REVIEW

Prognostic value of proliferation in invasive breast cancer: a review

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Breast cancer is the leading cause of death among solid tumours in women, and its incidence is increasing in the West. Adjuvant chemotherapy and hormonal treatment improve survival but have potentially serious side effects, and are costly. Because adjuvant treatment should be given to high risk patients only, and traditional prognostic factors (lymph node status, tumour size) are insufficiently accurate, better predictors of high risk and treatment response are needed. Invasive breast cancer metastasises haematogenously very early on, so many breast cancer prognosticators are directly or indirectly related to proliferation. Although studies evaluating the role of individual proliferation regulating genes have greatly increased our knowledge of this complex process, the functional end result—cells dividing—has remained the most important prognostic factor. This article reviews the prognostic value of different proliferation assays in invasive breast cancer, and concludes that increased proliferation correlates strongly with poor prognosis, irrespective of the methodology used. Mitosis counting provides the most reproducible and independent prognostic value, and Ki67/MIB1 labelling and cyclin A index are promising alternatives that need methodological fine tuning.

woman over the age of 50 years,³ but preferentially detect slowly growing and more well differentiated tumours that inherently have a better prognosis and miss fast growing aggressive tumours, which often present as interval cancers.^{4 5} Therefore, the overall effect is still a matter of debate.⁶

“Adjuvant treatment should only be given to high risk patients, which requires good prognostic factors to indicate high risk and additional factors to predict response to treatment”

Adjuvant chemotherapy and hormonal treatment have been shown to improve survival in patients with breast cancer but have potentially serious side effects, and are costly. Therefore, adjuvant treatment should only be given to high risk patients, which requires good prognostic factors to indicate high risk and additional factors to predict response to treatment (for example, steroid receptors⁷ and HER-2/neu⁸). Traditional prognostic factors such as lymph node status and tumour size are not accurate enough. Therefore, to improve the indication for adjuvant treatment, additional predictive and prognostic factors are required. A multitude of prognostic factors have been identified for breast cancer. Many of them are directly (for example, cell cycle regulators⁹) or indirectly (for example, growth factors^{10–12} or angiogenesis¹³) related to proliferation. This is no surprise, because invasive breast cancer can conceptually be regarded as a disease that metastasises haematogenously in a very early phase. Hence, prognosis does not depend on the mere presence of distant metastases but on whether they grow or not.

Although many studies evaluating the role of individual genes regulating these processes have tremendously increased our knowledge of the complex process of proliferation, the functional end results of this process—a cell dividing—has remained the most important prognostic factor so far. Here we review the prognostic value of different proliferation assays in invasive breast cancer.

Assessment of proliferation

Different methods based on the concepts of the cell cycle have become available to assess the rate of proliferation, and have recently been reviewed extensively.^{14–19} Cellular proliferation takes place

Abbreviations: BrdU, bromodeoxyuridine; MAI, mitotic activity index; McM, minichromosome maintenance; PCNA, proliferating cell nuclear antigen

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Breast cancer is the leading cause of death among solid tumours in women, and the incidence is still increasing in the Western world, especially among younger woman. In the Netherlands, there are over 10 000 new cases of breast cancer every year in a population of about 16 million people. At this moment, the disease will affect 12% of all women and about 30–40% of patients will die from metastatic disease despite radical surgery. Although there are several recognised risk factors, such as early menarche, late menopause, nulliparity, and positive family history, there are at present no realistic options for primary prevention in patients not known to have a germline mutation in one of the hereditary breast cancer related genes, such as BRCA1 and BRCA2,¹ PTEN (Cowden's disease),² and p53 (Li Fraumeni syndrome).

