From the Department of Oncology-Pathology, Cancer Center Karolinska
Karolinska Institutet, Stockholm, Sweden

Prognostic markers in breast cancer associated with cell cycle control, proliferation and angiogenesis

Thomas Lindahl

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ABSTRACT

This thesis focuses on potential prognostic molecular breast cancer markers which are related to angiogenesis, proliferation or cell cycle control. A common denominator for all of the investigated markers in the thesis is either hypothesized or previously reported association with the tumor suppressor gene TP53. All patients in the present studies derived from a population based cohort of 311 Swedish breast cancer patients with known TP53 gene status.

In study nr I expression of the angiogenic cytokine Vascular Endothelial Growth Factor (VEGF) was compared with available TP53 data from cDNA based gene sequencing, a TP53 luminometric immunoassay and TP53 immunohistochemistry (IHC) in 224 patients. High VEGF expression was associated with mutated TP53. The highest VEGF levels were found in breast cancers with insertions, deletions or stop codon point TP53 mutations. These findings support the hypothesis that wild-type TP53 is involved in the regulation of VEGF. Significantly worse outcome for was observed for tamoxifen treated estrogen receptor (ER) positive patients with high VEGF.

In study nr II the cell cycle regulatory protein cyclin E was investigated by IHC in breast cancers from 270 patients. TP53 mutations were much more common in patients with high cyclin E expression compared with low cyclin E expression. In TP53 mutated breast cancers, high cyclin E content was associated with insertions, deletions and nonsense point mutations in the TP53 gene, whereas low cyclin E was linked to TP53 missense point mutations. Strong associations were found between a high cyclin E content and presence of several other tumor markers including aneuploidy. High cyclin E content was associated with poor survival. The results implicate that overexpression of cyclin E is associated with an aggressive tumor phenotype and specific types of TP53 mutations.

In study nr III the possibility of improving the indirect TP53 mutation screening with TP53 IHC, by co-analysis of two proteins associated with wild-type TP53, p21(waf1/cip1) and MDM-2 (murine double minute-2), was investigated. The expression of p21and MDM-2 was determined by IHC on 276 and 257 primary breast cancers, respectively, and compared with the TP53 mutation status. Expression of p21 and MDM-2 was strongly positively correlated, but no statistically significant correlations between TP53 gene status and expression of p21 or MDM-2 was observed. According to these results is determination of the IHC expression of p21 or MDM-2 not useful for identification of breast cancers with mutated TP53.

In study nr IV the proliferation marker Ki67 (MIB-1) was analyzed with IHC in 305 patients, in order to compare and validate Ki67 in relation to S-phase fraction (SPF), other breast cancer markers and patient outcome. A graticula based method for reading the Ki67 sections was also developed. High Ki67 correlated with several other breast cancer prognostic markers, although only moderately to SPF. Using a grid graticule for Ki67 scoring gave comparable results to individual cell counting. Patients with high Ki67 had shorter survival.

In summary, VEGF content was associated with TP53 status and gave predictive information for adjuvant tamoxifen therapy. Indirect p53 mutation screening with IHC is not improved by co-analyses of p21 and MDM-2. Among the tumor markers investigated in this thesis cyclin E and KI-67 had the highest prognostic value for patient outcome.

LIST OF PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

I. Linderholm, B., **Lindahl, T**., Holmberg, L., Klaar, S., Lennerstrand, J., Henriksson, R., Bergh, J. ”The expression of vascular endothelial growth factor correlates with mutant TP53 and poor prognosis in human breast cancer.” Cancer Research, 2001, March 1;61(5):2256-60

II. **Lindahl, T**., Landberg, G., Ahlgren, J., Klaar, S., Holmberg, L., Bergh, J. ” Overexpression of cyclin E protein is associated with specific mutation types in the TP53 gene and poor survival in human breast cancer.” Carcinogenesis, 2004 March;25(3):375-380. [Electronic publication ahead of print Nov 21, 2003]

III. **Lindahl, T**., Blomqvist, C., Lindgren, A., von Boguslawski, K., Norberg, T., Bergh, J. “Immunohistochemical expression of p21(waf1/cip1) and MDM-2 in human breast cancer – no correlation with the TP53 gene status”. 2004, Submitted.

IV. **Lindahl, T**., Linderholm, B., Lindgren, A., Nordgren, H., Holmberg, L., Bergh, J. “Ki-67 (MIB-1) compared with S-phase and other breast cancer prognostic factors and the development of a rapid screening method for KI-67”. 2004, Manuscript.

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ABBREVIATIONS

ALF	Automated Laser Fluorescence
ASCO	American Society of Clinical Oncology
BCSS	Breast Cancer Specific Survival
CAF	Cyclophosphamide, Doxyrubicin, 5-flourouracil
CDK-2	Cyclin-Dependent-Kinase-2
C.I	Confidence Interval
CMF	Cyclophosphamide, Methotrexate, 5-flourouracil
DNA	DeoxyriboNucleic Acid
ECM	Extra Cellular Matrix
ER	Estrogen Receptor
FGF	Fibroblast Growth Factor
HR	Hazard Ratio
HER2	Human Epidermal Growth Factor-2
HGF	Hepatocyte Growth Factor
IHC	ImmunoHistoChemistry
IL	InterLeukin
Ki-67	Kiel Antigen-67
MDM-2	Murine Double Minute-2
MIB-1	Molecular Immunology Borstel-1
mRNA	Messenger RiboNucleic Acid
NPI	Nottingham Prognostic Index
OS	Overall Survival
PAI-1	Plasminogen Activator Inhibitor Type-1,
PCR	Polymerase Chain Reaction
PgR	Progesterone Receptor
pRB	Retinoblastoma Protein
RFS	Recurrence Free Survival
RH	Relative Hazard
SPF	S-Phase Fraction
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TNM	Tumor, Node, Metastasis
uPA	Urokinase Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor

BACKGROUND

Breast cancer is the most common malignant disease in women. The estimated global annual incidence is 1.05 million new cases per year. (Schwartsmann et al., 2002) The estimated total number of women living with breast cancer is 3.9 million. (Schwartsmann et al., 2002) The breast cancer incidence is 23% higher in more developed countries compared with less developed countries. (Schwartsmann et al., 2002) According to the most recent information 6623 women (and 34 males) were diagnosed with breast cancer in Sweden 2002. (The National Board of Health and Welfare, 2003) The corresponding figure 1970 was 3390, thus only around half that number of patients just 30 years ago. The increased incidence is only partly explained by an aging Swedish population and very likely also due to increased early diagnosis via the population based screening programmes. The age-adjusted breast cancer incidence 2002 was 129 new cases per 100 000 inhabitants compared with 81/100 000 in 1970. (The National Board of Health and Welfare, 2003) Despite the rising breast cancer incidence does the breast cancer mortality rate remain around 1500 female breast cancer fatalities each year, with 1487 fatalities in 2002. (The National Board of Health and Welfare, 2003) The number of prevalent female breast cancer cases in Sweden 1997 was 61442 which translates into a prevalence of approximately 1350 cases per 100 000 women. (The National Board of Health and Welfare, 2003)

The primary treatment of breast cancer is surgical tumor removal. The overall patient survival can also be significantly improved by adjuvant therapy with chemotherapy and/or endocrine therapy. (EBCTCG, 1998; EBCTCG, 1998) Breast cancer survival is statistically significantly improved by the use of screening mammography. (Nyström et al., 2002) Postoperative adjuvant radiotherapy reduces the risk of local recurrences by approximately two thirds, and breast cancer survival, although the effect by radiotherapy on overall survival is controversial. (EBCTCG, 1995; Overgaard et al., 1997; Overgaard et al., 1999; Ragaz et al., 1997) According to the most recent review of 15 randomized clinical trails pooling 9422 patients, are radiotherapy with modern strategies after breast conserving surgery associated with a small decrease in the risk of overall mortality. (Vinh-Hung and Verschraegen, 2004)

Breast cancer is a heterogeneous disease, with marked variations in malignant potential and metastatic capacity. The risk of having relapse, for many patients resulting in a fatal outcome, from breast cancer vary a lot between patients from almost no risk at all for dying for patients with in situ cancer (Wärnberg et al., 1999) to 70% risk or more for relapse within five years from diagnosis, using conventional treatment modalities. (Bergh et al., 2000) These figures highlight the importance of proper selection of patients for different treatment modalities. Ideally we should also be able to estimate the likelihood for response to a selected therapy. Prognostic and predictive tumor markers are tools that may allow for more precise treatment allocation of patients. A prognostic marker (or factor) is a disease characteristic associated with clinical outcome, ideally for untreated patients. A predictive marker is associated with the probability of response to a specific treatment. In theory, these markers should allow for separation of a large heterogeneous patient population into smaller more homogenous groups. (Hayes et al., 1998)

Cell cycle control and TP53

The proliferation of eukaryotic cells is tightly controlled by several checkpoints during the process of DNA duplication and mitosis, the cell cycle. Loss of cell cycle control result in unrestricted cellular proliferation, genetic instability and inappropriate cell survival, allowing proliferation and evolution of cells with genetic damage. (Malumbres and Barbacid, 2001) Escape from cell cycle control is essential for tumor development. (Hartwell and Kastan, 1994)

The tumor suppressor gene TP53 has a key role in the cell cycle control system (Figure 1). The human TP53 gene is located at the short arm of chromosome 17 (17p13.1). (McBride OW, 1986) The TP53 gene has 11 exons (the first is not translated) and encodes a 53kDa nuclear transcription factor. (Lamb and Crawford, 1986) The open reading frame of TP53 is 393 amino acids long, and the central region contains the DNA-binding domain. (Cho et al., 1994) A mutation in the TP53 gene is the most common mutation found in malignant cells, and somatic mutations are present in 20-30% of all breast cancers. (Soussi and Beroud, 2001; Soussi et al., 2000) Expression of the TP53 gene is induced by diverse forms of cellular stress such as hypoxia or DNA damage caused by carcinogens, ionizing radiation and UV light. (Graeber TG, 1994; Hall et al., 1993; Harris, 1996; MacCallum et al., 1996)) Induction of TP53 triggers either cell cycle arrest to allow for DNA repair, or execution of programmed cell death if the DNA damage is beyond repair (reviewed in (Schwartz and Rotter, 1998)). The TP53 dependent cell cycle arrest is mediated through TP53 dependent induction of p21(waf1/cip1/CDKN1). The p21 gene is located at chromosome 6p21.2 and contains a transcription responsive TP53 binding site in its promoter. (el-Deiry et al., 1993) p21 inhibits G1-cyclins/cyclin dependent kinase-complexes to facilitate cell cycle arrest at the G1/S-phase checkpoint. (Gartel et al., 1996) TP53 dependent induction of p21 is also necessary for maintained arrest at the G2 checkpoint after DNA damage. (Bunz et al., 1998)

Programmed cell death proceeds through at least two main pathways, which both can be regulated at multiple levels. The extrinsic apoptotic pathway consists of cell surface receptors (death receptors), their inhibitory counterparts (decoy death receptors) and downstream cytoplasmic proteins such as caspase activators (reviewed in (Peter and Krammer, 2003)). The intrinsic apoptotic pathway is focused on the mitochondria, which contains several apoptogenic factors. (Kroemer, 1999) TP53 can induce apoptosis through both pathways by activating transcription of pro-apoptotic genes, although the intrinsic pathways contribution to TP53-mediated cell death is not clearly defined (reviewed in (Fridman and Lowe, 2003)). The best described link between TP53 and apoptosis is the TP53 mediated regulation of transcription of pro-apoptotic members of the Bcl-2 family, Bax, Bid, Noxa and Puma. (Miyashita et al., 1994; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002) The exact action of these proteins downstream of TP53 is not clearly defined, but the net effect is to increase the ratio of pro- versus anti-apoptotic Bcl-2 proteins. (Fridman and Lowe, 2003)

MDM-2 (Mouse Double Minute 2) is a TP53 induced phosphoprotein that acts as a major regulator of the TP53 by targeting its destruction, thus forming an autoregulatory loop with TP53. (Piette et al., 1997) The MDM-2 gene is mapped to the 12q13-q14 region and encodes a 90kDa protein. (Oliner et al., 1992) Binding of MDM-2 to TP53 results in ubiquitination and rapid degradation of TP53. (Piette et al., 1997) During DNA damage is TP53 phosphorylated at amino acid ser15, which induces a conformational change that makes MDM2 unable to bind TP53 and results in the relief of the inhibitory effect of MDM2 on TP53. (Shieh et al., 1997)

