Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now?

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Received 18 January 2005; revised 11 April 2005; accepted 12 April 2005

In the last few decades, proliferative markers have been broadly evaluated as prognostic and predictive factors for early stage breast cancer patients. Several papers evaluating one or more markers have been published, often with contradictory results. As a consequence, there is still uncertainty about the role of these proliferative markers. The present paper critically reviews the current knowledge about the following markers: thymidine labeling index, S phase fraction/flow cytometry, Ki 67, thymidine kinase (TK), cyclins E, cyclin D, the cyclin inhibitors p27 and p21, and topoisomerase IIα. For each marker, the prognostic and predictive role was separately analyzed. Only papers published in English in peer-reviewed journals before June 2004 that include at least 100 evaluable patients were selected. In addition, the prognostic and predictive role of the proliferative markers had to be assessed through multivariate analyses. One hundred and thirty-two papers fulfilled these criteria and 159,516 patients were analyzed. Unfortunately, several methodological problems in the research to date prevent us from including any one of these proliferative markers among the standard prognostic and predictive factors. Early incorporation of translational research and new technologies with clinical trials are needed to prospectively validate biological markers and allow their use in clinical practice.

Key words: breast cancer, proliferative markers, prognostic markers, predictive markers

Introduction

Invasive breast carcinoma (BC) is the most frequent carcinoma and is the second cause of death from malignant disease among women in the western world. Significant improvements in disease-free survival (DFS) and overall survival (OS) have been obtained with the extensive use of adjuvant systemic therapies, which are largely empirically based. The possibility to have strong prognostic and/or predictive markers is of utmost importance for clinicians in order to identify patients at higher risk of relapse and to select the most appropriate systemic treatment for an individual patient. Prognostic factors are those that predict the risk of recurrence or of death from BC independently of treatment. Predictive factors are those that distinguish between patients who are more or less likely to respond to a given therapy. However, the distinction between the prognostic and predictive value of each marker is not straightforward. The retrospective nature of the great majority of these studies may jeopardize their results. Hundreds of papers evaluating several prognostic and predictive factors have been published in the last 30 years. However, the only validated prognostic factors are tumor (T) size, lymph node (N) status, hormone receptor (HR) status, histologic grade, and age [1]; and, as predictive factors: HR status and human epidermal growth factor receptor-2 (HER2) status [2] for endocrine and trastuzumab therapy, respectively.

Tumor cell proliferation has been widely investigated in BC for its association with neoplastic growth, progression, and metastatic potential; the present article is a review of the knowledge gathered on tumor cell proliferative markers in the past decade, with a critical assessment of their prognostic and/or predictive value.

Materials and methods

A computerized literature search through Medline was performed, applying the words ‘BC proliferative prognostic markers’ and ‘BC proliferative predictive markers’ and each one of the following: thymidine labeling index, S phase fraction/flow cytometry, Ki 67, thymidine kinase (TK), cyclins E, cyclin D, cyclin inhibitors p27 and p21, and topoisomerase IIα. Articles were also identified by back-referencing from original and relevant review papers. We chose not to include histological grade since it is an already accepted standard marker, and similarly, mitotic index, which is an important component of all histological grading systems and has recently been reviewed [3].

Selected for the present review were papers published in English in peer-reviewed journals before June 2004, which included at least 100 evaluable patients and in which the prognostic and predictive role of each marker was assessed through multivariate analyses. When more than one proliferative marker was evaluated, we referred to the most relevant one. The levels of
evidence were provided according to the Tumor Marker Grading Utility System proposed by Hayes et al. [4] as shown in Table 1, and we arbitrarily decided to consider as large retrospective studies those including ≥200 patients. We cannot exclude the fact that some papers that did not find significant correlations for a certain proliferative markers, have never been published and, therefore, this could hamper our findings.

Measurement of cells in the S phase

Cell kinetics can be evaluated by detecting cells undergoing DNA synthesis. It is possible either to measure the fraction of cells that incorporates labeled pyrimidine bases into newly synthesized DNA or to estimate the percentage of cells in the S phase of the cell cycle (S-phase fraction) by flow cytometry while simultaneously determining DNA content.

3H-thymidine labeling index

Description

3H-thymidine labeling index (TLI) was one of the first methods utilized to evaluate the proliferative activity of BC. The number of tumor cells undergoing DNA synthesis can be measured using in vivo or in vitro assays for 3H-thymidine uptake [5], which can be visualized by autoradiography. The TLI expresses the ratio between thymidine-labeled cells and the total number of tumor cells and has been shown to be reliable and stable over time [6]. Labeled pyrimidine bases other than [3H] thymidine, such as the halogenated analogue bromodeoxyuridine (BrdU), have also been used successfully [7, 8]. Of note, incorporated BrdU can be revealed by immunohistochemistry (IHC). The technique, however, has limitations that have hampered its acceptance as a standard method. In fact, fresh tumor tissue is needed and a complex and time-consuming radioactive assay or in vivo administration of labeled substances is required.