Improvements in survival can therefore be expected from early detection and adjuvant treatment. Mammographic screening strategies result in the early diagnosis of breast cancer and a 25–30% decrease in breast cancer mortality in

through a defined process in which several phases can be recognised. From the resting (G0) phase they join the active cycling population after appropriate stimuli and enter the first gap (G1) phase. Both phases have a highly variable duration. In G1, the cell prepares for the synthesis (S) phase, in which DNA synthesis and doubling of the genome take place. The S phase is followed by a period of apparent inactivity known as the second gap (G2) phase, in which the cell prepares for further separation of chromatids during the mitotic (M) phase. After the M phase, each daughter cell may enter G0 phase or move on to the G1 phase to repeat the cell cycle. The interphase, which comprises the G1, S, and G2 phases, forms the largest part of the cell cycle, but cells in these phases cannot be morphologically recognised. However, cells in the mitotic phase can easily be identified because of the typical appearance of the chromosome sets during the different subphases of the M phase. This has been the basis for light microscopical counting of mitotic figures, the oldest form of assessing proliferation.

However, the duration of the mitotic phase can vary, especially in aneuploid tumours, so the number of mitoses is not linearly correlated with the rate of proliferation. Therefore, cell biologists in particular have explored other methods. An optimal assessment of the proliferation rate of a tumour includes measurements of the growth fraction, in addition to measurements of the cell cycle time.¹⁴ Cell cycle time is difficult to assess, but preliminary studies have assessed argyrophilic nuclear organiser regions in Ki67 positive cells, with promising results.²⁰ The growth fraction can be more easily assessed by immunohistochemistry of proliferation associated antigens, such as Ki67, Ki-S1 topoisomerase II α , proliferating cell nuclear antigen (PCNA), geminin,²¹ or minichromosome maintenance (McM) proteins,²² or by DNA flow cytometric²³ or image cytometric (two dimensional²⁴ or three dimensional²⁵) assessment of the S phase fraction. Incorporation techniques (for example, with bromodeoxyuridine (BrdU) and tritiated thymidine) theoretically provide the gold standard of cellular proliferation. All these methods have their good and bad points from a cell biological or practical point of view.¹⁴ However, incorporation techniques are impractical because fresh material is needed, patients need to be injected intravenously, and/or radioactivity is involved, making them unattractive in daily practice. The percentage S phase is hampered by pronounced intratumour heterogeneity.²⁶ Therefore, mitosis counting and the Ki67 index are the most practical methods. Mitosis counting has been best studied from a methodological point of view and larger retrospective and prospective studies have been used (see below).

Prognostic value of proliferation in breast cancer

The different methods to assess proliferation have all been tested for prognostic value in invasive breast cancer. Most studies have been performed on sporadic patients, and a few on BRCA1/2 related cases, which in general show higher proliferation, compatible with their worse prognosis.²⁷⁻²⁸

Incorporation techniques

A high thymidine labelling index has been shown to be associated with poor prognosis in lymph node positive and negative patients with breast cancer,²⁹⁻⁴⁴ and patients with a high thymidine labelling index benefit from adjuvant chemotherapy.⁴⁵ For BrdU, only few clinical studies have been published. Thor *et al* compared BrdU with the mitotic index and Ki67 index, and found comparable prognostic value for these three techniques,⁴⁶ and Weidner *et al* confirmed the good correlation between BrdU and mitotic index.⁴⁷ Goodson *et al* found BrdU to be slightly superior to Ki67.⁴⁸ However, as stated above, incorporation methods are impractical for routine use, which hampers their worldwide

application, despite the very good prognostic value of the thymidine labelling index.

Flow cytometric S phase

With regard to the flow cytometric S phase fraction, most studies that used fresh/frozen material and a sufficient number of patients found a relation between high S phase fraction and an unfavourable prognosis.⁴⁹⁻⁵² However, in view of the high intratumour heterogeneity of the S phase fraction, this feature cannot be used for individual patients.²⁶

Proliferation associated antigens

The monoclonal antibody Ki-S1 is thought to recognise a cell cycle associated antigen, related to the mitotic count,⁵³ but only a few clinical studies have been reported using this antibody, and most have revealed no prognostic value.⁵³⁻⁵⁵

