current study	63	36	50-74	2	yes	univariate multivariate	tumour size ER expression tumour size

[1] *adjusted for tumour size.*
[2] *adjusted for age and tumour size.*

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7

VALIDATION OF THE 4B5 RABBIT MONOCLONAL ANTIBODY IN DETERMINING HER2/NEU STATUS IN BREAST CANCER

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van der Vegt B[1]
de Bock GH[2]
Bart J[1]
Zwartjes NG[1]
Wesseling J[3]

Departments of Pathology[2] and Epidemiology[2] University Medical Center Groningen, University of Groningen, and Department of Pathology, The Netherlands Cancer Institute/ Antoni van Leeuwenhoek Hospital, Amsterdam[3], The Netherlands

Abstract

HER2 overexpression in breast cancer is associated with worse clinical outcome. To select patients for anti-Her2 based therapy immunohistochemistry is commonly performed as a first step to assess Her2 status. However, interobserver and interlaboratory variability can compromise adequate assessment of Her2 status significantly. In addition, immunohisto-chemistry does not always result in an unambiguous test result requiring additional testing for Her2 gene amplification. This study aimed to improve the reliability of Her2 immunohisto-chemistry by using rabbit monoclonal antibody 4B5 as an alternative of mouse monoclonal antibody CB11 routinely used in our laboratory. Therefore 283 breast adeno-carcinomas were included in a tissue microarray. Immunohistochemistry using the 4B5 and CB11 antibodies, and fluorescence- and chromogenic in situ hybridisation (FISH or CISH) were performed. Immunohistochemistry was scored by two independent investigators. We found that 4B5 staining was more distinct than CB11 staining. For the CB11 staining there were 12% (BV) and 5% (JW) 2+ scores compared to 4% (BV) and 2% (JW) for 4B5. There was a strong trend towards higher interobserver agreement for 4B5 compared to CB11 (4B5: κ 0.87, 95% CI 0.79–0.96; CB11: κ 0.77, 95% CI 0.66 – 0.88). There were no significant differences in sensitivity, specificity, and predictive values between CB11 and 4B5. Our results indicate that the 4B5 antibody provides more robust assessment of immunohistochemical Her2/neu status and will reduce the number of gene amplification tests compared to CB11. However, for tumours with a 2+ score additional gene amplification measurement using FISH or CISH remains necessary.

Introduction

The status of the Her2 receptor is an important factor in prognosis and treatment choice in primary breast carcinoma.[1] Mortality and recurrence are reduced in patients with Her2/neu positive breast carcinoma that receive adjuvant therapy with both the humanized anti-Her2/neu monoclonal antibody therapy trastuzumab and chemotherapy.[2-5]

Trastuzumab therapy however is associated with cardiotoxicity, in 2 to 4.7% percent of patients when used as monotherapy, but in up to 27% when given concomitantly with anthracycline and cyclophosfamide therapy.[6] Therefore this treatment is only given to patients with confirmed Her2/neu positive breast carcinoma and adequate left ventricle ejection fraction. Several methods are used to assess Her2/neu status in breast cancer. Measurement of gene amplification using fluorescence in situ hybridisation (FISH) or chromogenic in situ hybridisation (CISH) is considered to be the 'gold standard' in the assessment of Her2/neu status.[7-9] However the most commonly used first line method to determine Her2/neu status is immunohistochemistry. Immunohistochemistry is relatively inexpensive and a routinely used technique in pathology laboratories which makes it easy to implement. Immunohistochemistry results in a Her2/neu score ranging from 0 (no expression) to 3+ (strong complete tumour cell membrane expression).[10] This semi-quantitative scoring system does not always result in a clear positive or negative Her2/neu amplification status. It is generally agreed that when a Her2 score is ambiguous (2+ score) a gene amplification measurement has to be performed. [7; 11; 12] Another setback of Her2 immunohistochemistry is the significant interobserver variation and poor interlaboratory reproducibility.[13-16] Since accurate diagnostic assessment of HER2/neu is essential for the appropriate application of trastuzumab containing treatment regiments, the concordance between immunohistochemistry and the 'gold standard' gene amplification assessment needs to be as high as possible. Therefore immunohistochemistry staining should be improved to optimize accurate estimation of the HER2/neu status.

In recent years, rabbit monoclonal antibodies have been developed which show higher affinity and specificity than mouse monoclonal or rabbit polyclonal antibodies resulting in more reliable staining results.[17; 18] To improve

Her2 immunohistochemistry reliability in our laboratory we tested the potential of the rabbit monoclonal antibody 4B5 directed against Her2/neu as an alternative to the mouse monoclonal CB11 directed against Her2/neu using both a CISH array for Her2/neu and a Her2/neu FISH array as reference.

Materials and Methods

Patients

To determine the size of the series, a power analysis was performed. In this power analysis, we considered HER2/neu immunohistochemistry negative if the staining pattern and intensity is equivalent to score '0' or '1+' and positive if it is equivalent to score '3+'. 2+ cases are not taken into account in this power analysis, because they are clinically uninformative. We assumed 10% 2+ cases. We also assumed that the rabbit monoclonal antibody has a better sensitivity and specificity than the mouse mouse monoclonal antibody CB11. Furthermore a loss of 20% cases due to unavailability of tumour material or uninterpretability of one or more of the stainings was assumed. Based on these assumptions, a sample size of 280 was required to achieve 90% power to detect an odds ratio of 3.000 using a two-sided McNemar test with a significance level of 0.05000. The odds ratio is equivalent to a difference between two paired proportions of 0.100 which occurs when the proportion T_N negative versus T_0 positive is 0.150 and the proportion T_N positive versus T_0 negative is 0.050. 283 consecutive female patients treated for a primary operable invasive carcinoma of the breast at the University Medical Center Groningen between January 2002 and December 2005 were included in this study. Afterwards 8 patients were excluded because no representative material was available in the tissue microarray blocks. The analyses have been performed on the resulting group of 275 patients. Patient and tumour characteristics were obtained retrospectively from hospital records and are summarized in table 1.

Tissue microarray construction

From the patient's tumour paraffin block, three 0.6 mm core samples of the

most representative tumour area were included in a tissue microarray. The technique of tissue microarray production has been described and validated for breast carcinoma by others.[19; 20] In the latter study authors showed that the concordance between the tissue microarray with the whole tissue sections was over 97% percent if three 0.6 mm core samples per tumor were included in the TMA. In brief tissue microarrays were compiled as follows: the most representative tumour area was marked on the original hematoxylin- and eosin (H&E) stained section. Using this section as an orientation, three 0.6 mm core punches were taken from the selected area in the donor blocks and mounted in a recipient block, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD, USA). In total seven tissue microarray blocks, each containing tumour cores from 40 to 50 patients, were made. Using a standard microtome 3µm sections were cut from these tissue microarray blocks.

Immunohistochemistry, FISH and CISH

Immunohistochemistry for rabbit monoclonal antibody 4B5 (PATHWAY® HER-2/neu (4B5) rabbit monoclonal antibody, Ventana Medical Systems, Illkirch, France) and mouse monoclonal antibody CB11 (PATHWAY® HER-2/neu (CB11) mouse monoclonal antibody, Ventana Medical Systems, Illkirch, Cedex, France) was performed on the tissue microarray sections using the automated Benchmark® platform (Ventana Medical Systems, Illkirch, Cedex, France) and according to the manufacturers recommendations. FISH (PathVysion HER-2 DNA Probe Kit, Vysis Inc., Downers Grove, IL, USA) and CISH (SPoT-Light® HER2 CISH™ Kit, Zymed, Carlsbad, CA, USA) assays were performed according the manufacturers recommendations.