Table 1. Levels of evidence for grading clinical utility of tumor markers

<table>
<thead>
<tr>
<th>Level</th>
<th>Type of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Evidence from a single, high-powered, prospective, controlled 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, controlled randomized trial in which diagnostic and/or therapeutic clinical decisions in one arm are determined at least in part on the basis of marker results, and diagnostic and/or therapeutic clinical decision in the control arm are made independently of marker results. However, study design may also include prospective but not randomized trial with marker data and clinical outcome as primary objective.</td>
</tr>
<tr>
<td>II</td>
<td>Evidence from a study in which marker data are determined in relationship to a 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 the protocol as secondary objectives.</td>
</tr>
<tr>
<td>III</td>
<td>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.</td>
</tr>
<tr>
<td>IV</td>
<td>Evidence from small retrospective studies that do not have prospectively dictated therapy, follow-up, specimen selection, or statistical analysis. Study design may use matched case-control, etc.</td>
</tr>
<tr>
<td>V</td>
<td>Evidence from small pilot studies designed to determine or estimate distribution of marker levels in sample population. Study design may include ‘correlation’ with other know or investigational markers of outcome but is not designed to determine clinical utility.</td>
</tr>
</tbody>
</table>

Prognostic role

Several papers, mostly retrospective and sometimes based on large series of BC patients, have reported a significant correlation between high TLI and poor clinical outcome in terms of relapse-free survival (RFS)/DFS, distant metastases-free survival (DMFS) and/or OS independently of N status, T size, histological grade, HR status and menopausal status [9–17]. However, even if the results of these studies have consistently shown the feasibility and the high reproducibility of TLI as a measure of the tumor proliferative activity [18, 19] it has never been accepted as a standard prognostic marker for the reasons described above.

Predictive role

Three studies have evaluated the predictive role of TLI. In a retrospective study, 285 out of 403 women with more than three N-positive BC randomized to receive, as adjuvant chemotherapy, alternating or sequential courses of doxorubicin and CMF (cyclophosphamide, methotrexate, 5-fluorouracil) were analyzed [20]. The median values and ranges of TLI for the two arms were superimposable. Patients with high TLI had a significantly worse 12-year RFS, DMFS and OS and benefited significantly more from the sequential administration of doxorubicin and CMF. In two multicenter prospective phase III adjuvant trials, N-negative BC patients with high TLI, regardless of HR status, were randomized to receive chemotherapy (CT) or no further systemic therapy. In the first trial [21], the adjuvant CT consisted of six courses of the ‘classic’ CMF regimen. At a median follow-up of 81 months, a statistically significant increase in DFS was obtained with CT. Similar results were reported in the other trial utilizing an anthracycline-based regimen [cyclophosphamide, epirubicin and 5-fluorouracil (FEC)] for six courses [22]. The analysis of relapse sites showed that CT reduced significantly all loco-regional relapses and led to a decrease in the number of distant metastases and contralateral tumors, although these differences were not significant. Despite the achievement of
level 1 evidence by these two trials, TLI was not adopted in clinical practice given the technical problems reported above.

Flow cytometry

Description

Flow cytometric measurement of the nuclear DNA content of tumor cells provides simultaneous information about DNA ploidy and proliferative activity as represented by the S-phase fraction (SPF). This technique can be performed on fresh-frozen tumors or on paraffin-embedded tissue. Paraffin blocks provide the advantage of using archival tissue stored for many years, but there is a risk of finding more debris than in frozen or fresh tissue, which results in a relatively high proportion of tumors for which SPF cannot be determined. However, the utilization of fresh-frozen material requires compliance to a number of different technical steps and is therefore cumbersome [23].

Prognostic role

Since the first publication in 1987 [24], numerous papers have addressed the technological aspects of the method, the relationships of SPF with other standard prognostic factors and its association with clinical outcome. In 1992, the DNA Cytometry Consensus Conference supported by the National Cancer Institute concluded that the literature clearly showed a link between high SPF values and increased risk of recurrence and death for patients with primary BC, but the lack of a standardized procedure to prepare and to analyze tumor samples precluded recommending this method as a routine way to determine prognosis or to select treatment in the adjuvant or metastatic settings [25]. Attempts to standardize the method have been made by several groups [23, 26–30], some of which have also prepared guidelines and recommendations and shown the possibility to increase interlaboratory reproducibility [31]. Nonetheless, the Tumor Marker Panel of the American Society of Clinical Oncology recently did not include SPF among the standard markers to use for treatment decision-making in BC patients [2]. Interestingly, high SPF has been associated with HR-negativity, larger T size, N-positivity and high grade in several reports, and has been found to be a significant independent prognostic factor for early BC patients in 41 out of 49 published papers enrolling a large number of patients (range 131–709) [23, 32–77]. In the majority of the studies, high levels of SPF predicted worse DFS/RFS; in nine this was true only for OS [32, 42, 58, 65–68, 74, 77] and in 11 for both DFS and OS [23, 33, 37, 40, 51, 59, 61, 69]. However, only three studies were prospective [45, 49, 76], and several weaknesses in the design, conduct, and interpretation of the results of these studies hamper the clinical utility of SPF for BC patients. First, the tumor tissue utilized differed across the studies, and while more often paraffin-embedded tissue blocks were processed (26 studies), in others fresh/frozen material was used. Secondly, the characteristics of patients enrolled in the different studies varied according to stage, menopausal status and treatment received. Thirdly, the median follow-up fluctuated from 26 months to 27 years. Fourthly, there was substantial variability in the assay methodology and in the selection of cut-off values. In fact, in some papers, one cut-off point was selected to separate high versus low SPF, while in others, two cut-off points were used in order to provide three categories as suggested by the DNA Cytometry Consensus Conference [25].