Topoisomerase II α is a recently established marker of proliferating cells.⁵⁶ In one study, topoisomerase II α and Ki67 scores closely paralleled one another, indicating that the topoisomerase II α labelling index reflects the proliferative activity of tumour cells.⁵⁷⁻⁵⁸ Topoisomerase II α provided independent prognostic value in two studies.⁵⁷⁻⁵⁸

Cyclin A is expressed in the late S, G2, and M phases of the cell cycle, and is therefore one of the most useful markers of proliferating cells.⁵⁹ Indeed, cyclin A labelling appeared to have prognostic value in invasive breast cancer.⁶⁰⁻⁶¹

Ki67 labelling correlates with the S phase fraction⁶² and mitotic index.⁶²⁻⁶³ Using frozen sections, the Ki67 labelling index was prognostically relevant in several studies in invasive breast cancer.⁶⁴⁻⁶⁶ The MIB1 antibody, which is reactive against Ki67 and can be used on paraffin wax embedded tissues, confirmed the prognostic value of Ki67 on archival material,³⁰⁻⁴⁸⁻⁵¹⁻⁵⁸⁻⁶⁴⁻⁶⁷⁻⁸¹ including tissue from lymph node negative patients,⁸²⁻⁸³ and there was a good correlation between Ki67 and MIB-1 staining.⁸² In predominantly in situ cancers, even the Ki67 labelling index of the in situ parts seems to have prognostic value.⁷¹ A pronounced decrease in the Ki67/MIB-1 labelling index is associated with a good response to preoperative treatment.⁸⁴⁻⁸⁶ However, not all studies on Ki67/MIB1 reached significance.⁵² Few studies have dealt with the methodological issues, such as sampling strategies, intratumour heterogeneity, and reproducibility, most studies are retrospective, and thresholds vary.⁸⁷

Because of its conflicting results, PCNA immunohistochemistry does not provide a prognostically relevant assessment of proliferation in breast cancer.⁸⁸⁻⁹³ For geminin and McM proteins, no prognostic results have been described to date.

Mitosis counting

Several studies have shown that the mitotic count is the most important constituent of histological grade,⁹⁴⁻⁹⁵ but there are well known problems with reproducibility of grading because of the lack of strict protocols.⁹⁶⁻¹⁰² In different studies from our group, we have shown that a highly standardised way of assessing the mitotic activity index (MAI; counting at $\times 400$, magnification in an area of 1.6 mm², in the highest proliferative invasive area in the periphery of the tumour) provided a very strong prognostic factor, with additional prognostic value to tumour size and lymph node status in several retrospective studies¹³⁻¹⁰³⁻¹¹⁷ and two prospective studies.¹⁰¹⁻¹⁰² Several other groups from different countries have confirmed the prognostic value of mitosis counting in primary invasive breast cancer,⁴⁻⁴⁶⁻⁵¹⁻⁷⁸⁻⁸²⁻⁹⁰⁻⁹⁵⁻¹¹⁸⁻¹⁵¹ including prospective studies.¹⁵² Elkhuijzen *et al* found that patients who had undergone breast conserving treatment and had a recurrence after an interval of more than two years, but who had a high mitotic count, had an equally poor prognosis as those patients with local recurrence detected after a short interval.¹⁵³ The threshold in the different studies varies

Table 1 Overview of different studies on the prognostic value of mitotic activity in invasive breast cancer