Evaluation of immunohistochemistry, FISH and CISH

Scoring of the CB11 and 4B5 immunohistochemistry stainings was performed independently by an experienced pathologist (JW) and a senior resident (BV). Her-2/neu expression was graded as recommended by the HercepTest™ scoring guidelines: 0: no staining at all or membrane staining in ‹10% of the tumour cells; 1+: a faint/barely perceptible partial membrane staining in ›10% of the tumour cells; 2+: weak to moderate complete membrane staining in ›10% of

the tumour cells; 3+: strong complete membrane staining in >10%.

FISH was scored according the ASCO guidelines: the ratio of 20 cells was calculated. A ratio <1.8 was considered negative, a ratio >2.2 was considered positive. For the equivocal cases another 20 cells were counted. In these cases a ratio .2 was considered positive. A ratio <2 was considered negative. For CISH, tumours with at least 5 signals in more than 50% percent of the tumour cells were considered positive.

Data analysis

All statistical analyses were performed using the SPSS 14.0 software package.

The feasibility of both staining methods was compared. The number of informative test results and the number of 2+ scores were calculated for each of the staining methods. Agreement between immunohistochemistry results and in-situ hybridisation results were calculated in a cross tabulation using a Pearson chi-square test. Sensitivity and specificity were calculated using two methods, including and excluding 2+ scores. In the first method 2+ scores were considered a positive test result. This method is most commonly used in literature and was performed to compare our results to those found in other studies. 2+ scores are however clinically uninformative and were therefore eliminated from analysis in the second method. The positive predictive value (PPV) was calculated by dividing the number of cases is the immunohistochemistry 3+ group with amplification on FISH or CISH by the total number of cases with an immunohistochemistry 3+ score. The negative predictive value (NPV) was calculated by dividing the number of cases immunohistochemistry score 0 or 1+ without amplification on FISH or CISH by the total number of patients with an immunohistochemistry 0 or 1+ score. For sensitivity, specificity and predictive values a 95% CI interval was calculated. Interobserver agreement was calculated in a cross tabulation using Cohen's kappa test.

Results

Feasibility

In figure 1 an example of both a 4B5 and a CB11 staining for 1 case is shown. In general the 4B5 staining was more distinct compared to the CB11 staining. Also the 4B5 staining showed less non-specific cytoplasmic staining.

Test characteristics

Of the 275 cases included 262 (95%) of the cases were adequately represented in the tissue microarray. Immunohistochemistry could be evaluated in 83% (JW) and 90% (BV) of the cases for 4B5, in 87% (JW) and 91% (BV) of the cases for CB11. Differences in evaluation percentages between authors have been caused by disagreement on the overall percentage of tumour available in the core punches and the relative percentages of invasive tumour vs. in-situ tumour available in the core punches. FISH could be evaluated in 90% and CISH in 92% of the cases.

Agreement

In 230 of the 240 assessable cases (96%) there was agreement between FISH and CISH scoring (κ 0.837, 95% CI 0.737 – 0.937) . There was an agreement of 80% (BV) and 81% (JW) between the scoring results of 4B5 and CB11 (table 2). The disagreement between scores is mainly caused by the 2+ scores. There was a reduction of more than 50% for the number of cases scored as 2+ when comparing CB11 with 4B5; respectively 28 cases (12%) vs 13 cases (4%) (BV) and 12 cases (5%) vs 5 cases (2%) (JW). When excluding the 2+ cases from analysis there was no difference between both antibodies for the classification of cases as amplified or not amplified (McNemar's test p=1.0, data not shown) e.g. there would be no clinical consequences when using either of the antibodies. In tables 3 and 4 the results for the concordance between immunohistochemistry and respectively FISH and CISH are shown. Sensitivity (including and excluding 2+ scores), specificity (including and excluding 2+ scores), PPV and NPV of 4B5 and CB11 using both FISH and CISH as reference are shown in table 5. Sensitivity, specificity, PPV and NPV did not show any significant differences between both antibodies and between both observers. However, there was a near significant difference in specificity (including 2+

scores) for the lesser experienced observer (BV) between 4B5 and CB11: (4B5: 0.99, 95% CI 0.95-1.0; CB11: 0.93, 95% CI 0.88-0.96). Although not significant, there was a trend towards an increased concordance between observers for 4B5 compared to CB11 (4B5: κ 0.87, 95% CI 0.79– 0.96; CB11: κ 0.77, 95% CI 0.66 – 0.88) (Table 6).

Discussion

In this study we tested the potential of the 4B5 anti Her2 rabbit monoclonal antibody by comparing it to the CB11 anti-Her2 mouse monoclonal antibody on a consecutive series of invasive breast adenocarcinomas using both FISH and CISH as reference. We found that 4B5 staining was more distinct and showed less nonspecific cytoplasmic background staining, which led to a more than 50% reduction of the number of 2+ scores for 4B5 compared to CB11. In this well powered study we found no significant differences in sensitivity, specificity, predictive values and interobserver concordance between 4B5 and CB11. However there was a trend towards a higher specificity for 4B5 for one of observers (BV) and a trend towards an increased interobserver concordance for 4B5. The – non-significant - differences in the test results of both observers would have no consequences for clinical decision making regarding the Her2 status of the carcinomas investigated.

Gene amplification measurement is considered to be the 'gold standard' for Her2/neu status assessment[7-9] although it suffers from variability between institutes[21; 22] and the sensitivity varies between detection methods used.[9] Traditionally gene amplification measurement has been performed using FISH assays for Her2/neu gene amplification. This method however has several disadvantages: FISH is expensive, is time demanding[8], is not readily accommodated in most pathology laboratories and is accompanied by technical challenges[23]. More recently CISH assays have been developed. Instead of the fluorogens used in FISH, this technique uses chromogens. This has several advantages[24]: where FISH requires a fluorescence microscope for interpretation, CISH can be interpreted using a normal bright field microscope. CISH allows analysis of tumour morphology, making it possible to interpret tumour

heterogeneity and gene copy number in different components of the tumour (an invasive and an in-situ component). Also FISH signals are labile and fade over time, but CISH produces a permanent staining.

Many authors have compared CISH with FISH (reviewed in 24). Most studies find an agreement of both methods of more than 90 percent. In a multicenter study, where pathology laboratories blindly performed CISH on cases from each other this method was validated.[25] That study also reported an intra- and interobserver agreement of over 90 percent and concluded that CISH is a viable alternative to FISH. In the current study the agreement between FISH and CISH was 96 percent, which is in line with the conclusions of those previous studies. Of the 10 discordant cases in our series 5 cases contained an in situ component. This tumour heterogeneity might have led to the discordance because distinction between the invasive and the in-situ component can be difficult in FISH where tumour morphology is not readily recognized in all cases.