Predictive role

We found only one retrospective study evaluating the predictive role of SPF for adjuvant tamoxifen therapy in stage I–III BC patients with progesterone receptor (PgR)-positive tumors [78]. While with univariate analysis, tamoxifen improved the clinical outcome of patients with high SPF more than in those with low SPF, this effect disappeared with multivariate analysis.

Tymididine kinase

Thymidine kinase (TK) is a salvage enzyme implicated in DNA synthesis: it catalyses the phosphorylation of deoxythymidine to deoxythymidine monophosphate. Its activity increases after the G1-S transition checkpoint and then declines rapidly in G2. High levels of TK have been reported in BC patients [79–82] and refer to the fetal isoform, which is located in the cytoplasm and is cell cycle-regulated [83]. TK enzyme activity is determined using a radioenzymatic assay on cytosol and is expressed as mU/mg protein.

Prognostic role

We found only one prospective study [82] of 290 N-negative and N-positive BC patients in whom high TK activity was significantly correlated with high grade and PgR-negativity and predicted a worse RFS in the pre/peri-menopausal subset and worse OS in the postmenopausal subgroup.

Predictive role

The predictive value of TK was retrospectively evaluated in two studies [81, 84], of which one analyzed 1692 BC patients [84]. In both studies, high levels were correlated with large T size and HR-negativity [84] or PgR-negativity [81]. In the first, all N-positive patients received CT with a regimen containing fluorouracil [CMF or CAF (cyclophosphamide, doxorubicin and 5-fluorouracil)] and no endocrine therapy [81]. High levels of TK were predictive of worse RFS and OS independently of the treatment, as though the overexpression of this enzyme could allow tumor cells to escape the effects of fluorouracil and methotrexate. In the second study, high TK was predictive of better disease specific survival (DSS) and distant relapse-free interval in N-negative patients treated with anthracycline-based CT (FAC or FEC) in comparison with those untreated [84].

Proliferation associated antigens

(Ki-67, MIB 1)

Description

Cell proliferation can also be assessed through IHC measurement of the expression of proliferation-associated antigens in
tumor tissues. This method is cheap and easy to perform in virtually every pathology department, but it has some shortfalls mainly related to the subjectiveness of the evaluation, the differences in reactivity among the various antibodies available for detecting a given antigen, and other sources of inter-observer variability, such as different reactivity of the detection kits or methods used (e.g. epitope unmasking techniques). Several monoclonal antibodies reacting with different proliferating cell nuclear antigens have been described, such as PCNA, Ki-67 and MIB 1, KiS1 and others. The Ki-67 monoclonal antibody, identified in 1983, reacts with a nuclear protein present exclusively in proliferating cells and the function of which remains unknown. A detailed cell cycle analysis revealed that the antigen was present in the nuclei of cells in all phases as well as in mitosis, while quiescent or resting cells in the G0 phase did not express it. For more detailed information on the protein we refer to two review articles [85, 86]. Since this protein is present in all proliferating cells (normal and tumor), it became evident that it could be an important marker to evaluate the growth fraction of a given cell population. However, initially the Ki-67 monoclonal antibody could only be used on fresh or frozen tissue, since fixation greatly reduced or abolished immunostaining; this problem could be overcome by the preparation of another equivalent monoclonal antibody MIB-1, which can be evaluated on paraffin sections after antigen retrieval using microwave-processing [87]. A good correlation between the two antibodies was demonstrated [88, 89] so that it was possible to investigate the prognostic potential of the MIB1 protein in retrospective studies using formalin-fixed and paraffin-embedded archived pathologic material. For the other antibodies, conflicting results have been reported [3], and therefore they have no role in clinical practice.

Prognostic role

In the last decade, a large number of papers have been published and, even after taking into consideration the inevitable publication bias in favor of positive trials, it is now clear that the Ki-67/MIB 1 protein has a prognostic value for many types of malignant tumors. In BC, all the selected studies [89–103] have shown a statistically significant correlation with clinical outcome (DFS and/or OS) as reported in Table 2, but all except one [104] are retrospective. The number of patients included ranged from 127 to 707, and the populations were heterogeneous, except in three studies [91, 96, 102], which analyzed only N-negative patients. The median follow-up length ranged from 31 months [90, 92] to 13.5 years [103], and other relevant differences among these studies were related to the type of antibody utilized, the cut-off value selected to define high versus low proliferative activity, and the number of cells counted. Although, the Ki-67/MIB 1 protein expressed as Ki-67 index (percentage of cells staining positive) is widely used in the routine assessment of prognostic markers, it is not considered a standard one [1, 105] due to the lack of an international standardization method for antigen retrieval, staining procedures and scoring methods (semi quantitative and quantitative).