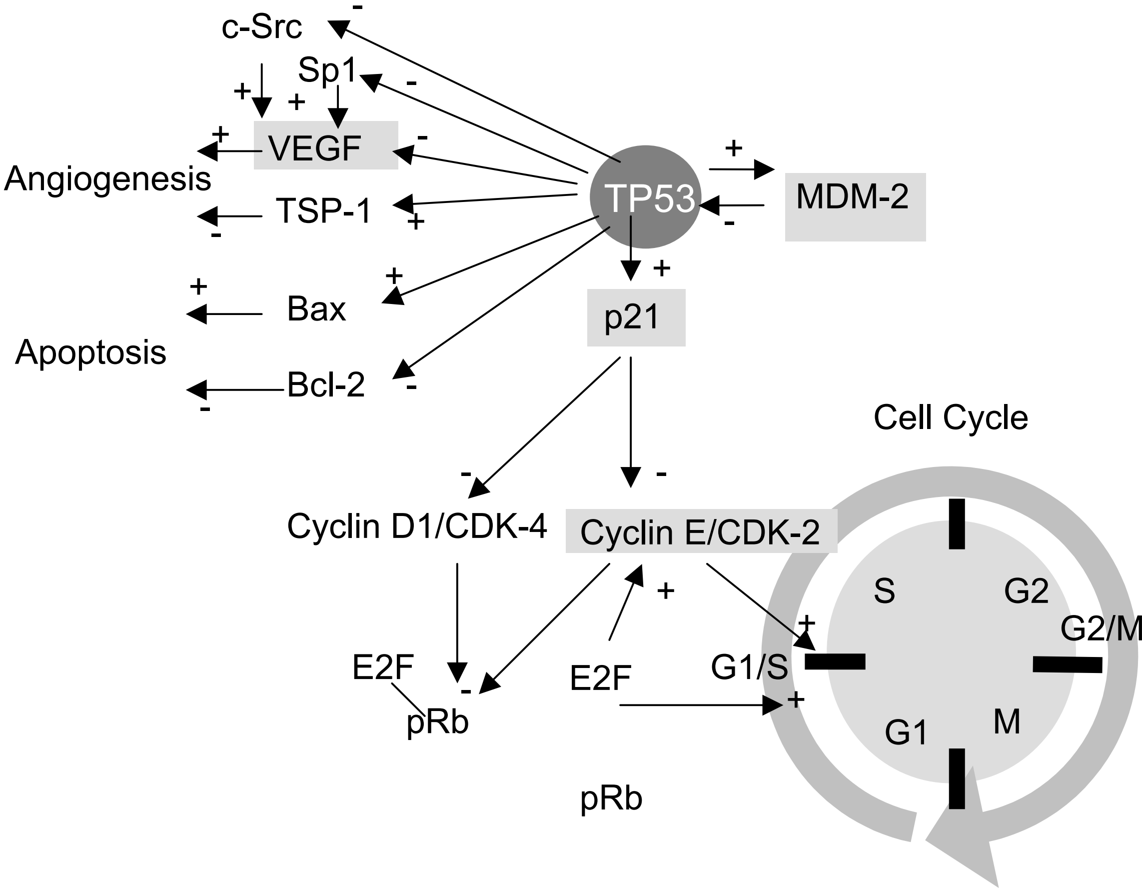


Figure 1. A schematic overview of the TP53 network and the links between TP53 and VEGF, p21, MDM-2 and Cyclin E.

The cyclin E protein is involved in cell cycle control downstream of TP53. Cyclin E is induced by the transcription factor E2F1 at the transition from G1 into S-phase, and rapidly degraded in early S-phase by an ubiquitin-mediated degradation. (Pestell et al., 1999) The cyclin E gene is positioned at 19q13 (Demetrick et al., 1995) and encode a protein that binds and activate a catalytic subunit, the cyclin-dependent-kinase-2 (CDK-2). (Sherr, 1994)

During the G1 phase, phosphorylation of the retinoblastoma protein (pRB) by mainly cyclin D- cyclin-dependent kinase-4 complexes, releases pRb from E2F. (Morris et al., 2000) Cyclin E/CDK-2 may induce E2F by phosphorylation of pRb which abolishes pRb binding to E2F response elements. (Keenan et al., 2003) Cell cycle progression into S-phase is suggested to be facilitated through E2F mediated recruitment of the p300/CBP family of co-activators, which binding to E2F is stabilized by phosphorylation of E2F by cyclin E/Cdk2. (Morris et al., 2000) Both cyclin-E/CDK-2 and E2F can also initiate S-phase independent of one another. (Leone et al., 1999) Cyclin E/CDK-2 is also playing a role in the initiation of DNA replication (Krude et al., 1997) and centrosome duplication. (Hinchcliffe et al., 1999; Mussman et al., 2000)

Vascular Endothelial Growth Factor and TP53

The formation of new blood vessels, angiogenesis, is a necessity for growth of both primary and metastatic tumors, since tumor growth beyond 1-2mm^3 requires development of an adequate blood supply due to the oxygen diffusion limit between a capillary and cells. (Folkman, 1990) Angiogenesis is a highly regulated process involving sequential activation of series of receptors by various ligands, in order to initiate degradation of the basement membrane, endothelial cell proliferation, cell migration and tube formation. (Ferrara et al., 2003; Scott et al., 1998)

The existence of angiogenic factors was first proposed in 1939 by Gordon Ide (Ide, 1939) and colleagues after observing tumor growth accompanied by rapid and extensive neovascularisation in transplanted tumors (reviewed in (Ferrara, 2002)) Today a large number of molecules have been associated with pro-angiogenic capacity, including the vascular endothelial growth factor (VEGF) family, acidic fibroblast growth factor (FGF), basic FGF, transforming growth factor alpha (TGF-α), TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor alpha (TNF-α), angiogenin and interleukin-8 (IL-8). (Folkman and Shing, 1992) VEGF signalling has often been shown to be a critical rate limiting step in physiological angiogenesis and also associated with pathological angiogenesis, such as tumor neo-vascularisation. (Ferrara et al., 2003; Yancopoulos et al., 2000) VEGF is a polypeptide cytokine induced by hypoxia, with exclusive and potent mitogenic effect on endothelial cells. (Ferrara et al., 2003) There is a family of growing number of VEGF homologues (VEGF A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF), with specific affinity for three tyrosine kinase receptors, VEGFR1, R2 and R3. (Reviewed in (Clauss, 2000))VEGF A was the first discovered family member and the gene is located at 6p21.3. (Senger et al., 1986; Vincenti et al., 1996) Alternative splicing produce four different isoforms of which $VEGF_{165}$ is believed to be the predominate one. (Ferrara, 1996)

Wild-type TP53 inhibit angiogenesis in vitro through induction of the endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1). (Dameron et al., 1994) It has also been reported that TP53 down-regulate VEGF in vitro by inhibitory binding to the VEGF transcription factor Sp1 or by inhibiting c-Src dependent VEGF expression. (Pal et al., 2001)

Elevated VEGF has been demonstrated to be associated with a worse outcome for primary breast cancer in several retrospective studies, despite methodological differences. (Eppenberger et al., 1998; Gasparini et al., 1997; Gasparini et al., 1999; Linderholm et al., 2000; Linderholm et al., 1998; Linderholm et al., 2001; Linderholm et al., 2003)

MAIN CATEGORIES OF PROGNOSTIC AND PREDICTIVE BREAST CANCER MARKERS

In this section of the thesis some of the most well known examples of prognostic and predictive tumor markers for breast cancer are presented. They are divided into established or emerging markers based on their use in the routine clinical management of breast cancer and/or their level of documentation with emphasis on the conservative clinical practice guidelines (Bast et al., 2001) of the American Society of Clinical Oncology.

Established markers

Stage

Clinical stage according to the TNM (TumorNodeMetastasis) staging system is the most well established prognostic marker in breast cancer. The TNM system summarises information for three important tumor markers, primary tumor size, regional lymph node status and weather distant metastasis are present or not. (Singletary et al., 2002) Patients are stratified into different prognostic categories (stage I – IV) by the TNM system (outlined in Table 1). In a large epidemiological retrospective study by Bland and colleagues, including approximately 240 000 breast cancer patients, the 10 year survival for stage I patients was 88% compared with 7% for stage IV patients. (Bland et al., 1998) Tumor size is a well documented prognostic marker in breast cancer. Patients with primary tumor diameter less than 1.0 cm, and without positive lymph nodes or distant metastases, have been reported to have a 10-year disease-free survival of 91% (87% at 20 years). (Rosen et al., 1993) Axillary lymph node status is considered the single most powerful predictor of disease-free and overall survival in breast cancer. Up to 70% of patients with ipsilateral axillary lymph node metastases will suffer relapse in comparison with 20% to 30% in the node negative group (Reviewed in (Fitzgibbons et al., 2000)). Sampling of the axillary lymph nodes by either surgical dissection of the axilla, or sentinel lymph node biopsy is becoming part of the routine staging of primary breast cancer patients, while it decreases long-term arm morbidity. (Veronesi et al., 2003)Breast cancer with distant metastasis is in general not considered curable, although some 20-25% of the patients will be alive 4-5 years after the diagnosis of systemic metastasis (Lekberg et al, unpublished data). The median overall survival time for metatstatic patients (after recurrence, including all sites) have been reported to be 21 months. (Giordano et al., 2004)

TNM - Clinical stage classification			
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table1. Definition of TNM staging in breast cancer. (Singletary et al., 2002) Primary tumor: TX, primary tumor can not be assessed. T0, no evidence of primary tumor, Tis, carcinoma in situ. T1, tumor with <2 cm in greatest dimension. T2, tumor with > 2 cm but not > 5 cm in greatest dimension. T3, tumor with > 5 cm in greatest dimension. T4, inflammatory carcinoma or tumors with extension to skin or chest wall.

Regional lymph nodes (N): NX, not assessable. N0, no regional lymph node metastasis. N1, metastasis in ipsilateral axillary lymph nodes. N2, metastasis in ipsilateral axillary lymph nodes fixed or ipsilateral internal mammary lymph node metastasis. N3, metastasis in infraclavicular lymph node(s) or metastasis in both ipsilateral internal mammary lymph node and ipsilateral axillary lymph node, or in the ipsilateral supraclavicular lymph node alone.

Distant metastasis (M): MX, not assessable. M0, no distant metastasis. M1, distant metastasis present.

Histopathological grade

The postoperative histopathological examination and classification of breast cancers into different grades of malignancy, referred to as grade or tumor grade, has been routinely used in the clinical assessment of disease prognosis for several decades. The procedure is in relative terms affordable, accessible and time efficient and consequently widely used. The first published systematic morphologic classification of breast cancer differentiation was presented by R. Greenhough in 1925. (Greenhough, 1925) The best known and most widely used (Latinovic et al., 2001) grading system today was developed by Bloom and Richardson (Bloom and Richardson, 1957) and later modified by Elston and Ellis (Elston and Ellis, 1991). The histopathological grade according to the Elston and Ellis method is based on the sum of three separate morphological components in the breast cancer, tubule formation, nuclear pleomorphism and mitotic count. Several studies have demonstrated that this method yields independent prognostic information for survival in human breast cancer (Elston and Ellis, 1991; Henson et al., 1991; Latinovic et al., 2001; Simpson and Page, 1992) although the opposite finding has also been reported. (Younes and Laucirica, 1997) The methods level of clinical usefulness and its place in the clinical decision making process is still debated. The main critique against histopathological grading is that it is a subjective evaluation, dependent of the skill of the examining pathologist. Studies addressing this issue have found moderate to high interobserver variability and therefore questioned the reliability of the method. (Boiesen et al., 2000; Frierson et al., 1995; Gilchrist et al., 1985) Consensus today is moving towards the use of strict grading guidelines, which earlier has been shown to improve the interobserver agreement. (Dalton et al., 1994)

Estrogen receptor and Progesterone receptor

The link between growth of breast cancer tumors and sex-hormone activity was suggested as early as 1896 when Dr. GT Beatson reported drastic clinical response for three breast cancer cases after surgical removal of their ovaries. Although the concept of hormonal regulation was not known at the time, Bateson stated that the ovaries seemed to have control over proliferation of the local epithelium in the breast. (Beatson, 1896) Normal human breast epithelium in the non-pregnant, non-lactating woman is, unlike the endometrium, not very sensitive to proliferative stimulus from the sex-hormones estrogen and progesterone (reviewed in (Anderson et al., 1998)).

The receptors for estrogen and progesterone are the nuclear transcription factors estrogen receptor (Er) and progesterone receptor (PgR), respectively. Normal breast epithelial cells express no or very low basal levels of estrogen receptor (ER) or progesterone receptor (PgR) and immunohistochemical studies show that only 7% of these cells stain positively for ER or PgR (reviewed in (Lapidus et al., 1998)). In contrast, approximately 70% of all breast cancers overexpress ER and half of these tumors also overexpress PgR, as detected by ligand binding or immunohistochemical analysis. (Chebil et al., 2003; Harvey et al., 1999; McGuire, 1978),).