Most pathology laboratories use immunohistochemistry to assess Her2/neu status. Immunohistochemistry is not only less expensive than in situ methods, there is also much experience with immunohistochemistry, which makes it a method that is easily implemented in the daily practice of pathology laboratories. Immunohistochemistry however, has several disadvantages: immunohistochemistry is scored semiquantitively which leads to a 0 – 3+ score rather than to a clear amplification / no amplification outcome. Scores 0 and 1+ are considered non-amplified and score 3+ is considered amplified. An ideal Her2 antibody has a low number of indeterminate (2+) cases and a high PPV and NPV. We found that 4B5 staining compared to CB11 was more distinct and showed less nonspecific cytoplasmic background staining. This has led to an increase in interobserver concordance (4B5: κ 0.87, 95% CI 0.79– 0.96; CB11: κ 0.77, 95% CI 0.66 – 0.88) that showed a trend towards significance. Interobserver concordance is essential because it has far reaching consequences for the choice of therapy regimen and response to therapy. A few studies have assessed interobserver concordance for CB11. Two of those studies found an interobserver concordance of 0.74 which is comparable to our results (0.77).[13; 15] In a study by Tsuda et al an interobserver concordance of only 0.29 was found for CB11. This study used a different method to

calculate concordance however. The concordance of 0.87 we found for 4B5 is considered almost perfect[26], which underlines that 4B5 is a safe method to assess Her2 status in breast cancer. To our knowledge no other studies have assessed interobserver concordance for 4B5. Interlaboratory concordance was not assessed in this study. One study mentions a perfect interlaboratory concordance on a very limited number of cases.[27] Future studies are needed to assess this issue.

In this series a cut-off of 10% staining was used for scoring Her2 immunohistochemistry. This cut-off was chosen based on Dutch guidelines, which differ from the ASCO guideline using a cut-off of 30%. However, this difference in cut-off did not cause a significant change in the results.

The number of cases that were scored as 2+ was lower for 4B5. For 4B5 observers scored 4% and 2% 2+ cases; for CB11 this was 12% and 5%. The number of CB11 2+ cases in this study is comparable the numbers in literature were the number of cases scored 2+ using an mouse monoclonal antibody ranges from 2 to 20.5%, and is usually is around 10%.[28] We expect that the introduction of 4B5 will reduce the number of FISH or CISH assays that will have to be performed, which leads to a reduction in costs. In this well powered study this decrease of indeterminate cases did not lead to a significant difference in sensitivity, specificity, or predictive power between CB11 and 4B5. Small numbers did not allow a statistically reliable subgroup analysis to assess differences in sensitivity, specificity and predictive values for the immunohistochemistry 2+ cases of both antibodies using FISH or CISH as a reference. In table 7 the results from other studies assessing sensitivity, specificity and predictive values of CB11 are shown. Most studies considered 2+ cases to be amplified for the calculations. As discussed before 2+ cases correlate with gene amplification very poorly.[7; 11; 12] We believe that 2+ cases should be excluded from analysis when assessing sensitivity, specificity and predictive values. The values for sensitivity, specificity and predictive values we found in this study are comparable to those found in literature. Powell et al performed a study comparing 4B5 with CB11 using FISH as a reference in two subsets of cases, one containing samples from a single institution, and one containing samples from a multicenter tissue bank.[27] In that study the levels of sensitivity were higher and the levels of specificity were lower than in the

current study. However, these differences were not significant for the subset best comparable to the current study group, i.e. the single institution subset. PPV and NPV show no significant difference between both antibodies, which underlines that the lower number of indeterminate cases which led to the increase in sensitivity and specificity did not cause a loss of predictive value of the test. In a group of 199 invasive breast cancers Egervari et al compared the 4B5 antibody with a number of antibodies including the CB11 antibody using FISH as a reference.[29] When recalculating their results using the method described earlier they a sensitivity (0.76 (0.5-0.92)), specificity (0.99 (0.96-1.0)), PPV (0.93 (0.64-1.0)) and NPV (0.97 (0.93-0.99)) that does not differ significantly to our results. Although they suggest a lower sensitivity of 4B5 compared to CB11 this result is not significant (table 7).

In conclusion we showed that the novel 4B5 rabbit monoclonal anti-Her2/neu antibody has a good agreement with both FISH and CISH and has a sensitivity, a specificity and predictive values comparable to CB11. We found a reduction of more than 50% in the number of indeterminate cases for 4B5 and an increase in interobserver concordance. Our results indicate that immunohistochemistry using the 4B5 antibody provides more robust and hence more reliable assessment of the Her2/neu status detection and will reduce the number of gene amplification tests compared to the CB11 antibody. However, for patients with a 2+ score additional gene amplification measurement using FISH or CISH remains mandatory.

Tables

Table 1. Patient and tumour characteristics.

	n (%)
Age at diagnosis	
median (range)	60 (29-90)
Menopausal status	
premenopausal	78 (28)
postmenopausal	197 (72)
Pathological tumour size (mm)	
median (range)	22 (1-140)
Pathological tumour type	
IDC	109 (40)
IDC and DCIS	110 (40)
ILC	25 (9)
ILC and LCIS	6 (2)
other	25 (9)
Grade of differentiation (invasive)	
well	82 (30)
moderate	114 (41)
poor	79 (29)

n: number of cases; %: percentage; mm: millimetre; IDC: invasive ductal carcinoma; DCIS: ductal carcinoma in situ; ILC: invasive lobular carcinoma; LCIS: lobular carcinoma in situ; other: other specific subtypes of invasive breast carcinoma.

Table 2. Correlation of 4B5 and CB11 for observer BV (A) and JW (B).

A

	CB11 BV				
4B5 BV		0	1	2	3	total
	0	149 (95)	20 (50)	4 (14)	0 (0)	173 (71)
	1	7 (4)	19 (48)	14 (50)	0 (0)	40 (16)
	2	1 (1)	1 (2)	8 (29)	0 (0)	13 (4)
	3	0 (0)	0 (0)	2 (7)	19 (100)	21(9)
	total	157 (100)	40 (100)	28 (100)	19 (100)	244 (100)
	χ^2: p=<0.001					

B

	CB11 JW				
4B5 JW		0	1	2	3	total
	0	106 (96)	28(35)	0 (0)	0 (0)	134 (60)
	1	5 (4)	51 (64)	6 (50)	0 (0)	62 (28)
	2	0 (0)	1 (1)	4 (33)	0 (0)	5 (2)
	3	0 (0)	0 (0)	2 (17)	20 (100)	22 (10)
	total	111 (100)	80 (100)	12 (100)	20 (100)	223 (100)
	χ^2: p=<0.001					

CB11: mouse monoclonal antibody CB11; 4B5: rabbit monoclonal antibody 4B5; BvdV: Bert van der Vegt; JW: Jelle Wesseling.

TABLE 3. COMPARISON OF IMMUNOHISTOCHEMISTRY WITH FISH.

		AMPLIFICATION	NO AMPLIFICATION	TOTAL			AMPLIFICATION	NO AMPLIFICATION	TOTAL
IHC CB11 BvdV	0	3 (8)	150 (74)	153 (64)	IHC CB11 JW	0	2 (5)	116 (59)	118 (51)
	1	2 (5)	38 (19)	40 (16)		1	5 (14)	78 (40)	83 (36)
	2	13 (35)	15 (7)	28 (12)		2	10 (27)	2 (1)	12 (5)
	3	19 (52)	0 (0)	19 (8)		3	20 (54)	0 (0)	20 (8)
	total	37 (100)	203 (100)	240 (100)		total	37 (100)	196 (100)	233 (100)
	χ^2: p=<0.001					χ^2: p=<0.001			
IHC 4B5 BvdV	0	5 (14)	165 (81)	170 (71)	IHC 4B5 JW	0	3 (8)	131 (69)	134 (60)
	1	4 (11)	36 (18)	40 (17)		1	7 (20)	57 (30)	64 (28)
	2	7 (19)	3 (1)	10 (4)		2	4 (11)	1 (1)	5 (2)
	3	21 (56)	0 (0)	21 (8)		3	22 (61)	0 (0)	22 (10)
	total	37 (100)	204 (100)	241 (100)		total	36 (100)	189 (100)	225 (100)
	χ^2: p=<0.001					χ^2: p=<0.001			

IHC: immunohistochemistry; CB11: mouse monoclonal antibody CB11; 4B5: rabbit monoclonal antibody 4b5; BvdV: Bert van der Vegt; JW: Jelle Wesseling.