Predictive role

Ki-67 has been retrospectively [106–109] and prospectively [110] assayed in a few trials of primary systemic therapy

### Table 2. Studies evaluating Ki-67 as prognostic marker

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Number of patients</th>
<th>Median FU</th>
<th>Stage</th>
<th>Prognostic value in multivariate analysis</th>
<th>Analysis type</th>
<th>Evidence level</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>170</td>
<td>66.5 months</td>
<td>I–IIIb</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[89]</td>
</tr>
<tr>
<td>IHC</td>
<td>327</td>
<td>Mean 2.7 years</td>
<td>I–III</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[90]</td>
</tr>
<tr>
<td>IHC</td>
<td>212</td>
<td>Mean 8.3 years</td>
<td>T1, N-</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[91]</td>
</tr>
<tr>
<td>IHC</td>
<td>385</td>
<td>31 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[92]</td>
</tr>
<tr>
<td>IHC</td>
<td>135</td>
<td>Up to 46 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[93]</td>
</tr>
<tr>
<td>IHC</td>
<td>177</td>
<td>NR</td>
<td>&lt;5 cm</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[94]</td>
</tr>
<tr>
<td>IHC</td>
<td>341</td>
<td>128 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[95]</td>
</tr>
<tr>
<td>IHC</td>
<td>674</td>
<td>60 months</td>
<td>N-</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[96]</td>
</tr>
<tr>
<td>IHC</td>
<td>140</td>
<td>70 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[97]</td>
</tr>
<tr>
<td>IHC</td>
<td>186</td>
<td>88 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[98]</td>
</tr>
<tr>
<td>IHC</td>
<td>707</td>
<td>66 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[99]</td>
</tr>
<tr>
<td>IHC</td>
<td>322</td>
<td>NR</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[100]</td>
</tr>
<tr>
<td>IHC</td>
<td>486</td>
<td>62 months</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[101]</td>
</tr>
<tr>
<td>IHC</td>
<td>441*</td>
<td>41 months</td>
<td>N-</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[102]</td>
</tr>
<tr>
<td>IHC</td>
<td>434</td>
<td>13.5 years</td>
<td>N-, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[103]</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; N-, node-negative; N+, node-positive; NR, not reported; R, retrospective analysis.

*aOnly premenopausal patients.
(PST) in an attempt to identify biological markers of response that could be surrogates for long-term outcome, as shown in Table 3. The only prospective study conducted did not find any significant correlation between high proliferative activity and response to PST, but Ki67 was determined on fine-needle aspirations of the invasive primary tumor obtained before starting CT and repeated before the second cycle [110]; furthermore, the chemotherapeutic regimen utilized (mitoxantrone, methotrexate, ± mytomicin C) cannot be considered a standard one, and the follow-up is relatively short. Conflicting results have been reported in the retrospective studies. In one of the two positive studies, for example, the cut-off value chosen was quite high (>40%) [106] while, in the other, only a reduction of Ki-67 of ≥36% in two consecutive measures was predictive of response rate (clinical CR or PR) [109]. Other differences regarding tumor tissue preservation, monoclonal antibody and scoring system used, chemotherapeutic regimen, evaluation methods of clinical responses, and length of follow-up could partially explain these results. Furthermore, intratumor variations of MIB 1 scoring were reported in the assessment of individual cancers in a limited set of patients, questioning the reliability of monitoring treatment induced changes in proliferation by this technique in the clinic [111].

### Cyclins E

#### Description

Eukaryotic cells are driven through the cell cycle by successive activation and inactivation of cyclin-dependent kinases (Cdks). The Cdks are regulated by different proteins, including cyclins that bind and activate the Cdks to form a serine/threonine kinase holoenzyme complex. The different cyclins, so called because their concentration rise and fall at specific stages throughout the cell cycle, have a temporally distinct and highly regulated pattern of expression, i.e. they are synthesized and degraded at specific stages of the cell cycle. Cyclin E is the limiting factor for G1 phase progression and S phase entry. It associates with Cdk2 and activates its kinase activity shortly before entry of cells into the S phase. More detailed information on cyclin E can be found in two recently published reviews [112, 113]. Two proteins, codified by two different genes but with high homology, called cyclin E1 (formerly cyclin E) and cyclin E2 have been identified [114–116]. Cyclin E2 like cyclin E1 associates with Cdk2 and activates its kinase activity at the G1/S boundary [117]. Cyclin E2 shares 47% overall similarity to cyclin E1, and it remains to be determined if structural features outside the conserved regions impart unique functions [117]. Recently, several splice variants of cyclin E1, which are not present in normal cells, have also been discovered; importantly, they seem to stimulate cells to progress through the cell cycle much more efficiently than the full length cyclin E1 through a mechanism not yet completely elucidated [113, 118], but probably different from kinase activation [112]. They are generated post translationally through cleavage by elastase, and it is possible that a balance between elastase activity and an endogenous inhibitor of elastase, elafin, controls their expression [119]. Cyclin E protein, as a proliferative marker, has been analyzed in BC patients, and the determination method more frequently used has been IHC followed by western blot (which allows assessment of both total and isoform-specific expression), while cyclin E mRNA has been measured by real time reverse transcription polymerase chain reaction (RT-PCR). Increased expression of cyclin E1 has been reported in approximately 40% of cases [120, 121], and of cyclin E2 in 38% of primary ER-negative BC [122]. Elevated levels of both cyclins were more frequently found in ER-negative than in ER-positive tumors [123].