The ER and PgR genes are located at chromosome 6q25.1 and 11q22, respectively. (Menasce et al., 1993; Rousseau-Merck et al., 1987) Expression of ER and PgR are associated with slightly better short-term survival and a high response rate to hormonal therapy, ranging from 80% in ER+/PgR+ patients, 30% % in ER+/PgR- down to <10% in ER-/PgR- breast cancers (reviewed in (Lapidus et al., 1998)) Thus, ER and PgR are predictive and prognostic tumor markers for breast cancer. The clinical values of ER and PgR as tumor markers are well established and also recommended to be analysed in all primary breast cancers according to the American Society of Clinical Oncology (ASCO) guidelines for tumor markers. (Bast et al., 2001)

Emerging markers

TP53

The majority of genetic alterations in the TP53 gene found in breast cancer tumors are point mutations leading to translation of a stable, mal-functional protein with extended half-life which accumulates in the cell, and is therefore detectable by imunohistochemistry (IHC) (reviewed in (Borresen-Dale, 2003)).

During the initial studies on the prognostic value of TP53 for breast cancer mainly IHC was used for TP53 determination, as an accessible substitute for direct mutation detection. Some studies using IHC reported that TP53 overexpression was associated with worse outcome, especially in node negative patients. (Allred et al., 1993; Silvestrini et al., 1993) However, overall only one-third of such studies observed a positive association (reviewed in a meta-analysis including over 9000 patients by Barbareschi, et al (Barbareschi, 1996)). A very likely explanation for these results is that mutation types such as insertion, deletions or stop codon point mutations may result in truncated proteins, undetectable by IHC, as shown by Klaar (formerly Sjögren) et al (Sjögren et al., 1996) comparing TP53 IHC with cDNA based sequencing of all coding exons in the TP53 gene. Elevated expression of wild-type TP53 due to DNA damage, or unspecific antibody-binding may also influence TP53 determination by IHC. (Borresen-Dale, 2003) Klaar et al observed that IHC produced a rate of 33% false negative and 30% false positive cases when compared with TP53 gene sequencing data. (Sjögren et al., 1996) Studies directly correlating TP53 gene mutations with patient prognosis, such as Bergh et al (Bergh et al., 1995) or Blaszyk et al (Blaszyk H, 2000), have in general observed strong associations between TP53 and outcome.

A meta-analysis with inclusion of data from 2319 patients from eleven studies, investigating the association between somatic TP53 mutations and outcome demonstrated the combined relative hazard (RH) of 2,0 (CI95% 1,7-2,5) for overall fatal outcome for patients with TP53 mutated breast cancers. (Pharoah et al., 1999)

TP53 is also a potential predictive marker for breast cancer. Adjuvant radiotherapy along with systemic adjuvant therapy, especially tamoxifen, has been reported to be of less effect in lymph-node positive patients with mutated TP53. (Bergh et al., 1995) Tamoxifen resistance has also been associated with TP53 mutations that affect the DNA-binding region or mutations in the zinc-binding domain L3 (Berns et al., 1998) and with concomitant VEGF overexpression and mutated TP53 in advanced breast cancer. (Berns et al., 2003; Linderholm et al., 1998; Linderholm et al., 2001; Linderholm et al., 2003)

Opposing results on the predictive value of TP53 for tamoxifen therapy have also been published (Archer et al., 1995; Elledge et al., 1997) thus suggesting further validation before clinical recommendation. Smaller studies have observed associations between mutated TP53 and effect of chemotherapeutic agents, such as low response to doxorubicin (Geisler et al., 2001) , 5-fluorouracil and mitomycin (Geisler et al., 2003) or paclitaxel (Schmidt et al., 2003)) while others demonstrated opposite results. (Bertheau et al., 2002)

Although the biological properties of TP53 suggest potential clinical usefulness and many study results are promising, but the documentation of the predictive and prognostic value for TP53 is not yet solid enough, for the recommendation to include TP53 determination in routine clinical management of breast cancer patients. (Bast et al., 2001)

HER2(neu/c-erbB-2)

Human epidermal growth factor-2 (HER2), also known as neu or c-erbB-2) is one of four members of the HER family of receptor tyrosine kinases, expressed on the cell membrane. (Kirschbaum and Yarden, 2000) Activation of HER tyrosine kinases in a normal cell triggers a network of signalling pathways that control cell proliferation, differentiation, motility and adhesion (reviewed in (Menard et al., 2003)). The HER2 gene is located at chromosome 17q21 (Kaneko et al., 1987) and has been shown to be amplified in many breast cancer cases. (Zhou and Hung, 2003) HER2 gene amplification results in HER2 protein overexpression, which can readily be detected by immunohistochemistry. The highest frequency of HER2 overexpression is found in inflammatory breast cancer (60%) compared to 20-30% in breast cancer in general. (Cooke et al., 2001; Menard et al., 2003; Sjögren et al., 1998) HER2 overexpression is associated with shorter patient survival in breast cancer, at least in node positive patients were the prognostic value of HER2 has been widely demonstrated. (Menard et al., 2003; Sjögren et al., 1998)

The primary interest in HER2 at present is focused on its potential predictive capabilities. Overexpression of HER2 is associated with a worse outcome using tamoxifen therapy, while the aromatase inhibitors letrozole and anastrozole have been demonstrated in small randomized studies to result in higher response rates compared with tamoxifen in patients with receptor positive breast cancer. (Dowsett, 2003; Ellis et al., 2001; Ellis et al., 2003)
Breast cancers overexpressing HER2 has been demonstrated to have better response to anthracyclin-containing chemotherapy (CAF, cyclophosphamide, doxyrubicin, 5-flourouracil) (Di Leo et al., 2002; Paik et al., 1998) HER2 is also a target for therapy. The relatively novel monoclonal anti-HER2 antibody Trastuzumab targets amplified and overexpressed HER2. (Cobleigh et al., 1999) When given as a single agent for first-line treatment of HER2-overexpressing metastatic breast cancer, Trastuzumab is associated with 15 - 40% objective response rates (Cobleigh et al., 1999, Cardoso et al., 2002), and prolongs, in combination with chemotherapy, the overall survival compared with chemotherapy alone. (Slamon et al., 2001)

Determination of HER expression is recommended in all breast cancers to identify patients whom may benefit from anthracyclin-based chemotherapy or trastuzumab, according to the latest update (Bast et al., 2001) of the ASCO clinical practice guidelines. A later, more conservative publication (Goldhirsch et al., 2003) from the International Expert Consenus Panel on the primary therapy of early breast cancer, state that the predictive utility of HER2 for tamoxifen and anthracyclin-based chemotherapy may become clinically useful but need further confirmation.

Urokinase plasminogen activator (uPA)

Degradation of the extracellular matrix is a prerequisite for invasive tumor growth and metastasis. (Duffy, 2002) Urokinase plasminogen activator (uPA) is a 53-kDa protease that converts plasminogen into active plasmin protease. (Andreasen et al., 1997) Plasmin targets a wide range of protein substrates including most components of the extra cellular matrix (ECM). Plasmin can also mediate the activation of latent matrix metalloproteases, which allows further proteolysis of the ECM (reviewed in (Duffy, 2002)). There are several findings from studies in vitro supporting a causal link between uPA activity and cancer invasion and metastasis, including prevention or reduction of metastasis with antibodies or inhibitors against uPA. (Duffy, 1996) In vivo, a large number of retrospective studies have reported shorter disease free survival for breast cancer patients with high uPA activity in their primary tumors (Duffy et al., 1999; Schmitt et al., 1997) The clinical usefulness of uPA (together with it's inhibitor Plasminogen Activator Inhibitor Type-1, PAI-1) has recently been validated with two Level of evidence-1 studies (for definition see page 26) (Janicke et al., 2001; Look, 2000) something still very rare for molecular tumor markers. The first of the studies was a pooled analysis of 18 retrospective datasets including more than 8300 patients. uPA was found to be an independent prognostic marker for outcome, although less powerful than axillary node status. (Look, 2000) The second study was a prospective randomised multicenter study of almost 556 breast cancer patients. (Janicke et al., 2001) The patients' adjuvant treatment was based on the uPA and PAI-1 concentrations in their primary tumors. Node negative patients with low uPA and PAI-1 did not receive any systemic chemotherapy whereas patients with high uPA/PAI-1 where randomised to either observation alone or adjuvant cyclophosphamide-methotrexate-flourouracil (CMF). The recurrence rate was significantly lower in the patients with low uPA/PAI-1, 6,7% versus 14,7% in the high uPA/PAI-1 group. However, the study results on the predictive value of uPa/PAI-1 may be less reliable since 42% (n=133) of the patients in the high risk group (n=315) refused to be randomized. (Janicke et al., 2001) uPa or PAI-1 is at present not recommended for routine clinical use. (Bast et al., 2001)

DNA analysis: S-phase fraction

By measuring the number of cells actively synthesizing DNA by automated DNA analysis with flow cytometry, it is possible to assess the intratumoral fraction of cycling cells in S-phase, thus getting an estimate of proliferation (reviewed in (Fitzgibbons et al., 2000)). Several hundred publications have addressed the relationship between S-phase fraction (SPF) and outcome, other prognostic factors and various technological issues with flow cytometry. (Wenger and Clark, 1998) A consensus conference in 1993 reviewing 43 reports on the clinical utility of SPF concluded that the literature supports an association between SPF and breast cancer patient outcome, despite lack of standardization and suboptimal SPF measurement in the reviewed studies. (Hedley et al., 1993) In a second review of 237 reports by Wenger & Clark (1998) (Wenger and Clark, 1998) they made similar conclusions, high SPF is generally associated with worse outcome, but standardization and quality control must be improved before SPF can be recommended for general clinical use. The overall median SPF ranged between 2,6% and 4,8% in diploid tumors and between 7,6% and 17,3% in aneuploid tumors. The corresponding median cut-points for high SPF was 5,0% and 11,0% in diploid and aneupliod cancers, respectively. It has later been demonstrated that interlaboratory agreement can be improved using standardization guidelines. (Baldetorp et al., 2003)

Malmström, et al (2001) have suggested that SPF may histological grade as selection instrument for adjuvant treatment in lymph node negative breast cancer, after observing better association between SPF (single cut-point for high SPF, ≥12%, overall survival relative hazard 5,7, p=0,005) and outcome than Nottingham Prognostic Index (NPI) in a prospective study on premenopausal lymph node negative patients. SPF is a potential predictive marker for chemotherapy in breast cancer. Studies have reported better response for both CMF (Stal et al., 1994) and FEC (Hietanen et al., 1995) in patients with high SPF. At present however SPF is not recommended for routine clinical use according to the ASCO guidelines on prognostic and predictive tumor markers for breast cancer. (Bast et al., 2001)

DNA ploidy analysis

DNA ploidy analysis measurers the degree of DNA content abnormality. The normal cell non proliferating cell contain two sets of homologous chromosomes, it is *diploid.* Abnormal DNA content is called aneuploidy. Measuring DNA content with flowcytometry give an estimate of the nuclear DNA content (reviewed (Fitzgibbons et al., 2000)) The flowcytometry DNA content analysis also allows for assessment of SPF, and it is generally agreed that the two are strongly positively correlated. (Wenger and Clark, 1998) Flow cytometry DNA ploidy analysis suffer from the same potential errors as SPF such as confounding by dilution of the tumor sample by normal cells or cell debris and interpretation issues. (Fitzgibbons et al., 2000) The prognostic value of DNA ploidy has not been validated so far.

The DNA Cytometry Consensus Conference in 1993 (Hedley et al., 1993) concluded that DNA ploidy status fail to achieve independent prognostic status in multivariate analysis, but there are later reports supporting independent prognostic value of DNA ploidy analysis locally advanced breast cancer. (Pinto et al., 2003) There is no general recommendation for clinical use of DNA ploidy analysis. (Bast et al., 2001)

Cyclin E

The cell cycle regulatory protein cyclin E has recently been described as a strong prognostic factor E for breast cancer. Keyomarsi et al (Keyomarsi et al., 2002) reported that patients with high cyclin E values had more than thirteen times higher risk for breast cancer caused death (Relative hazard 13,3 95%CI: 5,8-30,2) compared with patients with low cyclin E. In addition, among the 112 stage I patients in the study none of the 102 patients with low cyclin E had died of breast cancer at five years follow up, whereas all 12 patients with high cyclin E had died from breast cancer within that period. In multivariate analysis, including lymph node, high cyclin E was the strongest prognostic marker.

When reviewing the literature on cyclin E and breast cancer outcome, conflicting results have been reported (Table 2). Nine (Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kuhling et al., 2003; Lindahl et al., 2003; Loden et al., 2002; Nielsen et al., 1996; Porter et al., 1997; Rudolph et al., 2003) out of twelve (Bukholm et al., 2001; Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kim et al., 2001; Kuhling et al., 2003; Lindahl et al., 2003; Loden et al., 2002; Nielsen et al., 1996; Porter et al., 1997; Rudolph et al., 2003; Span et al., 2003) studies have observed statistically significant association between cyclin E and overall outcome. In four (Han et al., 2003; Keyomarsi et al., 2002; Lindahl et al., 2003; Porter et al., 1997) of nine (Bukholm et al., 2001; Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kuhling et al., 2003; Lindahl et al., 2003; Porter et al., 1997; Rudolph et al., 2003) multivariate survival analyses did cyclin E remain statistically significant (Table 2).