First author	Ref	P/R	N	Subgroup	Overall survival	Significance	Independent value
Aaltomaa	121	R	293	LN-	0.005	-	Yes
	121	R	224	LN+	0.004	-	Yes
	122	R	106	All	<0.001	<0.001	Yes
	119	R	281	LN-	0.0115	0.0007	Yes
	118	R	688	All	<0.0001	<0.0001	Yes
	120	R	611	All	<0.001	<0.001	Yes
Back	104	R	271	Ductal	-	<0.001	Yes
	105	R	82	Ductal	-	0.0254	Yes
	159	P	576	LN-, <55	<0.0001	<0.0001	Yes
Barbareschi	124	R	178	LN-	0.03	-	No
Biesterfeld	125	R	104	All	-	<0.0001	Yes
	152	R	108	LN+	-	0.0093	Yes
Bos	107	R	153	All	0.046	0.017	Yes
Chen	155	R	255	LN-	NS	NS	No
Clahsen	82	R	441	LN-	<0.01	-	-
Clayton	127	R	378	LN-	-	<0.0001	Yes
	126	R	399	LN+	-	<0.0001	Yes
Collan	108	R	120	All	-	0.001	Yes
Colpaert	128	R	104	LN-	<0.0001	-	No
Jong	13	R	112	All	-	0.0009	Yes
Eskelinen	129	R	216	All	0.01	-	Yes
Groenendijk	4	R	387	All	<0.0001	-	-
Ikpat	130	R	300	All	-	<0.0001	Yes
Jannink	112	R	186	All	-	<0.001	Yes
	113	R	189	All	-	<0.001	Yes
Joensuu	131	R	311	All	-	<0.0001	-
Kato	156	R	70	LN-	-	NS	No
	132	R	422	All	<0.0001	<0.0001	Yes
Keshgegian	133	R	126	All	0.0003	-	-
Kronqvist	135	R	364	All	<0.0001	-	Yes
	134	R	202	All	-	0.0001	Yes
Ladekarl	138	R	71	Ductal	-	0.1	Yes
	137	R	98	LN-	-	0.0005	Yes
Laroye	157	R	76	All	-	NS	Yes
Le Doussal	95	R	1262	Ductal	<0.0001	0.002	Yes
Linden	160	P	195	All	0.001	-	Yes
	114	R	156	All	0.001	0.005	Yes
Lipponen	139	R	111	All	0.001	0.001	Yes
	141	R	363	All	0.004	0.001	Yes
	140	R	202	All	0.012	-	Yes
Liu	142	R	791	All	<0.0001	<0.0001	Yes
Mandard	144	R	281	LN-, prem.	<0.001	<0.001	Yes
Manders	145	R	137	All	0.0070	0.0017	Yes
Page	146	R	311	LN-	NS	0.01	Yes
Pietilainen	78	R	191	All	-	0.0025	Yes
Russo	90	R	646	All	<0.0001	-	Yes
Simpson	147	R	560	LN+	0.004	-	Yes
Theissig	148	R	92	All	-	<0.0001	Yes
Thor	46	R	486	All	0.0056	-	Yes
Toikkanen	149	R	217	Lobular	-	0.0001	Yes
Tosi	150	R	350	All	0.025	-	Yes
Uyterlinde	115	R	63	Ductal	-	0.008	Yes
	116	R	225	Ductal	-	<0.0001	Yes
	117	R	295	Ductal	-	<0.0001	Yes
Van Diest	111	R	211	<55	-	<0.0001	Yes
	110	R	20	LN+	-	0.004	-
	9	R	148	All	-	0.0001	Yes
Younes	151	R	300	Ductal	-	0.0032	Yes

Not all these studies used independent patient groups.
LN, lymph node; NS, not significant; P, prospective, R, retrospective.

slightly, but there seems to be a consensus threshold at about 10–12 mitosis/2 mm², as in histological grading. Mitosis counting in lymph node metastases also provides some prognostic value.¹⁵⁴

“We have shown that a highly standardised way of assessing the mitotic activity index provided a very strong prognostic factor, with additional prognostic value to tumour size and lymph node status”

Table 1 provides an overview of the different studies on the MAI in breast cancer. The total number of patients investigated is difficult to estimate because not all of the

studies shown used independent patient groups, but the table makes it clear that the MAI has been studied in thousands of patients with usually strong independent prognostic value. Only a few smaller studies failed to reveal prognostic value.^{155–157} In several studies, mitotic count has been shown to have additional prognostic value to tumour size and lymph node status, a combination denoted the multivariate prognostic index.^{104 108 111 120 150 158} Not many data have been published on other subgroups, such as oestrogen receptor positive and negative patients, but in general it can be stated that mitosis counting has independent prognostic value, even from the oestrogen receptor status.