#### Prognostic role

The prognostic role of cyclin E (E1) has been retrospectively evaluated in several studies [120, 124–132] shown in Table 4. The majority of these studies included patients with early stage BC, but in two of them [120, 124], stage IV patients were also entered. Few studies analyzed only N-negative patients [126–128] and in two of them the same patient population was evaluated with just 59 tamoxifen-treated patients added [126]. In some studies no systemic adjuvant treatment was administered, while in others, all patients or only a subgroup of them received CT and/or endocrine therapy. IHC was the most common evaluation method even if the antibodies utilized and the cut-off values were different. Western blotting analysis was performed in two studies [120, 124], while RT-PCR was chosen in another one [132]. Cyclin E was prognostic in seven out of 10 studies [120, 125–130]. Keiomarsi et al. found that the overexpression

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### Table 3. Studies evaluating Ki-67 as predictive marker

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Number of patients</th>
<th>Median FU</th>
<th>Stage</th>
<th>Predictive value in multivariate analysis</th>
<th>Analysis type</th>
<th>Evidence level</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>125</td>
<td>34 months</td>
<td>T2–3 N–, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[106]</td>
</tr>
<tr>
<td>IHC</td>
<td>157</td>
<td>52.7 months</td>
<td>T2–4 N0–1</td>
<td>No</td>
<td>R</td>
<td>IV</td>
<td>[107]</td>
</tr>
<tr>
<td>IHC</td>
<td>399</td>
<td>NR</td>
<td>T2–4d N0–N2</td>
<td>No</td>
<td>R</td>
<td>III</td>
<td>[108]</td>
</tr>
<tr>
<td>IHC</td>
<td>106</td>
<td>NR</td>
<td>N–, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[109]</td>
</tr>
<tr>
<td>IHC</td>
<td>158</td>
<td>48 months</td>
<td>I–II</td>
<td>No</td>
<td>P</td>
<td>II</td>
<td>[110]</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; N–, node-negative; N+, node-positive; NR, not reported; P, prospective analysis; R, retrospective analysis.
of cyclin E was accompanied by the appearance of low molecular weight (LMW) isoforms, and both were a reliable prognostic marker in stage I–III BC patients [120]. In fact, the hazard ratio for death due to BC in patients with high levels of cyclin E was higher than the hazard ratio associated with any other biological marker examined (seven times higher than the hazard ratio associated with N metastases). All N-negative patients with high levels of cyclin E (12 out of 114) died of BC. In some studies a correlation between cyclin E and high histological grade [126–129, 131] or ER negativity [124, 126–129, 131, 132] has been reported, while an inverse correlation with p27 was observed in a study where both factors were prognostic [133]. No definitive conclusion about the prognostic role of cyclin E can be derived from these studies. In fact, a standardization of evaluation methods and scoring systems and large prospective studies are required. No data on the potential prognostic role of cyclin E2 in BC have been published.

Predictive role

The role of cyclin E1 as a predictive marker has been evaluated only in one study by quantitative RT-PCR technique [132]. High levels of cyclin E1 were predictive of resistance to tamoxifen adjuvant therapy in 108 node-positive BC patients, independently of ER status.

Cyclin D1

Description

D-type cyclins are other key regulator proteins of the G1 phase progression. There are at least three cyclins in this family with differential effects on the development of the normal mammary gland [123]. Cyclins D bind to Cdks 4 and 6 and phosphorylate downstream substrates, mainly pRb, and these complexes can also sequester Cdk inhibitors (p21 and p27) in the G1/S transition. Furthermore, cyclin D1, the first member identified, can have Cdk independent functions and can act as a co-factor for ERα independently of the ligand [134]. Cyclin D1 is the product of a gene (CCND1) located on chromosome 11q13. The protein is synthesized in response to growth factors; its levels reach a maximum in the mid-G1 phase of the cell cycle and then begin to drop. It appears that the association of cyclin D1 to Cdk is crucial to drive cells to the restriction point where the cell is committed to divide [135]. Overexpression of cyclin D1 has been observed in many human tumors and is likely to promote cell proliferation and differentiation by shortening the G1/S transition [136]. Amplification of the gene has been detected in about 15% of BC, while overexpression of cyclin D1 at mRNA and protein levels is seen in up to 50% of primary BC, mostly ER-positive and well-differentiated tumors [123]. The overexpression of cyclin D1 has been evaluated by IHC utilizing different antibodies, or by RT-PCR or northern blot technique.

Prognostic role

As shown in Table 5, seven retrospective studies have analyzed the prognostic role of cyclin D1 in 1509 patients. In two of these studies stage I–IV BC patients were included [137, 138]; the median follow-up ranged from 75 months to 16.7 years. The most common evaluation method was IHC, with different antibodies and cut-off values utilized. Gene amplification and IHC were both assessed in two studies [139, 140]. A strong correlation between overexpression of cyclin D1 and HR-positivity has been reported in the majority of trials, but cyclin D1 does not appear to be a strong prognostic marker. In fact, its overexpression has been associated with better RFS in only one study [140] and with better RFS and OS in another one [137].