Keyomarsi et al (Keyomarsi et al., 2002) proposed that studies that does not determine cyclin E by methods that detect both full length cyclin E and the shorter isoforms, may underestimate the association between cyclin E and outcome. The five short cyclin E isoforms (size 34 to 49 kDa) are generated by proteolytic cleavage. They lack the amino terminus and are biologically hyperactive in inducing progression from G1 to S-phase. (Porter et al., 2001) Keyomarsi et al (Keyomarsi et al., 2002) used Western Blot with the HE12 mouse monoclonal anti-cyclin E antibody, which is directed at the C-terminal of the cyclin E protein. The use of cyclin E in clinical breast cancer management is not recommended by the ASCO guidelines. (Bast et al., 2001)

Table 2 Overview of the reported prognostic value of cyclin E

Study	No.cases		Cyclin E method	Univariate OS	Multivariate OS
Nielsen et al, 1996	100	Stage I-III	IHC	0,0002	N/A
Porter et al, 1997	278	Node negative	IHC*	0,001	RH 2,4 (1,1–5,2)
Bukholm et al, 2001	137	Unselected	IHC***	0,40	p=0,57, RH N/A
Kim et al, 2001	128	I-III	IHC*	0,32	N/A
Donnellan et al, 2001	157	Unselected	IHC	<0,0001	NS
Lodén et al, 2002	113	Stage I-IV	WB*	0,011	N/A
Keyomarsi et al 2002	395	Stage I-IV	WB*	0,0001	RH 4,3** (95% CI 2,2-8,4)
Lindahl et al, 2003	270	Stage I-IV	IHC*	0,0002	RH 2,4 (CI 1,3-4,5)
Kühling et al, 2003	332	Node negative	IHC	<0,0001[1]	NS
Rudolph et al, 2003	273	Node negative	IHC	0,0006/N.S[2]	NS
Span et al, 2003	277	Unselected	RT-PCR*	0,54	NS/RH 3,0***
Han et al, 2003	175	Node negative	TA-IHC*	0,028	RH 2,7 (95% CI 1,4-3,5)

Abbreviations: Overall survival (OS), Immunohistochemistry (IHC), Western blot (WB), Real time polymerase chain reaction (RT-PCR), Relative Hazards (RH), Confidence interval (CI), Not significant (NS), Tissue microarray immunohistochemistry (TA-IHC)

*Used antibody/method estimates content of both full length Cyclin E and shorter isoforms.

**Breast cancer specific HR 13,3 (95% CI 5,8-30,2)

***Type of anti-cyclin E antibody not disclosed.

**** Tamoxifen treated patients with high Cyclin E (HR 3,0, 95%CI 1,3-7,1). No statistically significant association for OS total study population.

1.Breast cancer specific survival

2. Breast cancer specific survival. p=0,0006 in postmenopausal patients. p=0,82 in premenopausal patients.

Ki67 (MIB-1)

The Ki67 protein is a proliferation marker expressed in cycling (non-G0) cells in the G1, S, G2 and M-phases of the cell cycle. Ki67 is a large nuclear protein (395kDa) and has been hypothesized to be involved in several different cellular functions such as cell cycle regulation, ribosomal RNA processing, organizing DNA or to have a structural role in the within the nucleus (reviewed in (Scholzen and Gerdes, 2000)). The function of Ki67 is still undefined. (Brown and Gatter, 2002)

Ki67 has been shown to worse patient outcome in a wide variety of malignancies such as breast cancer, sarcomas, lymphomas and gliomas. (Scholzen and Gerdes, 2000) The original monoclonal Ki67 antibody presented by Gerdes et al (Gerdes et al., 1983) in 1983 only works on frozen tumor sections why several additional monoclonal antibodies against the Ki67 antigen have been produced for use on paraffin embedded tumor specimens. (Rose et al., 1994) The most well-documented of these antibodies is the monoclonal murine antibody MIB-1, which has higher sensitivity for detecting Ki67 than other comparable antibodies. (Rose et al., 1994) A large number of published studies have observed significant association between Ki67 expression and clinical outcome for breast cancer, (reviewed in (Brown and Gatter, 2002)) although opposite findings also published (reviewed in (Daidone and Silvestrini, 2001))

Treatment predictive value of Ki67 has been described, such as better response to methotrexate and 5-flourouracil for patients with low Ki67 expression. (Sjöstrom et al., 2000) , although this study investigated the Ki67 status in the primary tumors, despite that the clinical study was on patients with metatstatic disease. A better response to neoadjuvant anthracyclin-based chemotherapy has been described for patients with high Ki67. (Faneyte et al., 2003) Overall the total evidence value of the reports addressing the prognostic and predictive value of Ki67 is not yet considered sufficient to warrant recommendation for clinical use of Ki67. (Bast et al., 2001)

Evidence value and clinical utility

Although there is a large number of breast cancer tumor markers that have been reported to have prognostic and/or predictive value, only three (ER, PgR and Her-2/neu) are recommended to be analyzed in primary breast cancer according to the guidelines by the American Society of Clinical Oncology (ASCO). (Bast et al., 2001)

At the time of the latest update of the ASCO guidelines, the documentation for all other markers were not considered satisfactory for recommendation for general clinical use. (Bast et al., 2001) The main factor behind the very conservative recommendations by the ASCO expert panel is the often low evidence value of the tumor markers studies. One may also add that data obtained from some European countries, especially the Nordic countries with population based cancer registry data, may not be fully appreciated by scientists working in an environment with no possibility to obtain population based data. Accordingly, the judgement of some studies and markers could have been different. Pharmaceutically driven clinical studies in general have thorough study designs to achieve highest possible level of evidence, in order to reach the goal of drug approval as fast as possible. In order to possible speed up the process for a tumor marker to be either fully recommended or rejected for clinical use, several of the authors of the ASCO guidelines have proposed a consensus system to study and evaluate the clinical utility of individual tumor markers, the Tumor Marker Utility Grading Scale (TMUGS). (Hayes et al., 1996)

One of the two principal components in the TMUGS system is determination of the reliability of the evaluated tumor marker data. The reliability is classified according to five levels of evidence (for definitions see Table 3).

After assessment of the level of evidence according to the TMUGS system, the author should then suggest the clinical utility of the studied marker by a six-grade scale (Table 4).

Table 3. Levels of Evidence for Grading Clinical Utility of Tumor Markers

Level	Type of evidence
I	Evidence from a single high-powered prospective study that is specifically designed to test marker or evidence from meta-analysis and/or overview of Level II or III studies. In the former case, the study must be designed so that therapy and follow-up are dictated by protocol. Ideally, the study is a prospective randomized trial in which diagnostic and/or therapeutic clinical decisions in one arm are determined based at least in part on marker results, and diagnostic and/or therapeutic clinical decisions in control arm are made independently of marker results. However, may also include prospective but not randomized trials with marker data and clinical outcome as primary objectives.
II	Evidence from study in which marker data are determined in relationship to prospective therapeutic trial that is performed to test therapeutic hypothesis but not specifically designed to test marker utility (i.e., marker study is secondary objective of protocol). However, specimen collection for marker study and statistical analysis are prospectively determined in protocol as secondary objectives.
III	Evidence from large but retrospective studies from which variable numbers of samples are available or selected. Therapeutic aspects and follow-up of patient population may or may not have been prospectively dictated. Statistical analysis for tumor marker was not dictated prospectively at time of therapeutic trial design.
IV	Evidence from small retrospective studies that do not have prospectively dictated therapy, follow-up, specimen selection, or statistical analysis. May be matched case controls, etc.
V	Evidence from small pilot studies designed to determine or estimate distribution of marker levels in sample population. May include "correlation" with other known or investigational markers of outcome, but not designed to determine clinical utility.

Modified from Hayes et al. *J Nat Cancer Inst.* 1996; 88:1456-66

Table 4. Tumor Marker Utility Grading Scale (TMUGS)

Utility scale	Explanation of scale
0	Marker has been adequately evaluated for a specific use and the data definitively demonstrate it has no utility. The marker should not be ordered for that clinical use.
NA	Data are not available for the marker for that use because marker has not been studied for that clinical use.
+/-	Data are suggestive that the marker may correlate with biological process and/or endpoint, and preliminary data suggest that use of the marker *may* contribute to favourable clinical outcome, but more definitive studies are required. Thus, the marker is still considered highly investigational and should not be used for standard clinical practice.
1	Sufficient data are available to demonstrate that the marker correlates with the biological process and/or endpoint related to the use, and that the marker results might effect favourable clinical outcome for that use. However, the marker is still considered investigational and should not be used for standard clinical practice, for one of three reasons: 1. The marker correlates with another marker or test that has been established to have clinical utility, but the new marker has not been shown to clearly provide any advantage. 2. The marker may contribute independent information, but it is unclear whether that information provides clinical utility because treatment options have not been shown to change outcome. 3. Preliminary data for the marker are quite encouraging, but the level of evidence is lacking to document clinical utility.
2	Marker supplies information not otherwise available from other measures that is helpful to the clinician in decision making for that use, but the marker cannot be used as sole criterion for decision-making. Thus, marker has clinical utility for that use, and it should be considered standard practice in *selected* situations.
3	Marker can be used as the sole criterion for clinical decision-making in that use. Thus, marker has clinical utility for that use, and it should be considered standard practice.

Modified from Hayes et al. *J Nat Cancer Inst.* 1996; 88:1456-66.

AIMS OF THE STUDY

I The primary aim was to determine the association between VEGF expression and TP53 status according to cDNA based gene sequence data, overexpression TP53 protein determined by immunohistochemistry and Luminometric Immunoassay in 224 breast cancer patients. The secondary aims were to investigate the clinical relevance of VEGF expression, alone and in combination with TP53 status according to cDNA based gene sequence data and overexpression TP53 protein determined by immunohistochemistry.

II The primary aim was to investigate the potential association between Cyclin E expression and TP53 status according to cDNA based gene sequence data, overexpression TP53 protein determined by immunohistochemistry. The secondary aims were to investigate prognostic properties of Cyclin E expression and potential associations between Cyclin E expression, and the expression of several previously determined known prognostic breast cancer markers.

III The primary aim of the study was to investigate the relationship between expression of p21 and MDM-2 with TP53 gene status determined by immunohistochemistry and sequencing, respectively.

IV The primary aim of the study was to compare and validate KI-67 in relation to S-Phase fraction and other breast cancer prognostic markers in relation to patient outcome.

PATIENTS AND METHODS

Patients

All studies in the present thesis were performed on a patient material consisting of breast cancer patients whom underwent surgery for primary breast cancer in the Uppsala County, Sweden. Breast cancer tumor material and clinical information for these patients was gathered in 1994 to perform a study on TP53 mutation detection by cDNA based sequencing of the entire coding region of the TP53 gene. (Bergh et al., 1995; Norberg, 2000) A population based approach was used aiming at including all 484 female patients diagnosed with breast cancer within the region during 1987-1989. Due to the requirement for available fresh frozen tumor tissue from the archives at the Department of Pathology, Uppsala Universtity Hospital, for the primary TP53 analyses 168 could not be included. Four of the included patients had inconclusive TP53 sequencing results and for one patient the survival data was not available, leaving 311 patients in the final patient material with known TP53 gene status. For detailed patient characteristics for all 311 patients see Table 5.

Table 5. Patient characteristics

	Study population	
n	311**	
Age at diagnosis (median)	63 years	
Primary tumor size (median)	20 mm	
Menopausal status		
Postmenopausal	189/234	81%
Premenopausal	42/234	18%
Perimenopausal	3/234	1%
TP53 mutated	69/311	22%
Estrogen receptor positive	241/304	79%
Adjuvant polychemotherapy*	34/308	11%
Adjuvant tamoxifen therapy	95/308	31%
No systemic adjuvant treatment	179/308	58%
Breast cancer recurrence	115/310	37%
Fatal outcome, any cause	136/311	44%
Fatal outcome, breast cancer	74/311	24%

*Cyclophosphamide, methotrexate and 5-flourouracil (CMF) except in single cases.

**Total numbers less than 311 are due to missing values

Therapy and follow-up

Information regarding clinical stage at the time of diagnosis, adjuvant therapy, length of disease free survival, overall survival time and cause of death were obtained from the patient records in 1994. A second follow up was performed in late 1999 reviewing the patient records to detect any additional recurrences within the patient population. Also, a new follow-up on overall and breast cancer specific survival was performed using the national population registry and the cause of death registry.

Surgery

All patients where operated. Primary surgical treatment consisted of breast conserving sector resection or modified radical mastectomy. (Sjögren, 1997) Axillary dissection was performed in all but 13 cases where the procedure was regarded inappropriate due to advanced age or co-existing serious illness.