Nevertheless, for practical reasons, it seems that the MAI by itself is preferred for clinical practice. The fact that (a) the

MAI has been shown to be reproducible in multicentre studies involving routine laboratories,¹⁶¹ and (b) the prognostic value of the MAI holds for premenopausal lymph node negative patients,^{111–117} which was confirmed in a nationwide prospective study in the Netherlands,^{160–162–163} has led to acceptance of the MAI by the Netherlands Society of Clinical Oncology as a high risk (MAI $\geq 10/1.59 \text{ mm}^2$) indicator for lymph node negative patients with invasive breast cancer necessitating adjuvant chemotherapy. According to this consensus,¹⁶⁴ lymph node negative patients with breast cancer who have tumours between 1 and 3 cm and a MAI lower than $10/1.6 \text{ mm}^2$ receive no adjuvant treatment, whereas patients with a MAI $\geq 10/1.6 \text{ mm}^2$ receive adjuvant chemotherapy and/or endocrine treatment, depending on their steroid receptor status. In addition, the College of American Pathologists' consensus statement 1999 mentions mitotic figure counting as a category I prognostic factor for breast cancer,¹⁶⁵ and the mitotic count has also been recognised by the UICC as an "essential prognostic factor".¹⁶⁶

"A prospective comparison between the prognostic and predictive value of mitosis counting and microarray expression analysis would be of great interest"

MAI is not seriously affected by fixation delay, although fixation delay does lead to worse morphology, which makes counting more difficult. Therefore, it is advisable to avoid fixation delay when possible, and to keep specimens in the refrigerator until fixation.¹⁶⁷ Ideally, mitosis should be counted before chemotherapy, but even after chemotherapy, the mitotic index has prognostic value.^{168–170} Mitoses should preferably be counted on excision biopsies or mastectomies to avoid sampling error, but even measurements on core biopsies seem to have some value. Several studies have shown that mitosis counting on biopsies can reach the highest score in the histological grading system, but the mitotic index as such is often underestimated in core biopsies.^{171–173} Currently, this issue is even more important, because neoadjuvant chemotherapy is planned based on the prognostic factors assessed on core biopsies.

The advantage of a section based morphological method to assess proliferation such as mitosis counting is that intratumour heterogeneity (for example, central and peripheral tumour parts) is relatively easy to deal with.¹⁷⁴ The MAI has been criticised for not correcting for cellularity, but correction for volume percentage epithelium or cellularity does not lead to a relevant increase in prognostic value, although it does

dramatically increase the time required for a proper assessment.^{112–113}

CONCLUSION

Proliferation plays an important role in the clinical behaviour of invasive breast cancer. Increased proliferation correlates strongly with poor prognosis, irrespective of the methodology used. However, of the different methods to assess proliferation, mitosis counting has been shown most convincingly to provide reproducible and independent prognostic value in invasive breast cancer. Therefore, the MAI is already used in clinical practice in several countries as a single prognostic marker, and is the most well established component of the histological grade. Ki67/MIB1 labelling and the cyclin A index are promising alternatives, which need further methodological fine tuning. In general, however, little attention has yet been paid to the value of these proliferation markers in predicting response to treatment. A prospective comparison between the prognostic and predictive value of mitosis counting and microarray expression analysis would be of great interest.

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Take home messages

- Because adjuvant treatment for invasive breast cancer should be given to high risk patients only, and traditional prognostic factors (lymph node status, tumour size) are insufficiently accurate, better predictors of high risk and treatment response are needed
- Most of the multitude of breast cancer prognosticators are directly or indirectly related to proliferation and increased proliferation correlates strongly with poor prognosis, irrespective of the methodology used
- Mitosis counting has the most reproducible and independent prognostic value
- Ki67/MIB1 labelling and the cyclin A index are promising alternatives, although they require further methodological fine tuning

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