TABLE 4. COMPARISON OF IMMUNOHISTOCHEMISTRY WITH CISH.

		AMPLIFICATION	NO AMPLIFICATION	TOTAL			AMPLIFICATION	NO AMPLIFICATION	TOTAL
IHC CB11 BvdV	0	2 (6)	156 (76)	158 (65)	IHC CB11 JW	0	1 (3)	121 (61)	122 (52)
	1	4 (11)	35 (17)	39 (16)		1	6 (17)	74 (37)	80 (34)
	2	11 (31)	15 (7)	26 (11)		2	9 (25)	3 (2)	12 (5)
	3	19 (52)	0 (0)	19 (8)		3	20 (55)	0 (0)	20 (9)
	total	36 (100)	206 (100)	242 (100)		total	36 (100)	198 (100)	234 (100)
	χ^2: p=<0.001					χ^2: p=<0.001			
IHC 4B5 BvdV	0	3 (8)	171 (83)	174 (71)	IHC 4B5 JW	0	2 (6)	134 (71)	136 (60)
	1	6 (17)	33 (16)	39 (16)		1	9 (25)	53 (28)	62 (28)
	2	6 (17)	3 (1)	9 (4)		2	3 (8)	2 (1)	5 (2)
	3	21 (58)	0 (0)	21 (9)		3	22 (61)	0 (0)	22 (10)
	total	36 (100)	207 (100)	243 (100)		total	36 (100)	189 (100)	225 (100)
	χ^2: p=<0.001					χ^2: p=<0.001			

Abbreviations: IHC: immunohistochemistry; CB11: mouse monoclonal antibody CB11; 4B5: rabbit monoclonal antibody 4B5; BvdV: Bert van der Vegt; JW: Jelle Wesseling.

TABLE 5. SENSITIVITY, SPECIFICITY AND PREDICTIVE VALUES FOR IMMUNOHISTOCHEMISTRY COMPARED WITH FISH AND CISH.

FISH							
	Sensitivity	Specificity	Sensitivity (excl 2+ scores)	Specificity (excl 2+ scores)	PPV	NPV	% 2+
4B5 BvdV	0.75 (0.57-0.87)	0.99 (0.95-1.0)	0.70 (0.50-0.85)	1.0 (0.98-1.0)	1.0 (0.81-1.0)	0.96 (0.92-0.98)	4
CB11 BvdV	0.83 (0.67-0.93)	0.93 (0.88-0.96)	0.79 (0.57-0.92)	1.0 (0.98-1.0)	1.0 (0.79-1.0)	0.97 (0.94-0.99)	12
4B5 JW	0.69 (0.52-0.83)	0.99 (0.96-1.0)	0.69 (0.50-0.83)	1.0 (0.98-1.0)	1.0 (0.82-1.0)	0.95 (0.91-0.97)	2
CB11 JW	0.81 (0.63-0.91)	0.99 (0.96-1.0)	0.74 (0.53-0.88)	1.0 (0.98-1.0)	1.0 (0.80-1.0)	0.97 (0.93-0.98)	5
CISH							
	Sensitivity	Specificity	Sensitivity (excl 2+ scores)	Specificity (excl 2+ scores)	PPV	NPV	% 2+
4B5 BvdV	0.76 (0.58-0.88)	0.99 (0.95-1.0)	0.70 (0.50-0.85)	1.0 (0.98-1.0)	1.0 (0.81-1.0)	0.96 (0.92-0.98)	4
CB11 BvdV	0.86 (0.70-0.95)	0.93 (0.88-0.96)	0.76 (0.54-0.90)	1.0 (0.98-1.0)	1.0 (0.80-1.0)	0.97 (0.93-0.99)	11
4B5 JW	0.72 (0.55-0.85)	0.99 (0.96-1.0)	0.67 (0.48-0.81)	1.0 (0.97-1.0)	1.0 (0.82-1.0)	0.94 (0.90-0.97)	2
CB11 JW	0.81 (0.64-0.91)	0.99 (0.96-1.0)	0.74 (0.53-0.88)	1.0 (0.98-1.0)	1.0 (0.80-1.0)	0.97 (0.93-0.98)	5

IHC: immunohistochemistry; CB11: mouse monoclonal antibody CB11; 4B5: rabbit monoclonal antibody 4b5; BvdV: Bert van der Vegt; JW: Jelle Wesseling; PPV: positive predictive value; NPV: negative predictive value.

Table 6. Concordance between observers.

	Kappa	95% CI
4B5	0.87	0.79 - 0.96
CB11	0.77	0.66 - 0.88

Table 7. Sensitivity, specificity, predictive and concordance for CB11 from literature.

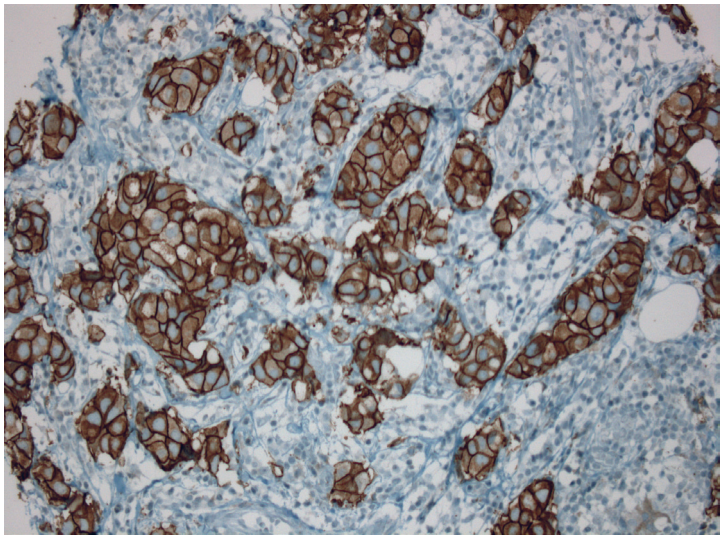
Author	Study size	Sens	Spec	Method	PPV	NPV
Kakar30	112	0.93(0.66-1.0)#	0.98 (0.90-1.0)#	2	0.88 (0.60-0.98)#	0.99 (0.92-1.0)#
Bartlett13	213	0.85 (0.70-0.94)#	0.69 (0.62-0.76)#	1	0.40 (0.30-0.51)#	0.95 (0.89-0.98)#
Press9	74	0.721 (0.56-0.85)	1.00 (0.95-1.0)	1		
Press21	64	0.95 (0.74-1.0)	0.84 (0.70-0.93)	1		
Ricardo31	190	0.52 (0.37-0.67)#	0.98 (0.93-1.0)#	2	0.92 (0.72-0.99)#	0.83 (0.75-0.89)#
Powell27	178*	0.92 (0.83-0.96)#	0.91 (0.82-0.96)#	1	0.92 (0.83-0.96)#	0.91 (0.82-0.96)#
	144*	0.74 (0.64-0.83)#	0.91 (0.80-0.97)#	1	0.93 (0.83-0.97)#	0.71 (0.59-0.80)#
Egervari29	199	0.83 (0.58-0.96)#	0.99 (0.96-1.0)#	2	0.94 (0.68-1.0)#	0.98 (0.94-0.99)#