Adjuvant therapy

Loco-regional postoperative radiotherapy was offered to all patients with node positive disease, sector resection, radical modified mastectomy and primary tumor diameter larger than 2cm located in the medial quadrant of the breast. Nineteen node positive patients did not receive adjuvant radiotherapy due to advanced age and/or concomitant disease or metastatic disease. Six node positive patients did not receive radiotherapy as part of a randomised study for patients with T2 tumors less than 3cm. (Bergh et al., 1995)

All premenopausal node positive patients were generally recommended adjuvant polychemotherapy with 6 to 9 cycles of cyclophosphamide, methotrexate and 5-flourouracil (CMF). All postmenopausal patients, irrespective of receptor status, with positive lymph nodes were routinely recommended antihormonal adjuvant treatment with tamoxifen. Single patients were not treated according to these guidelines and received other cytostatics due to locally advanced or metastatic disease or did not receive systemic adjuvant therapy. (Bergh et al., 1995) For three patients information on adjuvant treatment was not available.

The patients were normally followed by regular outpatient visits at least 5 years but often until 10 years after primary treatment. Blood tests and X-ray procedures were performed when clinically indicated.

Laboratory methods

TP53

The procedures behind the TP53 determination are described in detail in the primary TP53 reports. (Bergh et al., 1995; Sjögren et al., 1996) In brief, RNA was isolated from fresh frozen tumor tissue that had been stored at –70 °C since the primary surgical event. The tumor RNA was enzymatically converted into cDNA by a reverse transcriptase. The TP53 gene region was amplified from the tumor cDNA using the polymerase chain reaction (PCR) with four overlapping primers covering the entire coding region (exon 2 – 11) of the gene. After solid phase sequencing the sequence products were analysed using an automated laser fluorescence (ALF) DNA sequencer. Finally the complete sequence was compared with the wild-type TP53 sequence in order to detect any aberrations. All mutations were verified by re-amplification of the corresponding cDNA and sequencing of the new PCR product. In addition to the TP53 sequencing analysis the immunohistochemical (IHC) expression of TP53 protein was determined using the mouse monoclonal anti-TP53 antibody 1801 (Biozac AB, Järfälla, Sweden) on paraffin embedded primary tumor samples. (Sjögren et al., 1996) The immunostaining was performed in a Ventana ES Automated Immunohistochemistry Instrument (Annex, Helsinki, Finland).

Vascular Endothelial growth Factor (VEGF)

As part of a study on TP53 determination using the Luminometric Immunoassay (LIA) by Norberg et al (Norberg et al., 1998) fresh tumor cytosols was prepared from fresh frozen tumor tissue from 226 patients in the original study population. Out of these patients 224 had sufficient remaining tumor cytosol to allow analysis of VEGF content. A commercially available quantitative immunoassay kit for human $VEGF_{165}$ (Quantikine, human VEGF, R & D Systems, Minneapolis, MN, USA) was used to measure the intratumoral VEGF content. One hundred microliter of the cytosol sample diluted in 100µL buffer solution was added to a 96-well microtiter plate precoated with monoclonal mouse anti-human VEGF antibody and incubated at room temperature for 2 hours. A serially diluted standard solution (human VEGF, range 0 – 2000 pg/ml) was also added to the plate, before incubation, as reference. After incubation and subsequent washing a VEGF-specific polyclonal goat antibody was added and incubated for 2 hours. After the second incubation substrate solution was added and allowed to react for 25 minutes. The optical density was then measured with a microtiter plate reader (Multiscan MCC/340, Labsystems) at 450nm. The VEGF content in the cytosols was expressed as pg VEGF protein/mg total cytosol protein.

Cyclin E

The content of Cyclin E protein was determined by IHC on paraffin embedded primary breast cancer samples. Two hundred and seventy out of the original 311 patients had remaining unused paraffin sections derived from the archives at the Department of Pathology, Uppsala University Hospital, Uppsala, for Cyclin E analysis. The paraffin sections were deparaffinized and microwave treated. Immunostaining was performed using the monoclonal mouse anti-Cyclin E antibody HE 12 (Santa Cruz Inc., U.S.A.) and an automated Immunohistochemistry-staining machine (Ventana 320-202, Ventana Inc., AZ, U.S.A.). The IHC reactivity were divided into three levels, before statistical analysis, according to the percentage of tumor cells stained; low (0-4%), medium (5-49%) and high (50-100%). The cut-off levels were chosen in order to achieve distinct separation between patients with high and low Cyclin E expression. This selection was done before any statistical analyses were performed. All glasses were read without knowledge of previously determined tumor characteristics or patient outcome.

p21(waf1/Cip1) and MDM-2

Immunohistochemistry and paraffin embedded tumor samples was also used for determination of p21(waf1/Cip1) and MDM-2. Out of the 311 original patients 276 and 257 had tumor material available for IHC determination of p21 and MDM-2, respectively. The tumor sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water and then microwave treated in citrate buffer. Immunostaining was performed using a commercial Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) directed against mouse IgG. Blocking serum was applied for 15 minutes followed by overnight incubation with the diluted monoclonal primary antibody MDM-2 1:100 (clone IF2, Oncogene Research products, Cambridge, MA), p21 1:200 (WAF1 protein, clone 4D10, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). The sections were then incubated with the biotinylated second antibody and the peroxidase-labelled ABC for 30 minutes each. Sections stained with p21 and mdm2 were further stained with biotinyl tyramide and streptavidin conjugated to horseradish peroxidase, using these two reagents from a commercial amplification kit (Catalysed Signal Amplification, CSA, Dako, Glostrup, Denmark). Bound peroxidase was visualized in all slides with a 3-amino-9-ethyl-carbazole (AEC) solution. Finally, the sections were lightly counterstained in Mayer´s hematoxylin and mounted in Aquamount Mountant (BDH Ltd, Poole, United Kingdom).

Known positive sections for MDM-2 and p21 were included in every staining batch as positive controls. Slides stained with PBS substituting the primary antibody were used as negative controls. An experienced pathologist, without knowledge of previously determined tumor characteristics or patient outcome read all glasses. All breast cancers with nuclear p21 or MDM-2 immunostaining were regarded as positive. The percentage of tumor cells with positive nuclear staining was also noted for each slide.

Ki-67

Ki-67 immunostaining was performed on 4 µm thick serial sections from paraffin-embedded primary tumor tissue blocks. KI-67 was possible to determine in 305 out the original 311 patients. The paraffin sections were deparaffinized with xylene and pretreated for antigen retrieval with boric acid at 85° C for 16 hours. Staining was performed using the monoclonal murine anti-Ki67 E antibody MIB-1 (diluted 1:100) and an automated immunohistochemistry-staining machine (TechMate ™ Horizon, Daco). After immunostaining for KI-67 all glasses were evaluated by an experienced pathologist without knowledge of patient outcome or previously determined tumor markers. All breast cancer cells with positive nuclear KI-67 immunostaining were regarded as positive. In the 50 first cases KI-67 index were determined both with individual cell counting and with a grid graticula based method. The grid graticula method is based on a microscope grid with 25 randomly placed dots. The observer selects a region in the tumor section with as many positive cells as possible (hot spot) and subsequently rotate the grid in order to place the grid dots on as many positive tumor cells as possible. The tumor is scored according to the number of hits (0-25). We observed a high correlation between individual cell count and the grid graticule method ($p<0.001$; $r=0.89$), and for the following samples only the graticula method was used. All breast cancers were divided into three subgroups according to their KI-67 index score. We used the quartile distribution of KI-67 index score for subgroup classification; Low KI-67, quartile 1, Medium KI-67, quartile 2-3, High KI-67, quartile 4. Cut off values from low to high KI-67: 2, >2 to <6 and 6, respectively.

RESULTS AND DISCUSSION

Summary of paper I

The primary aim of study I was to investigate the potential association between VEGF Cyclin E expression and TP53 status, while the VEGF expression has been claimed to be partly regulated by TP53. We examined the expression of VEGF in 224 breast cancers (Table 6) from an original patient population of 315 patients from Uppsala. Cytosols from the fresh frozen tumor material were analysed for $VEGF_{165}$ content using a commercially ELISA-kit (Quantikine, human VEGF, R&D Systems, Minneapolis, MN, USA). The VEGF expression was compared with available TP53 data from cDNA based gene sequencing, TP53 data from a monoclonal antibody (Mab DO1 & 1801) based technique - luminometric immunoassay, and TP53 immunohistochemistry (Mab 1801). The patient VEGF level was classified as either high or low according to the median VEGF value (256,4pg/mg total protein) for the study population.

Table 6. Characterization of the 224 patients

Clinical parameters	Patients in study (n = 224)
Lymph node	
Negative	154
Positive	62
Positive/total	0.27
Tumor size	
Median	20
Mean	22
Range	2–65
S-phase fraction	
Low	172
High	43
High/total	0.19
Cut-off diploid/aneuploid (%)	7/12
ER (.0.1 fmol/mg DNA)	
Median	1.3
Mean	5.5
Range	0–207
PgR (.0.1 fmol/mg DNA)	
Median	1.5
Mean	9.0
Range	0–521
TP53 mutation frequency (%)	16.5

We observed statistically significant correlations between increased VEGF expression and mutated TP53. This finding was consistent for all methods for TP53 determination, although the strongest correlation was found between high VEGF and mutated TP53 detected with cDNA based sequencing. Seventy-three percent (n=27) of the patients with mutated TP53 had VEGF levels above median compared to 45,5% (n=85) in the wild-type TP53 group. The type of TP53 mutation also had impact on the VEGF content. Breast cancers with insertions, deletions or stop codon point mutations had increased frequency of high VEGF content compared to breast cancers with TP53 point mutations, 87,5% (n=7) vs 69% (n=20, p=0,004), respectively (Table 7).

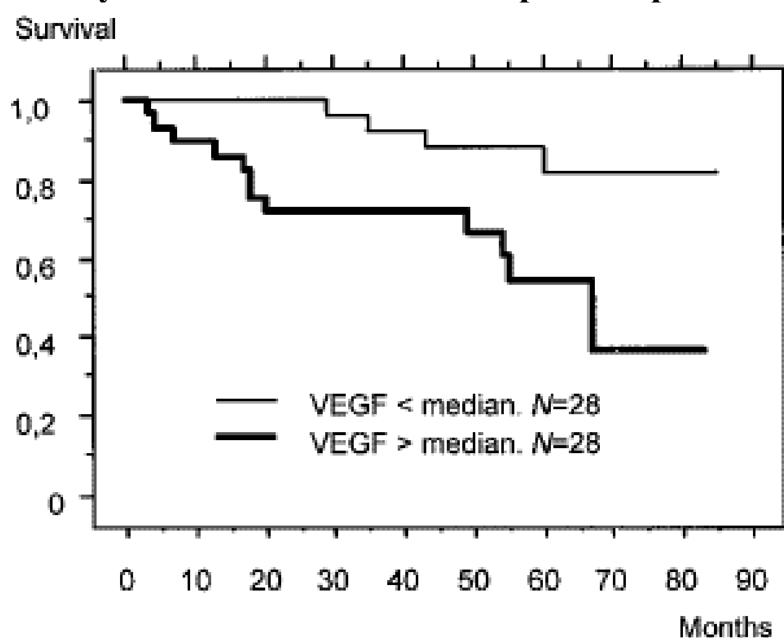
Table 7. Association between VEGF content and TP53 status according to sequence data and IHC data

	VEGF < median		VEGF > median		p-value
TP53 sequence based					
Mutant n = 37	n = 10	27,0%	n = 27	73,0%	0,0019
Wild-type n = 187	n = 102	54,5%	n = 85	45,5%	
Point mutations n = 29	n = 9	31,0%	n = 20	69,0%	0,0043
Severe mutations n = 8	n = 1	12,5%	n = 7	87,5%	
TP53 IHC based					
Positive n = 39	n = 12	30,8%	n = 27	69,2%	0,0062
Negative n = 183	n = 183	54,7%	n = 27	45,3%	

Such mutation types have a strong negative effect on TP53 translation, producing truncated protein or no protein at all, depending on the mutation-site. As shown by Klaar (formerly Sjögren) et al (Sjögren et al., 1996) the majority of breast cancers with this genotype has undetectable TP53 when determined by immunohistochemsitry (IHC). In this study all (of the few) patients with insertions/deletions or stop codon TP53 mutations where TP53 IHC negative.
The association between TP53 and VEGF has been observed earlier in vitro (Dameron et al., 1994; Mukhopadhyay et al., 1995; Pal et al., 2001) but to our knowledge no other study has previously confirmed this finding in vivo, using detailed sequencing data covering the whole coding region of the TP53 gene. There are breast cancer studies using other approaches (immunohistochemistry or immunoassay) for TP53 determination that support our finding. (Lee et al., 2002; Linderholm et al., 2000)

We observed a statistically non-significant trend towards shorter overall and breast cancer specific survival for patients with VEGF content above the median value (p=0,09 and p=0,07, respectively). VEGF alone was not an independent prognostic factor for overall or breast cancer specific survival in multivariate analysis. The strongest association between high VEGF and outcome was observed in a subgroup analysis of patients (n=66) whom received adjuvant tamoxifen therapy. Statistically significantly shorter recurrence free survival (RFS, p=0,04) overall survival (OS, p=0,015) and breast cancer specific survival (BCSS, p=0,009) was observed for patients with high VEGF compared with patients with VEGF levels below the median value. In this group 8 patients were found to be receptor negative and 2 had unknown receptor status. Exluding these patients did not alter the result in any significant way (RFS, p=0,04, OS, p=0,013, BCSS, p=0,006) (Figure 2). This finding has been confirmed in another breast cancer material by Linderholm et al., (Linderholm, JCO, 2000) and by a study on tamoxifen treatment in primary ER positive breast cancer patients with metastatic disease which showed VEGF to be a predictive marker for poor treatment response. (Berns et al., 2003) Our data suggests that VEGF adds predictive information to ER. Tamoxifen have an angoigenic effect in vitro by down-regulating transforming growth factor α (TGF α) which is a proangiogenic factor in ER-positive tumors. (Noguchi et al., 1993) Thus, it is possible that ER-positive patients with concomitant high VEGF would benefit from another systemic adjuvant treatment instead of tamoxifen.