Table 4. Studies evaluating cyclin E as prognostic marker

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Number of patients</th>
<th>Median FU</th>
<th>Stage</th>
<th>Prognostic value in multivariate analysis</th>
<th>Analysis type</th>
<th>Evidence level</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blotting</td>
<td>395</td>
<td>6.4 years</td>
<td>I–IV (92% I–III)</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[120]</td>
</tr>
<tr>
<td>Western blotting</td>
<td>114</td>
<td>53 months</td>
<td>100 pts(I–III)</td>
<td>No</td>
<td>R</td>
<td>IV</td>
<td>[124]</td>
</tr>
<tr>
<td>IHC</td>
<td>128</td>
<td>69.6 months</td>
<td>I–IIib</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[125]</td>
</tr>
<tr>
<td>IHC</td>
<td>332</td>
<td>99 months</td>
<td>N−</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[126]</td>
</tr>
<tr>
<td>IHC</td>
<td>273</td>
<td>99 months</td>
<td>N−</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[127]</td>
</tr>
<tr>
<td>TMA IHC</td>
<td>175</td>
<td>59 months</td>
<td>N-High risk</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[128]</td>
</tr>
<tr>
<td>IHC</td>
<td>270</td>
<td>122 months</td>
<td>N−, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[129]</td>
</tr>
<tr>
<td>IHC</td>
<td>247*</td>
<td>7.93 years</td>
<td>N−, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[130]</td>
</tr>
<tr>
<td>IHC</td>
<td>157</td>
<td>At least 3 years</td>
<td>N−, N+</td>
<td>No</td>
<td>R</td>
<td>IV</td>
<td>[131]</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>277</td>
<td>75 months</td>
<td>I–IIib</td>
<td>No</td>
<td>R</td>
<td>III</td>
<td>[132]</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; N−, node-negative; N+, node-positive; pts, patients; R, retrospective analysis.

*Askenazy Jewish.
Predictive role

Only one retrospective study has evaluated the predictive role of cyclin D1. At a median follow-up of 18 years, only patients with low/moderate levels of cyclin D1 and ERα-positive BC tumors benefited from tamoxifen, as though high levels of cyclin D1 affected tamoxifen response [134].

Cdk inhibitors

p27

p27 is a CDKI and belongs to the KIP family. The protein binds and inhibits cyclin E–Cdk2 and cyclin A–Cdk2 complexes [141–143] in the early G1 phase, but it also assembles cyclin D1–Cdks in the cytoplasm and facilitates the import of cyclin D1-complexes into the nucleus. During periods of cell proliferation, p27 remains in storage by binding to Cdk4 or Cdk6 in a non-inhibitory fashion. Anti-proliferative signals, including transforming growth factor-β (TGF-β) and cell to cell contact, mobilize the stored p27 so that it can inhibit Cdk2 [144]. This all happens in the nucleus because Cdk2 is a nuclear protein, and cytoplasmic localization of p27 would effectively partition it away from this target. Mechanisms of p27 regulation in normal cells and its deregulation in BC have been thoroughly reviewed [145].

Prognostic role

In some adjuvant studies, the prognostic role of p27 in BC patients has been retrospectively evaluated by IHC utilizing different monoclonal antibodies [133, 146–156]. Cells were considered positive only when nuclear staining was identified, and the percentage of immunoreactive cells was scored in the majority of studies as low or high using a cut-off value of 50%. The number of patients analyzed ranged from 102 to 830, and they received no adjuvant systemic treatment, CT alone, hormone therapy alone, or both. Low levels of p27 were associated with worse clinical outcome in eight out of 12 studies, but no impact on prognosis was reported in four of them including the two with the largest number of patients [152, 153, 155, 156] (Table 6). An inverse relationship was found between p27 levels and histological grade, and a positive one with ER status, in the majority of studies. Furthermore, an inverse correlation with cyclin E levels [121, 133] was observed, whereas higher concentrations of p27 were associated with increased concentrations of cyclin D1 and low tumor grade [151, 152, 157] or cyclin D expression and bcl-2 positivity [154]. A statistically significant correlation between low p27 protein expression and HER2 overexpression (158–160) could validate the hypothesis that one pathway for HER2 oncogenic activity seems to rely on down regulation of the cell-cycle regulator p27 [161]. Interestingly, a decrease in the p27 levels has been reported in a trastuzumab-resistant HER2-positive cell line, and the resistance could be reverted by proteasome inhibitors such as MG 132, which induced p27 expression [162]. An inverse correlation with p53 was shown in three out of five studies where it was analyzed [147, 154, 163]. In addition, in 202 Askenazi Jewish BC patients [149], BRCA 1/2 mutations were associated with low levels of p27, and both were identified as independent prognostic factors. The failure of some studies to find a prognostic value for p27 might reflect differences in tumor fixation, methods of staining and scoring, and also the prolonged storage time of the archival tumor blocks utilized in several studies [145]. Therefore, it is crucial to define a uniform methodology for tumor processing, staining and scoring and to evaluate p27 in large prospective trials.