Method 1: 2+ and 3+ scores considered positive; Method 2: 2+ cases excluded from analysis; #: calculated from data available in article;: two separate subsets were studied in article; sens: sensitivity; spec: specificity; PPV: positive predictive value; NPV: negative predictive value.*

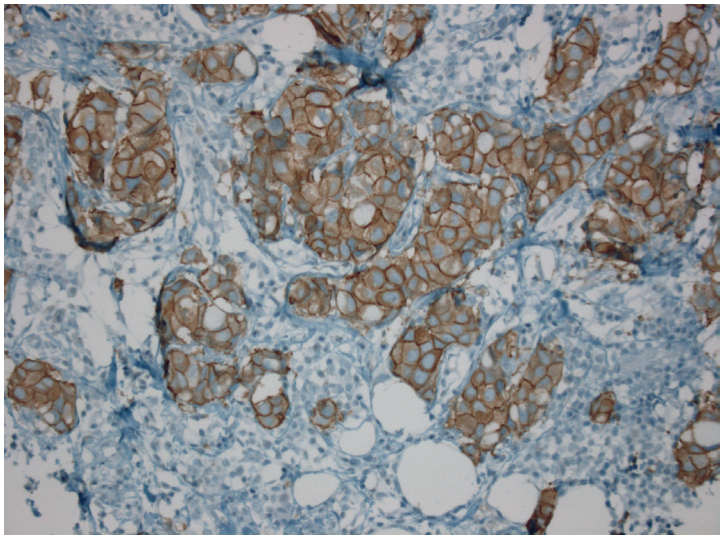
Figures

Figure 1. Comparison of 3+ case for 4B5 and CB11.

a) 4B5



b) CB11



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8

GENERAL DISCUSSION

This thesis explored the possibilities of the tissue microarray technique in the evaluation and validation of new prognostic indicators in breast carcinoma. Although many biomarkers have been introduced over the years few have actually made it to clinical practice. A marker that looks promising in an initial study may show inconsistent or contradicting results in subsequent studies. Many possible causes for this problem are given (summarized in[1]): differences in methods and assays, sample sizes, inappropriate patient selection due to the availability of patient material and the quality of patient material. In addition to these general causes one of the biggest challenges in breast carcinoma biomarker validation studies is study group homogeneity. There are several reasons for this: the early breast cancer profiling studies, for example the study by Perou et al[2], have shown us that breast carcinoma is not one entity but a very heterogeneous disease. Besides the histological subtypes of breast carcinoma that we knew, at least 3 different 'intrinsic' subgroups of breast carcinoma exist that cannotbe distinguished on histological criteria only: the ER positive types of breast cancer and at least two ER negative types: the basal type and the HER2 type of breast carcinoma.[3] While a candidate biomarker may play a role in the biology of one of the subgroups, this role might not be there for all the others. A second reason is the wide variety of treatment combinations used in breast cancer treatment. It is very difficult to test and validate candidate biomarkers in large study groups that have all received the same treatment regimen.

Because of these problems REporting guidelines for prognostic studies evaluating tumour MARKers (REMARK) have been proposed by the NCI-EORTC.[1] The most important items of this guideline are: a clear study objective and hypothesis (with as prerequisite a formal power analysis); a description of the marker, the study group (inclusion and exclusion criteria) and the analytical- and statistical methods used; univariate analysis to show the relation between the marker and standard prognostic factors and outcome; multivariate analysis to show the relation of the marker, compared with standard prognostic factors, and outcome. In addition to this it is important that the design of biomarker studies in breast carcinoma aims to reduce tumour-, patient- and treatment heterogeneity as much as possible.

With the introduction of the multigene profiles many authors believed that those profiles would replace conventional clinicopathogical parameters in the assessment of prognosis in breast carcinoma. Validation trails for the clinical use of the 21-gene Recurrence Score (Oncotype Dx) and the 70-gene profile (Mammaprint) in the assessment of breast cancer prognosis of ER positive breast cancer are currently ongoing.[4,5] Although both profiles have almost no genes in common, it has been shown that both profiles are able to identify similar risk groups. It is suggested that prognosis in ER positive breast carcinoma (the subgroup in which both of the profiles have been developed) is strongly directed by tumour proliferation and that although the genes included in both profiles are completely different both gene sets are a derivative of tumour proliferation.[3,6] Supportive evidence for this comes from a study by Cuzick et al who compared the 21-gene Recurrence Score with a score based on three commonly used immunohistochemical markers (ER, PR en HER2) combined with a proliferation marker (Ki-67).[7] In this study both scoring systems were able to identify similar risk groups. While the validation of the prognostic gene profiles is ongoing it has become apparent that 'classical' prognostic indicators remain an independent predictive factor in breast cancer prognosis models.[8] A recent study therefore developed a risk score based on a combination of the recurrence score of the Oncotype Dx profile with some classical prognostic indicators (age, tumour size and tumour grade.[9] The results of that study look promising: using the combined score some patients may be re-classified from the intermediate- to the low risk group, i.e. some additional patients that might not benefit from adjuvant chemotherapy were identified. However, this model still has to validated in independent data sets.[10]

The tissue microarray technique offers the opportunity to study cells and tissue at the protein level in a high throughput fashion. Because proteins are the executors of the majority of cellular functions and because not all (approximately 70%) mRNA is translated into protein it is highly relevant to use and analyse protein expression as candidate markers in prognosis and prediction of treatment response. In addition, posttranslational modifications such as phosphorylation or protein cleavage may play a role in protein function and those are not detectable at the mRNA level.[11] An advantage specific for

tissue microarray technique is the combination of protein expression with histology; therefore the location of the protein expression can be studied. As we have shown in chapters 5 and 6, the location of protein expression can lead to divergent effects in tumour behaviour. The stroma of a tumour has long been thought of as a passive bystander that supports the tumour cells. Recently however it was found that alterations, especially in tumour stromal fibroblast, may play a pivotal role in tumour development (reviewed in[12]) . They may shape the tumour microenvironment with matrix metalloproteinases that degrade the extracellular matrix and may be able to secrete cytokines and growth factors that stimulate tumour growth. It would be very interesting to use protein-based techniques to study the expression of markers specifically in the tumour stroma.

A problem of the currently used protein expression studies is that it can only be used to assess a small number of proteins, because only one protein can be evaluated in one test. This makes it unfit for genome wide screening studies. However, protein arrays compiled in much the same way as the DNA based techniques, with proteins fixed on a solid surface, are emerging. This makes it possible to assess the expression of large numbers of proteins in one experiment in a similar manner as the DNA based techniques.[11] Other techniques that are emerging are functional analysis like Chip-on-chip technique, and kinome profiling which give a genome wide view of respectively the DNA-binding proteins and kinase activity. These analyses provide insight in the molecular pathways that are activated or deactivated within a tumour. With the growing number of therapeutic monoclonal antibodies that bind specific targets in a tumour cell, patients with a certain tumour genotypes, that lead to altered molecular pathways in a tumour cell, might benefit from a therapy that targets that specific alteration. Examples are the use of the EGFR inhibitor gefitinib or more recently the ALK inhibitor crizotinib in specific lung carcinoma patients, or targeted therapies for melanoma patients with a BRAF mutation.