Figure 2. Cumulative probability of breast cancer specific survival by VEGF content in 56 ER-positive patients



Breast cancer specific survival, p=0,006

We also used the combination of VEGF and TP53 in order to stratify patients into three risk categories. Low risk: wild-type TP53 and low VEGF content. Intermediate risk: mutated TP53 or high VEGF. High risk: mutated TP53 and high VEGF. Using this stratification method yielded statistically significant differences in RFS, OS and BCSS for respective group, with 5 year overall survival ranging from 79% (low risk) to 50% (high risk). The combination of VEGF and TP53 also gave independent prognostic information for BCSS (Relative hazard 3.0, C.I:95% 1,3-6,95, p=0,01). In summary, the findings mentioned above supports the hypothesis that wild-type TP53 is involved in the regulation of VEGF. Also VEGF determination seems to be of prognostic value for breast cancer, at least in tamoxifen treated patients.

Summary of paper II

With the primary aim to investigate the relationship between cyclin E expression and TP53 gene status we determined cyclin E using immunohistochemistry (Mab HE 12) on breast cancers from 270 patients with known TP53 status from cDNA based sequencing. The HE 12 antibody targets the cyclin E protein's C-terminal and recognizes both the long and the shorter forms of the cyclin E protein without distinction. (Lees et al., 1992) Non-malignant breast cells lack cyclin E immunoreactivity, why IHC is considered valid for estimating cyclin E overexpression. (Scott and Walker, 1997)

High cyclin E protein content (detailed in methods) was observed in 10% (27/270) of all breast cancers and was significantly associated with mutated TP53, aneuploidy, high S-phase, negative ER status, presence of axillary node metastases, higher tumor grade and larger tumor size. The main finding in this study was the association between a high cyclin E content and insertions, deletions and nonsense point mutations in the TP53 gene. Although in small numbers, the predominant types of TP53 gene mutations in breast cancers with a high cyclin E content were insertions or deletions, and the prevalence of these mutation types increased in a dose-dependent manner with increasing cyclin E content (Table 8).

The majority of the TP53 insertions or deletions were found in the peripheral parts of the gene (exon 2-4 or 9-11) (Figure 3). Stop codon point mutations were also more commonly observed in high cyclin E tumors. All but one of seven tumors with a high cyclin E content and TP53 insertions/deletions had undetectable TP53 protein content by IHC, which implicate a strong negative effect on the TP53 protein expression as previously described by Klaar (formerly Sjögren) et al. (Sjögren et al., 1996)

The TP53 mutation pattern in low cyclin E tumors corresponded well to the reported overall frequency of different mutation types in the TP53 gene, with missense point mutations being predominant. (Hartmann et al., 1997; Hartmann et al., 1995; Hussain and Harris, 1998)

Seventy-four percent (200/270) of all breast cancers in this study had aneuploid DNA-content. The frequency of aneuploidy was markedly increased in tumors with a high cyclin E expression and a mutated TP53 gene, which were all aneuploid (n=15). Both wt-TP53/high cyclin E tumors and TP53 mutated/low cyclin E tumors displayed high frequencies of aneuploidy, 92% (1/11) and 96% (1/23) respectively. Deregulated expression of cyclin E affect the G1/S transition resulting in an uncoupling of DNA replication and cell cycle progression. (Mumberg et al., 1996) It is therefore likely that overexpression of cyclin E affect the fidelity of DNA replication and cause genetic instability. Spruck et al (Spruck et al., 1999) demonstrated that constitutive cyclin E overexpression in vitro increased the frequency of aneuploidy in vitro in immortalized rat fibroblasts and human breast epithelial cells. Our results support this finding in a human breast cancer material.

Table 8. Cyclin E in relation to the TP53 mutation pattern and DNA content

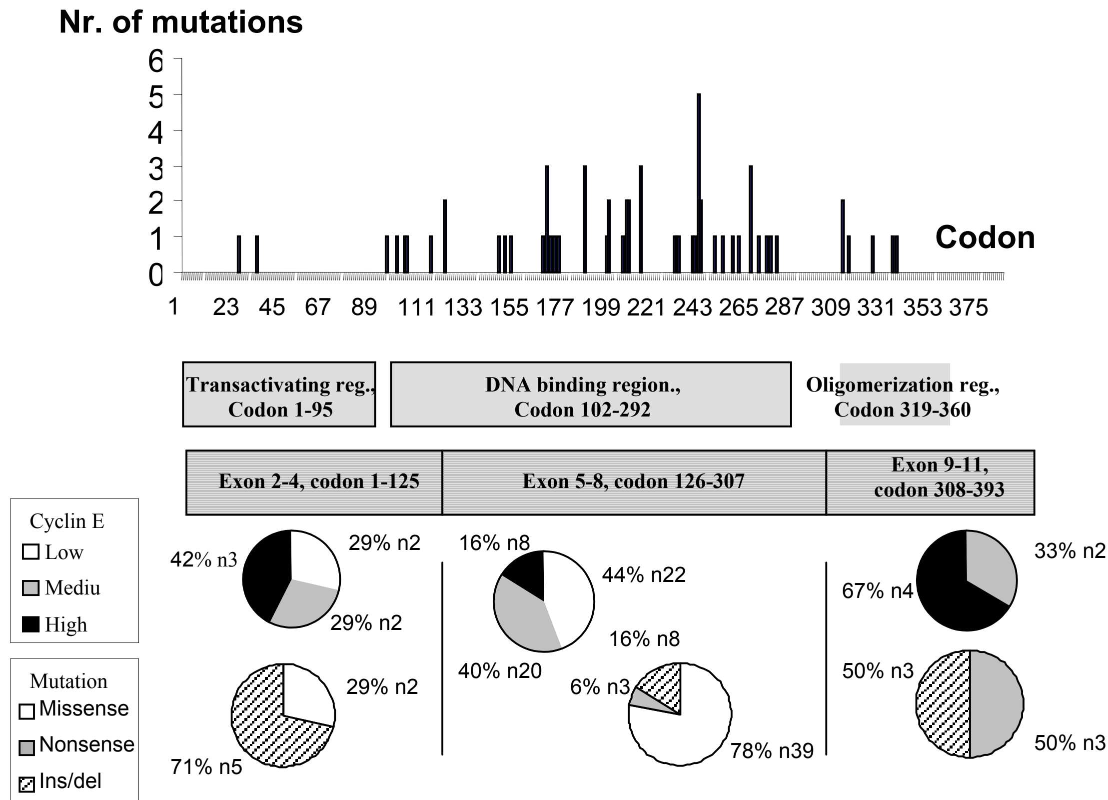
	Cyclin E content						
	Low		Medium		High		
n	171	63% (171/270)	72	27% (72/270)	27	10% (27/270)	p-values***
TP53 wild-type[1]	147	86% (147/171)	48	67% (48/72)	12	44% (12/27)	p<0.0001
TP53 mutation[1]	24	14% (24/171)	24	33% (24/72)	15	56% (15/27)	
TP53 IHC positive	27	16% (27/171)	21	29% (21/72)	8	30% (8/27)	p=0.03
TP53 IHC negative	144	84% (144/171)	51	71% (51/72)	19	70% (19/27)	
TP53 mutation types[1]							
Missense point mutation	20	83% (20/24)	17	71% (17/24)	4	27% (4/15)	p=0.005
Stop codon point mutation	1	4% (1/24)	1	4% (1/24)	4	27% (4/15)	
insertion or deletion	3	13% (3/24)	6	25% (6/24)	7	47% (7/15)	
Mutation inside exon 5-8[2]	22	92% (22/24)	20	83% (20/24)	8	53% (8/15)	p=0.01
Mutation outside exon 5-8[3]	2	8% (2/24)	4	17% (4/24)	7	47% (7/15)	
DNA content:euploid	50	29% (50/171)	19	26% (19/72)	1	4% (1/26)	p=0.02
DNA content:aneuploid	121	71% (121/171)	53	74% (53/72)	26	96% (26/27)	

The median-follow up for overall survival was 122 months. Patients with high cyclin E breast cancer had a statistically significantly reduced overall survival in univariate analysis (p=0.0002) (Figure 4). The 5-year overall survival for the low cyclin E group was 82% compared with 71% in the medium group and 41% for the breast cancers with high cyclin E. At 10 years follow-up, differences in overall survival rates were still present (Figure 4).

A high cyclin E content remained a strong negative prognostic factor for overall survival independent of TP53 status. We performed two multivariate Cox proportional hazard analyses (molecular and clinical variables, Table 9). High cyclin E content was an independent risk factor for shorter overall survival in both models (Relative Hazards 2.1 and 2.4, with corresponding CL95% 1.1-4.1 and 1.3-4.5, respectively).

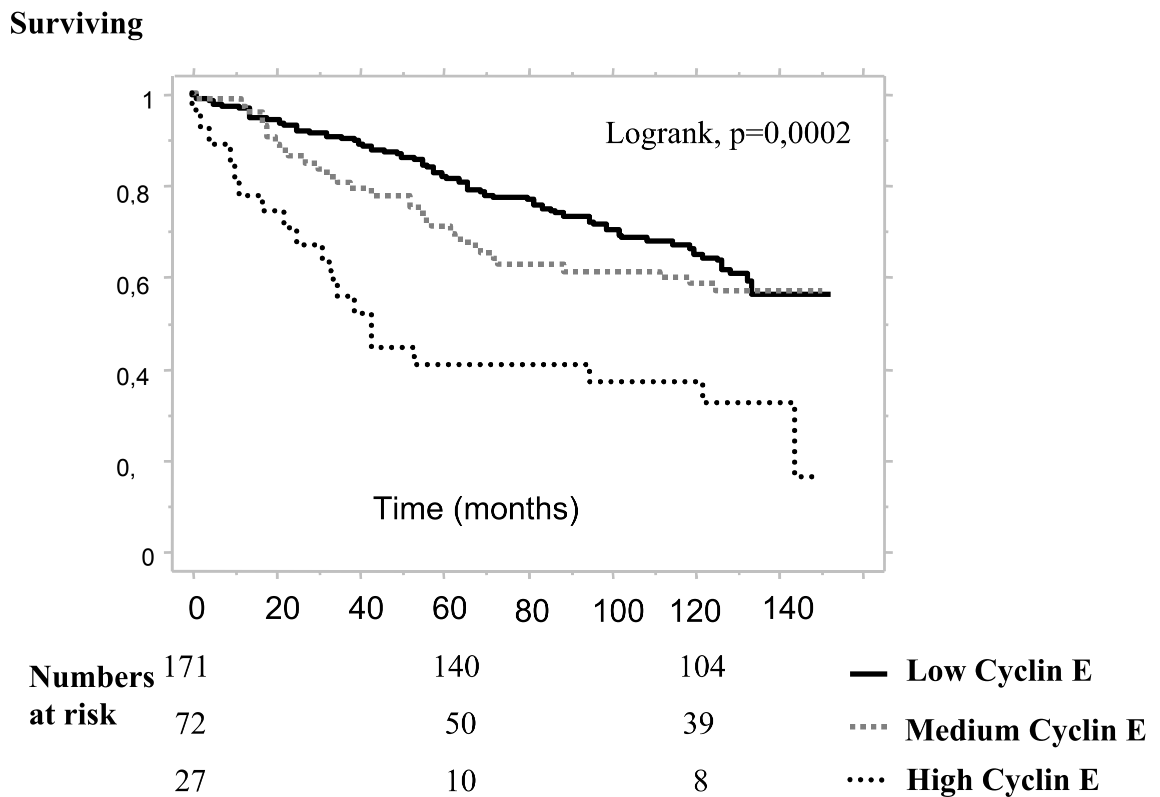
Figure 3. Location and frequency of TP53 mutations by codon number in relation to Cyclin E levels.