Predictive role

The predictive role of p27 has been retrospectively evaluated in two adjuvant studies shown in Table 7. In the first, the analysis focused on a group of premenopausal HR-positive BC patients with stage I–II disease enrolled into a randomized trial comparing goserelin plus tamoxifen to CT with CMF, and for whom a tissue sample was available [164]. High expression of p27 was observed in about 50% of patients in both arms and was found to be predictive of better RFS and OS, at a median follow-up of
5.5 years. The protein was also an independent predictor of responsiveness to endocrine therapy. Moreover, in patients with high p27 expression, endocrine therapy was superior to CT, although the benefit was statistically significant only for RFS. An unfavorable outcome was observed in patients with low p27 expression regardless of the adjuvant systemic treatment given.

In the other study [159], p27 immunoreactivity was analyzed in N-negative and N-positive BC patients enrolled in the International Breast Cancer Study Group (IBCSG) Ludwig trial V, but it did not show any significant prognostic value in terms of DFS and OS. However, in the N-negative population, the benefit from one course of perioperative CT (CMF i.v.) was confined exclusively to patients with tumors showing low levels of p27. In this subgroup of patients, low levels of p27 were associated with HER2 overexpression so, both factors should probably be analyzed to predict response to drugs that act on EGF/HER pathways. The usefulness of p27 in predicting response to systemic treatment needs to be investigated in large prospective trials.

### Table 7. Studies evaluating p27 as predictive marker

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Number of patients</th>
<th>Median FU</th>
<th>Stage</th>
<th>Predictive value in multivariate analysis</th>
<th>Analysis type</th>
<th>Evidence level</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>461</td>
<td>13.3 years (N−)</td>
<td>N−, N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[159]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.6 years (N+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC</td>
<td>512</td>
<td>5.5 years</td>
<td>I–II</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[164]</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; N−, node-negative; N+, node-positive; R, retrospective analysis.

*Premenopausal patients.

#### p21WAF1/CIP1

**Description**

The p21WAF1 (wild type p53 activated fragment 1) is a nuclear protein, also known as cyclin-dependent kinase-interacting protein 1 (CIP1) or senescent cell-derived inhibitor 1 (SDI1), that has been implicated in the mechanisms of cell-cycle arrest that allow cell DNA repair, differentiation and apoptosis. In response to DNA damaging agents, wild type p53 induces the expression of p21, which blocks the progression of the cell cycle at the G1/S transition by inhibiting the activity of Cdk2 and Cdk4/6; it can also serve as an assembly factor for cyclin D–Cdk4 complexes, increasing the efficiency of complex formation and Cdk4 activity. The integrity of G1 and G2 checkpoints requires the nuclear localization of p21WAF1. It has been shown that in cancer cells and in cell lines, p21WAF1 can localize in the cytoplasm where it inhibits apoptosis by binding and inhibiting the apoptosis signal-regulating kinase 1. In addition, other
factors can induce p21 expression and cell cycle arrest independently from p53, such as insulin-like growth factors [165], epidermal growth factor [166], or HER2 expression [167]. Upregulation of p21WAF1 occurs through the phosphatidylinositol 3-kinase/Akt (PI-3K/Akt) signaling. In HER2 overexpressing breast cancer cell lines, p21WAF1 is transcriptionally upregulated but also dislocated into the cytoplasm through a mechanism whereby Akt binds and phosphorylates p21 WAF1 in its nuclear-localization signal [167]. p21 WAF1 has also been identified as an anti-estrogen-regulated factor capable of inhibiting the Cdk4 activity present in extracts of estrogen-treated human BC cells [168].

**Prognostic role**

The prognostic role of p21WAF1 has been retrospectively analyzed in several adjuvant studies [169–178], with a number of patients ranging from 104 to 798, and a wide range of median follow-up times (Table 8). IHC was the evaluation method for p21WAF1 in all trials, and only tumor cells with detectable nuclear staining were considered positive. However, different monoclonal antibodies and scoring systems were adopted. When p21WAF1 was correlated with standard prognostic factors and other biological markers, contradictory results were obtained. Since p21WAF1 is a downstream effector in the p53-specific pathway of growth control, p53 was analyzed in all trials, although in the majority of them no association was found. Low expression of p21WAF1 was correlated with high histologic grade in three studies [171, 172, 178], while in one study the opposite was observed [170]. A positive association with proliferative activity was reported in three studies in which MIB-1 [175], Ki-67 [174] or cell nuclear antigen (PCNA) were measured; in contrast [177], an inverse association was seen with HR status in two [172, 175] and with N status in three studies [170–172]. Low levels of p21WAF1 were an independent prognostic factor for DFS only or for DFS and OS in three studies [171, 172, 178], even if in one of them it depended on the cut-off value chosen [178]. Moreover, low expression of p21WAF1 was prognostic only in combination with increased expression of p53 in three studies [170, 174, 178]. The combination of low levels of p21WAF1 and low SPF or low levels of cyclin A were associated with good prognosis on multivariate analysis in a study conducted in a relatively small number of patients [169]. The heterogeneity of the results reported in these studies calls for the necessity to standardize the evaluation method and scoring system for p21WAF1 determination.