In conclusion many high throughput methods for breast carcinoma have emerged in recent years. These methods have taught us much about the heterogeneity of breast cancer. Tissue microarrays can well be used to validate 'key' markers from genome wide arraying studies in larger cohorts. The results from these studies, perhaps by combining biomarkers with 'classical'

prognostic indicators, can aid to the development of clinically applicable prognostic scores that are able to better 'tailor' treatment for a specific breast carcinoma patient.

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APPENDICES

Summary in English

Breast carcinoma is the most common malignancy in women in the Western World. 'Classical' prognostic indicators like tumour size, tumour grade, estrogen receptor expression and Her2/neu receptor expression adequately predict outcome for large groups of breast carcinoma patients, but poorly predict outcome for the individual breast carcinoma patient. In recent years many new microarray techniques have been introduced to quickly assess the expression of many genes or proteins in one patient. One of these methods is the tissue microarray array technique. This technique makes it possible to compile material of tumours of up to 300 patients into one paraffin block, thereby enabling highly efficient testing of one protein in a large cohort of tumours in one experiment. The aim of this thesis, as formulated in chapter 1, was to examine the possibilities of the tissue microarray technique in the evaluation of new prognostic and predictive markers at the protein level in breast carcinoma.

In chapter 2 an overview of the most commonly used microarray methods (oligo- or cDNA arrays, CGH arrays, PCR arrays, and tissue microarrays) that are used to identify markers that are associated with tumour progression i.e. the development of metastatic disease, local recurrence or cancer related death is given. Oligo- and cDNA arrays are the most commonly used microarrays and make it possible to assess up to 50000 genes of interest in one experiment. Each experiment assesses a gene profile of one test sample. These techniques are very expensive (e.g. a single Mammaprint test costs €2,675,- (http://www.mammaprint.nl, accessed on 02-12-2011) and complex which makes it is only possible to use them in relatively small study groups. PCR and tissue microarray on the other hand are techniques that more easily allow larger study groups, but only allow the assessment of one gene of interest in one experiment. Rather than competitive these techniques can be complementary to each other; while the DNA microarray techniques can well be used to generate hypotheses, the PCR and TMA techniques are excellent methods to validate the 'key'-markers from these DNA microarray experiments in large patient groups and translate these results to clinically applicable tests.

In chapters 3 and 4, tissue microarrays are used to evaluate the prognostic significance of a candidate biomarker: the membrane-associated glycoprotein MUC1. The effects of this protein in breast cancer prognosis are conflicting as both positive and negative associations between MUC1 expression and tumour differentiation and outcome have been found. We applied a novel scoring system for MUC1 which takes the location of MUC1 expression in the cell into account. Five predefined MUC1 expression patterns were recognized: expression on the entire cell membrane, apical expression, focal cytoplasmic expression, diffuse cytoplasmic expression and inside-out expression. In chapter 3 this scoring system was applied to a series of 87 ductal in-situ carcinomas (DCIS). Only four of the five recognized expression patterns of MUC1 were seen in DCIS. The inside-out pattern, which is specific for micropapillary carcinoma was not seen. A difference was seen between expression of MUC1 on the membrane of the tumour cells and expression in the cytoplasm. Cytoplasmic expression of MUC1 was associated with a more aggressive biological profile (higher grade, larger lesion size and Her2/neu overexpression) and with local recurrence. This suggests that the localization of MUC1 expression to some extent also reflects the extent of tumour cell differentiation.

In chapter 4 the MUC1 scoring system was applied to a series of 243 invasive ductal carcinomas. MUC1 apical membrane expression was associated with smaller tumours, lower tumour grade, progesterone expression and increased overall survival, suggesting that this expression pattern, which is also seen in normal ducts, implies better tumour differentiation. MUC1 diffuse cytoplasmic expression was associated with Cyclin D1 positivity, and increased relapse free survival. Although the intracellular routing of MUC1 molecules in this pattern is incorrect (normally MUC1 is expressed at the cellular membrane), no differences in tumour grade or size were found. This might be explained by the common combination of apical- and diffuse cytoplasmic MUC1 expression. Indeed in a subgroup analysis showed that strict cytoplasmic expression of MUC1 was associated with clinico-pathological factors related to worse outcome. MUC1 negative tumours were associated with ER, PR and Cyclin D1 negativity. MUC1 negativity proved to be an independent predictor

of both decreased RFS and OS. This association was independent of the more 'classical' prognostic factors.

In chapters 5 and 6, the tissue microarray approach was used for studying differences between interval and screen-detected invasive and in situ ductal carcinomas. For DCIS the introduction of the breast cancer screening programme led to an increased incidence, being approximately 20% of all malignant lesions. Some authors suggest that at least part of the DCIS found in the screening programme should be considered as overdiagnosis, because DCIS will not always progress to an invasive carcinoma and part of the DCIS that do progress to an invasive carcinoma will not lead to a clinically detectable tumour in a patients lifetime, because the patient will die of competing causes of death before it becomes clinically apparent. In chapter 5 a series of screen detected DCIS are compared to DCIS clinically detected in the interval between two screening moments (interval DCIS). Screen-detected DCIS more often shows Her2 overexpression suggesting that screen-detected DCIS is biologically more aggressive than interval DCIS and should therefore not be considered as overdiagnosis.

For invasive ductal carcinoma the screening programme has also led to a decline in the rate of advanced breast carcinomas and breast cancer mortality. However, a number of women still present with a clinically symptomatic breast carcinoma between two screening moments, a so-called interval carcinoma. In participants in the Dutch Breast Cancer Screening Programme, 36% of the tumours emerge as interval carcinomas. In chapter 6 the clinico-pathological data and expression of well validated biological markers for tumour aggressiveness were compared in 'true' interval carcinomas versus screen detected- and missed carcinomas (carcinomas clinically appearing as interval carcinomas, that were in retrospect visible on the screening mammogram), hypothesizing that 'true' interval carcinomas express more parameters associated with aggressive tumour behaviour. Interval carcinomas were five times more often larger sized (>2 cm). This difference might be explained by radiological factors (e.g. the density of the breast tissue or lack of microcalcifications) or tumour growth rate. However none of the radiological- or tumour growth rate parameters measured in this study differed between both

groups. In univariate analysis screen-detected tumours more often showed oestrogen receptor expression. The results of this study therefore suggest that interval carcinomas are not a biologically more aggressive group of tumours, but that a part of the tumours found in the screening are small biologically indolent tumours for which it is debatable whether they would have led to a clinically symptomatic breast carcinoma in a patient's lifetime.