The upper pie-charts display the distribution of cyclin E expression divided by the location of the corresponding TP53 mutation. The lower pie charts display the distribution of mutation types by gene location



The reports on the prognostic value of cyclin E found in the literature are heterogenous (Table 2, page 23). The conflicting results have been proposed to be due to inappropriate cyclin E determination which fail to detect the shorter isoforms, thus underestimating the prognostic value of cyclin E. This methodology issue does not likely fully explain the heterogeneous cyclin E study results, since both Kim et al (Kim et al., 2001) and Span et al (Span et al., 2003) reported negative findings regarding cyclin E and overall survival, despite estimating total cyclin E. Span et al (Span et al., 2003) did however observe that high cyclin E predicted worse outcome for tamoxifen treated patients, and proposed that the differences in the literature on the prognostic value of cyclin E may be due to differences in treatment. In the present study we found strong associations between and cyclin E but it can be stated the potential prognostic value of cyclin E needs further evaluation.

Figure 4. Cyclin E and Overall survival in all patients.



In summary, we demonstrated that a high immunohistochemical cyclin E content is associated with insertions, deletions and nonsense point mutations in the TP53 gene, likely to have strong negative impact on the TP53 protein expression. We also observed independent strong associations between cyclin E, mutated TP53 and aneuploidy. Our results suggest that cyclin E is a strong prognostic marker for breast cancer which should be further investigated.

Table 9. Cox Proportinal Hazards multivariate overall survival analyses

Model with Cyclin E and other molecular tumor markers

Variable	Relative Hazard	95% RH Confidence limits	p-value
Low cyclin E (reference)	1.0		
Medium cyclin E	1.1	0.7-1.8	0.54
High cyclin E	2.4	1.3-4.5	<0.01
Mutated TP53 vs. wild-type	1.7	1.1-2.7	0.01
Anueploidy vs. Euploidy	1.1	0.7-1.7	0.68
ER negative vs. Positive	1.1	0.7-1.8	0.76
Age at diagnosis (yrs)	1.1	1.0-1.1	<0.01

Model with Cyclin E and clinical tumor markers

Variable	Relative Hazard	95% RH Confidence limits	p-value
Low cyclin E (reference)	1.0		
Medium cyclin E	1.0	0.6-1.6	0.94
High cyclin E	2.1	1.1-4.1	0.03
Node positive vs. Negative	2.1	1.4-3.2	<0.01
Tumor size (mm)	1.0	1.0-1.0	0.03
Low tumor grade (reference)	1.0		
Medium tumor grade	1.3	0.8-2.3	0.30
High tumor grade	2.2	1.1-4.5	0.03
Age at diagnosis (yrs)	1.1	1.0-1.1	<0.01

Summary of paper III

In study III we investigated if the reliability indirect TP53 mutation screening with TP53 IHC could be improved by concomitant IHC determination of p21(Waf1/Cip1) and MDM-2. The wild-type TP53 protein has a rapid turnover and is under normal conditions only detectable in very low amount in the cell. The majority of TP53 mutations (missense point mutations) result in extended half-life of the TP53 protein, (Ogretmen, 1997) which is the rationale behind indirect TP53 mutation screening protein based methods such as IHC. However, TP53 IHC has been shown to be unable to detect a substantial portion of mutations and furthermore produce a high number of false positive cases. (Sjögren et al., 1996) One theoretically possible way to filter out false cases could be through additional determination of TP53-associated proteins associated with wild-type TP53 gene status, such as p21(waf1/cip1) and MDM-2. We therefore determined the expression of p21 and MDM-2 according to IHC in 276 and 257 breast cancer patients, respectively, and compared the p21 and MDM-2 IHC data with previously determined TP53 IHC status, and cDNA –based sequencing data covering all protein coding exons in the TP53 gene.

We did not find any statistically significant correlations between TP53 gene status and the IHC expression of p21 or MDM-2. Positive immunostaining for both p21 and MDM-2 was frequently observed in breast cancers with a mutated TP53 (Table 10). Twenty-three out of 60 (38%) TP53 mutated tumors displayed p21 expression and 32/46 (70%) expressed MDM-2. As expected by the low correlation between p21 and MDM-2 expression and TP53 gene status in this study, did co-analyses of p21/MDM-2 and TP53 IHC not improve the sensitivity or specificity of TP53 IHC for detection of TP53 mutations.

Thus, determination of the IHC expression of p21 or MDM-2 is not useful for the identification of breast cancers with mutated TP53. The likely biological explanation for our findings is that there are alternate, TP53 independent regulators of p21 and MDM-2. p21 in vitro can be regulated independently of TP53 both on the transcriptional- and posttranscriptional level. (Li, 1996; Zeng, 1996) It has also been reported that the basal transcription of MDM-2 in murine tissue is TP53 independent, except under conditions of genotoxic stress (i.e. ionizing radiation). (Mendrysa, 2000) Our finding that p21 expression occurs in a relatively high frequency (38% 23/60) in tumors with TP53 mutations, have been observed in similar fashion by others investigating p21 expression in breast cancers. (Rey, 1998) Our results implicate TP53 independent regulation of both p21 and MDM-2 in vivo.

Table 10. TP53 gene status and expression of p21 and MDM-2

	p21			MDM-2		
	Positive	Negative	p-value	Positive	Negative	p-value
TP53 cDNA sequence data						
wild-type TP53	82% (103/126)	75% (113/150)	p=0,20	82% (149/181)	82% (62/76)	p=0,89
mutated TP53	18% (23/126)	25% (37/150)		18% (32/181)	18% (14/76)	
TP53 IHC						
Negative	83% (105/126)	78% (117/150)	p=0,27	83% (150/181)	83% (63/76)	p=0,99
Positive	17% (21/126)	22% (33/150)		17% (31/181)	17% (13/76)	
TP53 conserved regions						
Mutation in Cons. Reg. 2 & 5 *(1)*	83% (19/23)	86% (32/37)	p=0,68	88% (28/32)	93% (13/14)	p=0,58
Mutation in Cons. Reg. 3 & 4 *(2)*	17% (4/23)	14% (5/37)		12% (4/32)	7% (1/14)	
TP53 mutation types						
missense point mutation	70% (16/23)	62% (23/37)	p=0,51	63% (20/32)	57% (8/14)	p=0,24
nonsense point mutation	13% (3/23)	8% (3/37)		13% (4/32)	0% (0/14)	
insertion/deletion	17% (4/23)	30% (11/37)		25% (8/32)	43% (6/14)	
TP53 cDNA+IHC data						
wild-type/IHC-negative	48% (96/200)	52% (104/200)	p=0,64	70% (135/194)	30% (59/194)	p=0,38
mutation/IHC-negative	41% (9/22)	59% (13/22)		79% (15/19)	21% (4/19)	
mutation/IHC-positive	37% (14/38)	63% (24/38)	p=0,53	63% (17/27)	37% (10/27)	p=0,16
wild-type/IHC positive	44% (7/16)	56% (9/16)		82% (14/17)	18% (3/17)	

(1): Conserved region, Codon 117-142 & 270-286

(2): Conserved region, Codon 171-181 & 234-258

We observed a strong positive correlation between expression of p21 and MDM-2. Eighty-nine percent (99/111) of all p21 positive breast cancers were MDM-2 positive ($p<0.0001$, Table not shown). This finding was consistent regardless of TP53 status; wild-type TP53: 90% (87 /97), vs. mutated TP53: 86% (12/14). These data indicate a TP53 independent direct or indirect link between p21 and MDM-2. To our knowledge has no such candidate been described. It is possible that further mapping of the cell cycle regulation will reveal a novel connection between p21 and the expression of MDM-2.

Table 11. Probability of overall survival at 5 and 10 years after diagnosis.

The patients are categorized accordning to thier IHC levels of TP53, p21 and MDM-2.

		5 year		10 year		
	n	**Survival**	**C.L.95%***	**Survival**	**C.L.95%**	**p-value**
TP53 IHC positive	126	0,72	0,60-0,84	0,59	0,46-0,73	p=0,52
TP53 IHC negative	150	0,75	0,70-0,81	0,60	0,54-0,67	
p21 IHC positive	54	0,69	0,60-0,77	0,53	0,44-0,62	p=0,03
p21 IHC negative	222	0,80	0,74-0,86	0,66	0,58-0,73	
TP53 IHC-/p21 IHC+	105	0,70	0,61-0,78	0,54	0,45-0,64	p=0.11
TP53 IHC-/p21 IHC-	117	0,80	0,73-0,87	0,66	0,57-0,74	
TP53 IHC+/p21 IHC+	21	0,62	0,41-0,83	0,48	0,26-0,69	
TP53 IHC+/p21 IHC-	33	0,79	0,65-0,93	0,67	0,51-0,83	

*95% Confidence limits

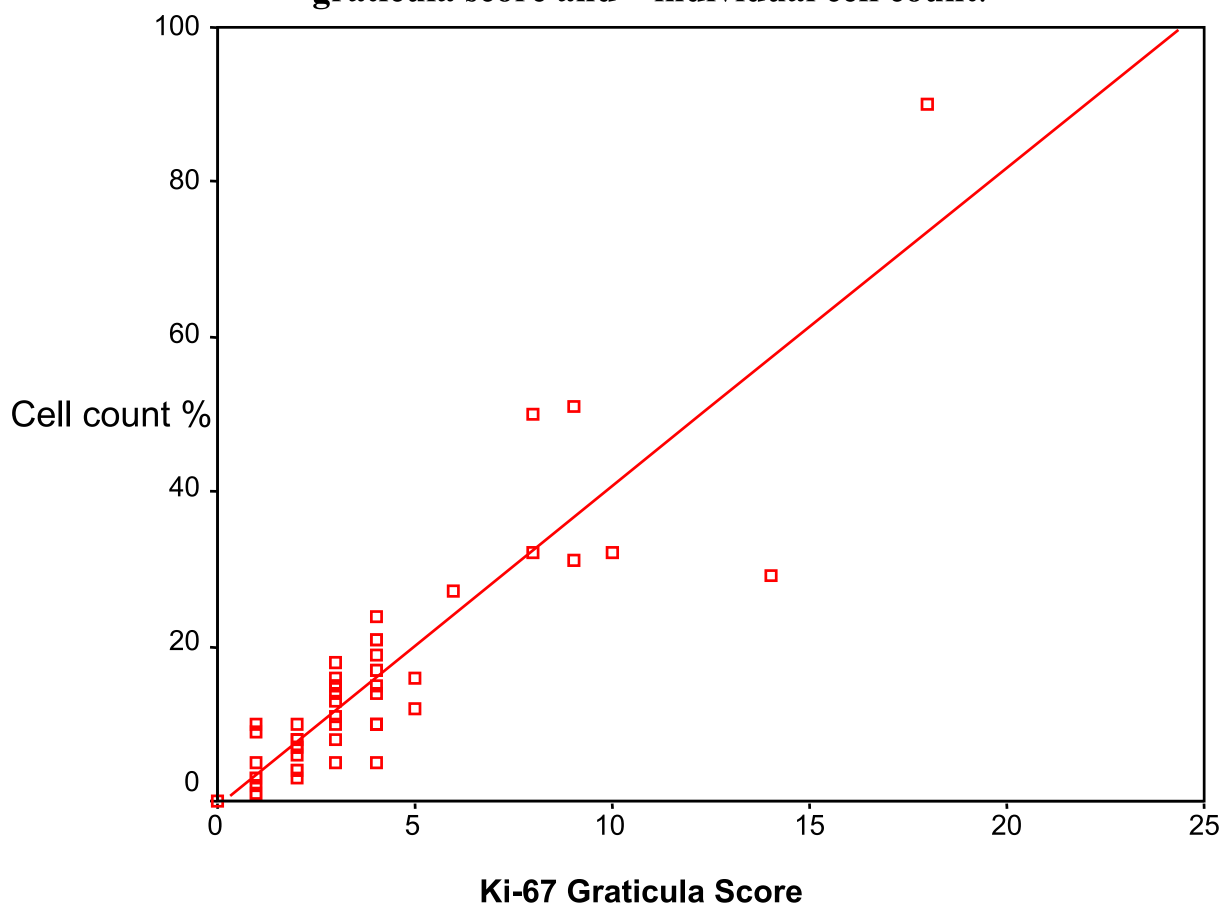
p21 expression was associated with negative outcome in univariate overall survival analysis with a 5 year cumulative probability for overall survival of 68% (0.68, C.I.95% 0.60-0.76) in p21 positive patients vs. 80% (0.80, C.L.95% 0.74-0.86) in negative cases (Table 11). The prognostic value of p21 was not statistically significant in the multivariate survival analysis (Relative Hazard 1,2, 95% CI 0,81 - 1,8, p=0,38). The IHC expression of MDM-2 was not associated with any specific clinical outcome in this study. To our knowledge there is also lack of a mechanistic explanation for a p21 associated negative effect on outcome. On the contrary, studies in vitro suggest that p21 is a likely tumor suppressor candidate due to its important role in TP53 dependent cell cycle arrest. Published reports on the prognostic value of p21 for breast cancer are heterogenous, reporting both favourable (McClelland, 1999) and worse (Thor, 2000) outcome for p21 positive patients. According to our data is p21 not a promising prognostic marker for breast cancer.