In chapter 7, tissue microarrays were used to test the robustness of HER2 testing using different antibodies as well as in situ hybridization. HER2 overexpression in breast cancer is associated with worse clinical outcome. Treatment with anti-Her2/neu monoclonal antibody trastuzumab leads to a reduction in mortality and recurrence, only in patients with Her2/neu-positive breast cancer. However, trastuzumab therapy is an expensive treatment (approximately €37,500,- /year /patient) and can lead significant cardiotoxicity in a small minority of patients. Because of the high treatment costs and the efficacy in HER2 positive tumours only, uncompromised, robust and reliable HER2-testing is required. To select patients for anti-Her2 based therapy immunohistochemistry is commonly performed as a first step to assess Her2 status. It is known that interobserver and interlaboratory variability can compromise the assessment of Her2/neu[6-9]. A new anti-Her2 rabbit monoclonal antibody (4B5) was compared to the commonly used CB11 anti-Her2 mouse monoclonal antibody using both fluorescent- and chromogenic in situ hybridisation methods as a reference. 4B5 staining was more distinct and showed less aspecific cytoplasmic background staining, which led to a more than 50% reduction of the number of 2+ scores for 4B5 compared to CB11. This might lead to a reduction in the number of additional in situ hybridisation tests and might therefore lead to a cost reduction. Although the study was well powered we found no significant differences in sensitivity, specificity, predictive values and interobserver concordance between 4B5 and CB11. However there was a trend towards a higher specificity for 4B5 and a trend towards an increased interobserver concordance for 4B5. These results therefore validate the 4B5 antibody for the assessment of HER2 expression status and suggest that it provides more robust staining results and therefore might reduce the number of gene amplification tests compared

to CB11. However, for tumours with a 2+ score additional gene amplification measurement using in situ hybridisation remains necessary.

In conclusion the tissue microarray technique can well be used to validate 'key' markers from genome wide arraying studies in larger cohorts and may aid to the development of clinically applicable prognostic scores that are able to better 'tailor' treatment for a specific breast carcinoma patient.

APPENDICES

Samenvatting in het Nederlands

Mammacarcinoom is de meest voorkomende maligniteit onder westerse vrouwen. 'Klassieke' prognostische indicatoren zoals tumorgrootte, tumorgraad en de expressie van hormoonreceptoren (ER en PR) en de HER2/neu receptor zijn goede voorspellers van uitkomst in grote groepen patiënten met mammacarcinoom. De uitkomst van een individuele patiënt valt echter slecht te voorspellen met behulp van deze indicatoren. In de afgelopen jaren zijn vele microarray-technieken geïntroduceerd. Deze technieken maken het mogelijk de expressie van vele genen of eiwitten in een patiënt te onderzoeken. Een van deze microarray-technieken is de tissue microarray (TMA)-techniek. Met behulp van deze techniek kan tumormateriaal van maximaal 300 patiënten in een paraffineblokje verzameld worden. Dit maakt het mogelijk om snel en efficiënt de expressie van een eiwit in grote series patiënten te testen. Het doel van dit proefschrift, zoals in hoofdstuk 1 geformuleerd, was om de toepasbaarheid van de tissue microarray-techniek in de evaluatie van nieuwe prognostische en predictieve eiwitmarkers voor het mammacarcinoom te onderzoeken.

In hoofdstuk 2 wordt een overzicht gegeven van de meest gebruikte microarray-technieken (oligo-/cDNA array, CGH arrays, PCR array en tissue microarrays). Deze technieken worden gebruikt om markers te identificeren die geassocieerd zijn met tumorprogressie (ontwikkeling van metastasen, een lokaal recidief of kanker-gerelateerd overlijden). Oligo-/cDNA arrays zijn de meest gebruikte microarrays en maken het mogelijk tot 50000 genen in een experiment te onderzoeken. Elk experiment levert een genprofiel op van een testsample. Deze technieken zijn zeer kostbaar (een Mammaprint experiment kost €2.675,-) en dusdanig complex dat het alleen maar mogelijk is deze technieken te gebruiken in relatief kleine studiegroepen. PCR- en TMA-technieken zijn daarentegen goed toepasbaar in grote studiegroepen, maar hebben als nadeel dat er slechts één gen of eiwit per experiment onderzocht kan worden. Daarom kunnen de verschillende microarray-technieken heel goed complementair aan elkaar zijn: DNA- microarray-technieken kunnen goed gebruikt worden om hypotheses te genereren. PCR en TMA daarentegen zijn goede methodes om de belangrijkste uitkomsten van deze DNA- microarray-studies te valideren in grotere studiegroepen en deze te vertalen naar klinisch toepasbare testen.

In de hoofdstukken 3 en 4 werd de TMA-techniek gebruikt om de prognostische significantie van het celmembraan-geassocieerde glycoproteïne MUC1 te onderzoeken. De rol van dit eiwit in de prognose van het mammacarcinoom zijn tegenstrijdig in de literatuur. Zowel positieve als negatieve associaties tussen MUC1-expressie en tumordifferentiatie en uitkomst zijn beschreven. In deze hoofdstukken wordt een nieuw scoringssysteem voor MUC1 toegepast. Dit scoringssysteem houdt rekening met de lokatie van de MUC1-expressie. Vijf vooraf gedefinieerde expressiepatronen werden herkend: expressie op de gehele celmembraan, apicale expressie, focale cytoplasmatische expressie, diffuse cytoplasmatische expressie en inside-out expressie. In hoofdstuk 3 wordt dit scoringssysteem toegepast op een serie van 87 ductale carcinomen in situ (DCIS). Slechts vier van de vijf beschreven patronen worden herkend in DCIS. Het inside-out patroon, dat specifiek is voor invasief micropapillair carcinoom, werd niet aangetroffen. Cytoplasmatische expressie van MUC1 was in DCIS geassocieerd met een agressiever tumorbiologisch profiel (hogere tumorgraad, grotere laesies en HER2/neu-overexpressie) en met het ontstaan van lokale recidieven. Dit suggereert dat de locatie van MUC1 tot op zekere hoogte een afspiegeling is van de mate van tumorceldifferentiatie.

In hoofdstuk 4 werd het MUC1 scoring systeem toegepast op een serie van 243 invasief ductale carcinomen. Apicale MUC1-expressie was geassocieerd met kleinere tumoren, lagere tumorgraad, PR-expressie en langere overall survival. Dit suggereert dat dit expressiepatroon, zoals ook gezien wordt in normale ducti, past bij een betere tumordifferentiatie. Diffuse cytoplasmatische expressie van MUC1 was geassocieerd met cycline D1-expressie en ziektevrije overleving. Hoewel de intracellulaire routing van MUC1- moleculen niet correct is werden geen verschillen gevonden in tumorgraad en -grootte. Dit verschil kan wellicht worden verklaard door de veelvoorkomende combinatie van apicale MUC1-expressie en cytoplasmatische MUC1-expressie. In een subgroep analyse waarbij er gekeken werd naar strikte cytoplasmatische expressie werden associaties met clinico-pathologische factoren gerelateerd aan slechtere uitkomst gevonden. Tumoren die geen expressie van MUC1 toonden

waren geassocieerd met ER-, PR- en Cycline D1-negativiteit. MUC1 was een onafhankelijke voorspeller voor kortere ziektevrije en totale overleving.

In de hoofdstukken 5 en 6 werd de tissue microarray techniek gebruikt om verschillen tussen interval- en screen-detected mammatumoren te onderzoeken. De introductie van het bevolkingsonderzoek borstkanker heeft geleid tot hogere incidentiecijfers van DCIS. Op dit moment zijn ongeveer 20% van de maligne laesies ontdekt bij het bevolkingsonderzoek DCIS. Sommige auteurs suggereren dat tenminste een deel van deze laesies beschouwd dient te worden als overdiagnostiek, omdat DCIS niet altijd uitgroeit tot een invasief carcinoom en dat een deel van de tumoren die uiteindelijk uitgroeit tot een invasieve tumor niet klinisch detecteerbaar wordt gedurende het leven van een patiënt, omdat deze voordat het een klinisch dectecteerbare tumor wordt zal komen te overlijden aan een andere doodsoorzaak. In hoofdstuk 5 werd een serie screen-detected-DCIS vergeleken met een serie DCIS welke klinisch ontdekt werd tussen twee screeningsmomenten (interval-DCIS). Screen-detected-DCIS laat meer HER2/neu-expressie zien. Dit suggereert dat screen-detected-DCIS tumorbiologisch agressiever is dan interval-DCIS en dat het daarom niet als overdiagnostiek beschouwd dient te worden.

Voor invasieve tumoren heeft de introductie van het bevolkingsonderzoek geleid tot een afname van het aantal vergevorderde mammacarcinomen en de mortaliteit van borstkanker. Er zijn echter nog steeds vrouwen die zich presenteren met een interval-carcinoom, een klinisch symptomatische tumor die ontdekt wordt in de periode tussen twee screeningsmomenten. 36% van de tumoren die gevonden worden in patiënten die deelnemen aan het bevolkingsonderzoek worden ontdekt als een intervalcarcinoom. In hoofdstuk 6 worden goed gevalideerde tumorbiologische markers vergeleken tussen 'ware' interval carcinomen en tumoren gevonden in het bevolkingsonderzoek en tumoren gemist in het bevolkingsonderzoek. De hypothese hierbij was dat 'ware' interval carcinomen meer parameters laten zien die geassocieerd zijn met agressiever tumorbiologisch gedrag. In deze studie hadden interval-carcinomen vijf keer zo vaak een tumordiameter van 2 cm of groter. Dit verschil kan wellicht verklaard worden door radiologische factoren (bijvoorbeeld de densiteit van het mammaweefsel of het ontbreken van microcalcificaties) of

de groeisnelheid van de tumor. Echter: geen van de radiologische parameters of parameters voor tumorgroeisnelheid verschilde tussen de beide groepen. In univariate analyse waren screen-detected-tumoren vaker ER-positief. Dit suggereert dat interval-carcinomen niet agressiever zijn dan tumoren ontdekt bij het bevolkingsonderzoek, maar dat dat een deel van de tumoren ontdekt bij het bevolkingsonderzoek klein en tumorbiologisch somnolent zijn.

In hoofdstuk 7 werden TMA's gebruikt om verschillende antilichamen en in-situ hybridisatie methoden voor Her2/neu te testen. Her2/neu-overexpressie bij mammacarcinoom is geassocieerd met een slechtere prognose. Behandeling met het anti-Her2/neu-monoclonale antilichaam trastuzumab leidt tot een reductie van mortaliteit en het aantal recidieven. Dit is echter alleen het geval bij patiënten met Her2/neu-positieve mammacarcinoom. Daarnaast is trastuzumab een zeer kostbare therapie (ongeveer €37.500,- /jaar / patiënt) en kan het in een klein deel van de patiënten cardiotoxiciteit geven. Om de juiste patiënten voor therapie te kunnen selecteren is robuuste en betrouwbare Her2/neu-diagnostiek noodzakelijk. Immunohistochemie wordt meestal als een eerste stap in de Her2/neu-diagnostiek gebruikt. Het is bekend dat immunohistochemie kan lijden onder interbeoordelaar- en interlaboratorium variabiliteit. In dit hoofdstuk werd een nieuw anti-Her2/neu-monoclonaal antilichaam, opgewekt in konijnen, getest en vergeleken met een veelgebruikt antilichaam tegen Her2/neu, opgewekt in muizen, waarbij fluorescente- en chromogene in-situ hybridisatie als referentiewaarden werden gebruikt. De 4B5 kleuring was meer uitgesproken en liet minder aspecifieke achtergrondkleuring zien. Hierdoor nam het aantal 2+ scores af met meer dan 50% in vergelijking met de CB11 kleuring. In de praktijk kan het gebruik van dit antilichaam wellicht leiden tot een reductie van het aantal in-situ hybridisaties en daar aan gekoppeld een kostenreductie. Ondanks een goede power van de studie werden geen significante verschillen gevonden in sensitiviteit, specificiteit, voorspellende waardes en interbeoordelaar variabiliteit tussen de twee vergeleken antilichamen. Er waren echter wel trends voor een hogere specificiteit en een toegenomen interbeoordeelaar-concordantie voor 4B5. De resultaten van deze studie valideren het 4B5 antilichaam in de Her2/neu-status bepaling. Er zijn aanwijzingen dat 4B5 robuuster is en mogelijk een reductie van het aantal in-situ hybridisatietesten kan geven in vergelijking met CB11.

Voor tumoren met een 2+ immunohistochemie score blijft in-situ hybridisatie echter noodzakelijk.

De conclusie van dit proefschrift is dat de tissue microarray-techniek goed gebruikt kan worden om de belangrijkste markers die uit genoom-brede arraying studies naar voren zijn gekomen te testen in grotere onderzoeksgroepen en dat deze techniek derhalve kan bijdragen aan de ontwikkeling van klinisch toepasbare prognostische scores die het mogelijk maken therapie beter toe te spitsen op een individuele borstkanker patiënt.

APPENDICES

List of publications

2004 van der Vegt B, Doting MH, Jager PL, Wesseling J, de Vries J. Axillary recurrence after sentinel lymph node biopsy. Eur J Surg Oncol 2004; 30(7):715-20.

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APPENDICES

Dankwoord

Hoe het begon…

Zeven en een half jaar geleden kwam ik op de afdeling Pathologie van het UMCG om er een wetenschappelijke stage te doen bij Jelle Wesseling en Truuske de Bock. Samen met Jaap de Vries had ik enig onderzoek verricht aan de sentinel node procedure bij het mammacarcinoom, en ik had bedacht dat het leuk zou zijn een iets meer basale wetenschappelijke stage te doen. Ik kon aansluiten bij het onderzoek dat Marnix de Roos verrichtte naar ductaal carcinomen in situ. We gingen daar een (in die tijd nog) revolutionaire techniek van tissue microarrays voor gebruiken. Samen met Tineke van der Sluis en Inge Plateel ging ik een dag naar het NKI/AVL om de techniek te leren. Ed Schuuring dacht mee over de tumorbiologische aspecten. We verzamelden veel data waarover ik een scriptie schreef.

Hoe het verder ging…

Na wat omzwervingen bij de chirurgie zag ik het licht en besloot ik te solliciteren voor de opleiding Pathologie. Ondertussen bleek dat de hoeveelheid data die ik had verzameld tijdens mijn wetenschappelijke stage genoeg zou kunnen zijn om uiteindelijk op te promoveren. Derhalve werd Harry Hollema uiteindelijk zowel mijn opleider als ook mijn promotor. Mijn onderzoekstijd is eigenlijk mijn opleidingstijd en die is prettig gekleurd door mede-AIOS, opleiders, analisten en vele andere medewerkers (zeker ook in Leeuwarden en Zwolle). Ook waren er zo nu en dan studenten, waarvan ik Nick Zwartjes wil noemen, die door middel van hun wetenschappelijke stage een bijdrage hebben geleverd aan dit proefschrift.

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