In summary, we did not find any statistically significant associations between expression of p21 or MDM-2 and the TP53 gene status. Thus, it is of no benefit to co-analyze the IHC expression of p21 and/or MDM-2 together with TP53 IHC to improve the sensitivity or specificity for TP53 mutation detection.

Summary of paper IV

The aims of this study was to compare and validate determination of Ki-67 (MIB-1) using imunohistochemistry, in relation to S-phase fraction (SPF), other breast cancer prognostic markers and patient outcome in the Uppsala patient material. In addition, we developed a graticula based method for reading the Ki-67 sections, making the method more rapid and user friendly. All samples were possible to analyse. For the first 50 samples we observed a high correlation between individual cell count and the grid graticule method ($p<0.001$; Pearson correlation coefficient =0.89), and for the following samples we only used the graticula method (Figure 5). SPF was determined by flow cytometry on fresh tumor material at the time of diagnosis, as part of the routine clinical management of breast cancer at the Uppsala university hospital. The cut off values for high S-phase were >7% in diploid tumors and >12% in aneuploid tumors. (Sjögren et al., 1996)

Figure 5. Presentation of the linear association between the graticula score and individual cell count.



Pearson correlation coefficient 0,89, p<0,001

We observed a high Ki-67 expression (highest quartile) in 26% (80/305) of the study population. Low (lowest quartile), or medium Ki-67 expression was found in 33% (101/305) and 41% (124/305) of the patients, respectively. The main finding in this study was the high association between high Ki-67 content and the presence of multiple known markers of negative prognosis for breast cancer (Table 12).

Table 12. Significant correlations between Ki-67 expression and other tumor markers

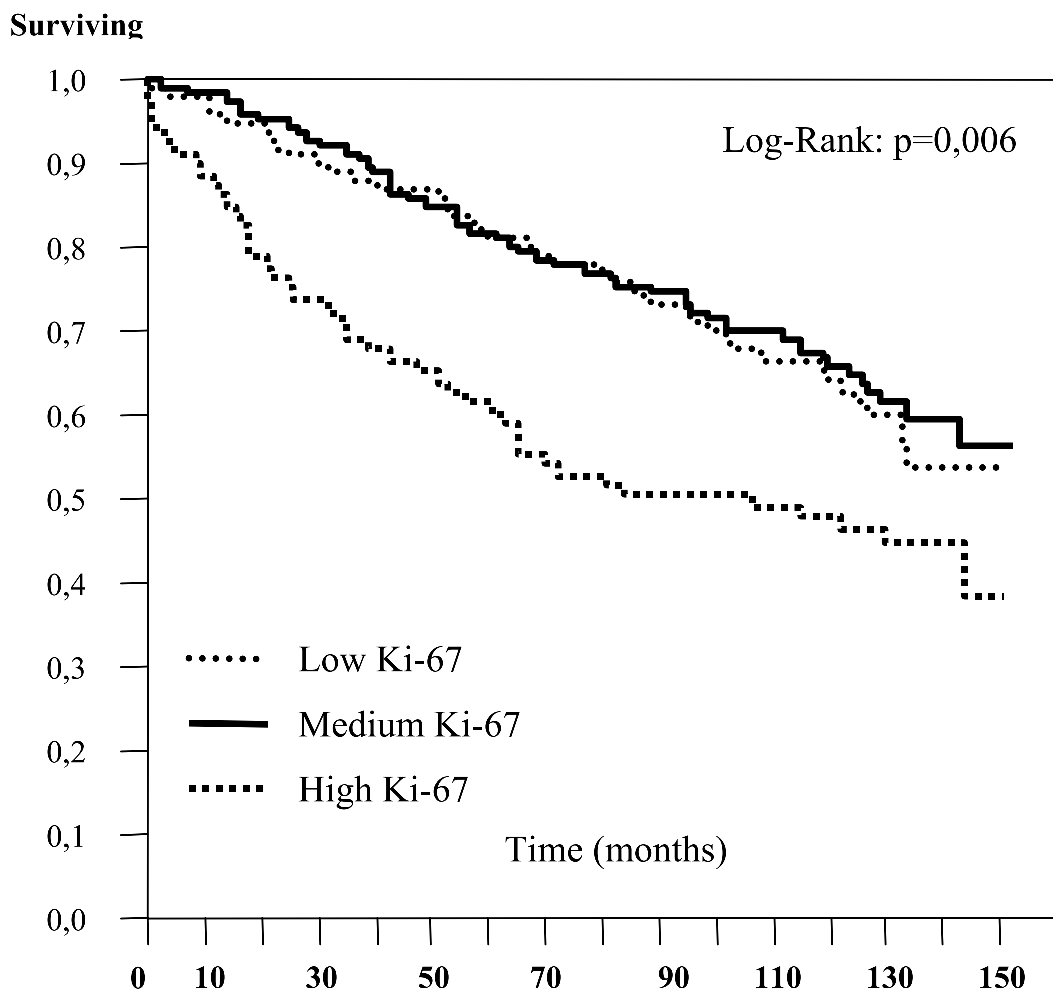
	High Ki-67	
	Pearson C*	p-value
High tumor grade	0.50	<0.0001
TP53 mutation	0.49	<0.0001
High CyclinE	0.48	<0.0001
ER negative	0.37	<0.0001
high VEGF	0.30	<0.0001
Premenopausal status	0.27	0.0001
aneupliody	0.26	<0.0001
High S-phase	0.17	0.0029
Larger tumor size	0.17	0.0098
Axillary node positive	0.17	0.0028
PgR negative	0.15	0.0103
HER2	0.13	0.0204

*Pearson correlation coefficient

The strongest correlations were observed between KI-67 and high tumor grade (r=0.50), TP53 mutations (r=0.49) and high cyclin E (R=0.48) (Table 12). High KI-67 expression was associated with high SPF (p=0.0029), but with a relatively weak linear correlation (r=0.17, Table 12). In addition, when comparing KI-67 and SPF according to the quartile KI-67 stratification, with SPF, no formal statistical difference was observed (p=0.27). A possible explanation for the relatively low level of concordance between the proliferation markers SPF and KI-67, is that SPF is a much more specific marker than KI-67. KI-67 is present in all phases of the cell cycle (G1, S, G2 and M) whereas SPF more specifically detects cells in S-phase by their DNA content. (Gerdes et al., 1984; Wenger and Clark, 1998) However, other studies have observed stronger correlation between KI-67 and SPF in breast cancer, using different methodology including lower cut-off values for SPF and antibodies for KI-67 determination. (MacGrogan et al., 1997; Pinto et al., 2001)

Determination of KI-67 gave statistically significant prognostic information for recurrence free survival (RFS), overall survival (OAS) and breast cancer corrected survival (BCSS) (Figure 6, RFS and BCss not shown). KI-67 also gave independent prognostic information for RFS, OAS and BCCS in multivariate survival analyses (Table 13).

Figure 6. Overall survival analysis. The patients are stratified according to their Ki-67-levels.



Numbers at risk:

Low Ki-67	**101**	**83**	**62**
Medium Ki-67	**127**	**105**	**78**
High Ki-67	**81**	**51**	**37**

Other studies (Jones et al., 2001; Pierga et al., 1996) have also reported the association between KI-67 expression and poor outcome, but there has also been conflicting results (Dettmar et al., 1997; Pinto et al., 2001) been reported regarding the prognostic properties of Ki-67, but methodology issues make the comparisons between these studies difficult. Pinto et al (Pinto et al., 2001) used IHC with the monoclonal anti-Ki67/7B11clone-antibody and a 10% cut-point for high proliferation, whereas both Dettmar et al (Dettmar et al., 1997) and Jones et al (2001) used MIB-1 but with different cut-points for high proliferation, 25% and 20% respectively.

Table 13. Cox Proportinal Hazards Multivariate survival analyses for Ki-67.

Multivariate overall suvival analysis*				
Covariate	**Hazard ratio**	**95% Confidence interval**		**p-value**
		Lower	Upper	
Age	1.06	1.04	1.08	<0.0001
Lymph node status	2.29	1.48	3.55	<0.001
Tumor size	1.02	1.01	1.03	0.004
High Ki67	2.07	1.25	3.43	0.005
High Cyclin E	2.09	1.09	3.99	0.025
SPF	n/a**	n/a	n/a	0.13
High tumor grade	n/a	n/a	n/a	0.48
Mutated p53	n/a	n/a	n/a	0.48
Medium tumor grade	n/a	n/a	n/a	0.58
Medium Ki67	n/a	n/a	n/a	0.79
Medium Cyclin E	n/a	n/a	n/a	0.85

Multivariate breast cancer specific suvival analysis*				
Covariate	**Hazard ratio**	**95% Confidence interval**		**p-value**
		Lower	Upper	
Lymph node status	2.94	1.65	5.25	<0.001
High Ki67	2.44	1.36	4.37	0.003
Tumor size	1.02	1.00	1.03	0.008
Age	1.02	1.00	1.05	0.03
Medium tumor grade	n/a**	n/a	n/a	0.20
Medium Ki67	n/a	n/a	n/a	0.20
SPF	n/a	n/a	n/a	0.44
Mutated p53	n/a	n/a	n/a	0.56
Medium Cyclin E	n/a	n/a	n/a	0.75
High tumor grade	n/a	n/a	n/a	0.83
High Cyclin E	n/a	n/a	n/a	0.91

Multivariate recurrence free suvival analysis*				
Covariate	**Hazard ratio**	**95% Confidence interval**		**p-value**
		Lower	Upper	
High Ki67	2,60	1,6	4,2	0,0001
Tumor size	1,02	1,0	1,03	0,006
Age	1,02	1,0	1,04	0,03
Lymph node status	n/a**	n/a	n/a	0,07
Medium Ki67	n/a	n/a	n/a	0,30
High tumor grade	n/a	n/a	n/a	0,31
Medium Cyclin E	n/a	n/a	n/a	0,40
Mutated p53	n/a	n/a	n/a	0,86
SPF	n/a	n/a	n/a	0,88
High Cyclin E	n/a	n/a	n/a	0,94
Medium tumor grade	n/a	n/a	n/a	0,96

*Cox Proportional Hazards Analysis. A stepwise forward procedure (Likelihood Ratio) was used for model fit.

**Hazard ratios were only calculated for covariates included in the final model.

There is still lack of consensus regarding KI-67 methodology guidelines on cell counting and scoring systems for tumor classification. Spyratos et. al (Spyratos et al., 2002) proposed a lower cut-off at 10 % and a higher cut-off at 25%, to exclude breast cancer patients from over- and under treatment, respectively. Although we used a grid graticula based method for scoring and predetermined cut-off levels according to the quartile score distribution, our cut off levels for low and high KI-67 score, 2 and 6, respectively, separates breast cancer patients with regard to presence of other negative prognostic markers and overall survival. When translating our grid graticula score into percent positive tumor cells (formula: score/number of grid dots) the result is 8% and 24% which is close proximity to the cut off levels suggested by Spyratos el al. (Spyratos et al., 2002) However, according to our results do patients with low and medium Ki-67 score have fairly similar outcome. Thus using a single cut-point around 25% for high Ki-67 may be sufficient for clinical use.

Determination of Ki67 with MIB-1 or any other method is not recommended (Bast et al., 2001) for clinical use at present. It is however possible that future evaluation of Ki67 using more precise guidelines for determination will produce stronger documentation. The results in the present study support the prognostic value of KI-67, when using cut-points for high KI-67 in close approximation with the values suggested by others. (Spyratos et al., 2002)

CONCLUDING REMARKS

I. We observed statistically significant correlations between high VEGF expression and shorter patient survival. We also saw a strong correlation between high VEGF expression and mutated TP53 which was consistent for all methods of TP53 determination with the highest levels of VEGF content were observed in patients with insertions, deletions or stop codon point mutations in the TP53 gene. These findings support the hypothesis that wild-type TP53 is involved in the regulation of VEGF.

II. We observed that a high immunohistochemical cyclin E content was associated with insertions, deletions and nonsense point mutations in the TP53 gene, likely to have strong negative impact on the TP53 protein expression. We also observed independent associations between cyclin E, mutated TP53 and aneuploidy. Cyclin E was strongly associated with patient outcome both in univariate and multivariate analyses. These findings implicate that cyclin E is a promising prognostic marker for breast cancer.

III. We observed a strong TP53 independent correlation between the expression of MDM-2 and p21, and therefore hypothesize that an previosly unknown link between p21 and MDM-2 might exist. We did not find any statistically significant correlations between the immunohistochemical protein content of p21 or MDM-2 and the TP53 gene status. It is therefore of no benefit to co-analyze the IHC expression of p21 and/or MDM-2 together with TP53 IHC, to identify breast cancers with mutated TP53.

IV. Ki-67 determination using MIB-1-IHC on paraffin embedded breast cancers gave significant prognostic information. Using a grid graticule for Ki-67 scoring gave comparable results to individual cell counting and may thereby be a time saving alternative. In the light of our data and previous studies, it seems that consensus for defining a high Ki-67 could be found at 25% or more immunopositive cells. The utility of this need further study.

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