**Predictive role**

Only one paper responding to our selection criteria could be found that retrospectively evaluated the predictive role of p21WAF1 [179]. In this study, 107 N-negative and N-positive BC patients were enrolled, but only 94 received adjuvant CT with CMF. A worse DFS was observed in patients treated with CMF if the tumor expressed high levels of p21 (either cytoplasmic or nuclear), and a positive association was found between high levels of p21 and HER2. These results seem to suggest that p21WAF1 may play a role in HER2-mediated CMF resistance.

**Topoisomerase IIα**

**Description**

Topoisomerases II (topo II) are essential nuclear DNA-binding enzymes that control and modify the topological states of DNA.

Table 8. Studies evaluating p21 as prognostic marker

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Number of patients</th>
<th>Median FU</th>
<th>Stage</th>
<th>Prognostic value in multivariate analysis</th>
<th>Analysis type</th>
<th>Evidence level</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>104</td>
<td>58 months</td>
<td>N−, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[169]</td>
</tr>
<tr>
<td>IHC</td>
<td>261</td>
<td>73 months</td>
<td>N−, N+</td>
<td>No</td>
<td>R</td>
<td>III</td>
<td>[170]</td>
</tr>
<tr>
<td>IHC</td>
<td>106</td>
<td>4.3 years</td>
<td>I–III</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[171]</td>
</tr>
<tr>
<td>IHC</td>
<td>104</td>
<td>Mean 37.1 months</td>
<td>N−, N+</td>
<td>Yes</td>
<td>R</td>
<td>IV</td>
<td>[172]</td>
</tr>
<tr>
<td>IHC</td>
<td>115</td>
<td>86 months</td>
<td>N−</td>
<td>No</td>
<td>R</td>
<td>IV</td>
<td>[173]</td>
</tr>
<tr>
<td>IHC</td>
<td>160</td>
<td>60.5 months</td>
<td>N−, N+</td>
<td>No</td>
<td>R</td>
<td>IV</td>
<td>[174]</td>
</tr>
<tr>
<td>IHC</td>
<td>798</td>
<td>16.3 years</td>
<td>N−, N+</td>
<td>No</td>
<td>R</td>
<td>III</td>
<td>[175]</td>
</tr>
<tr>
<td>IHC</td>
<td>435</td>
<td>Observation period: 191 weeks</td>
<td>N−, N+</td>
<td>No</td>
<td>R</td>
<td>III</td>
<td>[176]</td>
</tr>
<tr>
<td>IHC</td>
<td>307</td>
<td>82 months</td>
<td>T1–4 N0–2</td>
<td>No</td>
<td>R</td>
<td>III</td>
<td>[177]</td>
</tr>
<tr>
<td>IHC</td>
<td>222</td>
<td>Mean 69 months</td>
<td>N−, N+</td>
<td>Yes*</td>
<td>R</td>
<td>III</td>
<td>[178]</td>
</tr>
<tr>
<td>IHC</td>
<td>420</td>
<td>57.2 months</td>
<td>I–III</td>
<td>No</td>
<td>P</td>
<td>II</td>
<td>[203]</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; N−, node-negative; N+, node-positive; P, prospective analysis; R, retrospective analysis.

*Prognostic in a subgroup p21−/p53+.

*Trend in node-positive patients.

*Prognostic only if cut-off >3%.
by combining nuclease, helicase and ligase activities. In human cells, two distinct isoforms of topo II exist, termed α and β. These share considerable homology (72%), but are products of different genes located on chromosomes 17q21 and 3p, respectively. They differ in molecular weight, pattern of expression, function, and their apparent sensitivity to antineoplastic drugs. The β isoforms show no dependency on cell cycle phase, and its function is still largely unknown; in contrast, topo I expression is cell cycle-dependent (G2/M) and reduces DNA supercoiling and twisting by creating a double-strand nick that enables the passage of a second DNA double-strand through the break and subsequent religation of the cleaved one. Topo I is also essential for carrying out functions in the segregation and condensation of newly replicated chromosome pairs in dividing cells. Topo I protein levels are markedly higher in exponentially growing than in quiescent cell lines in tissue culture, and can be down-regulated by growth of cells at high density or in serum-free conditions [180]. Reduced levels of topo I have been found in cells induced to differentiate [181, 182] so that it can be regarded as a marker of cell proliferation. Topo I is the target enzyme for topo II inhibitors such as anthracyclines, epipodophyllotoxins, actinomycin, mitoxantrone and other drugs, and in vitro data have demonstrated a direct correlation between the intranuclear topo II levels and the degree of sensitivity to these agents. Moreover, the topo II gene (TOP 2A) is located close to the Her2 oncogene on the 17th chromosome. This could explain the very high incidence of TOP 2A aberrations in Her2 amplified tumors, with about 40% co-amplification and approximately 40% of TOP 2A deletions [183]. Only about 8%–10% of TOP 2A aberrations occur in Her2 negative patients [184, 185]. The exact mechanism by which Her2 amplification leads to TOP 2A deletion is not clear. There is some evidence that deletion of TOP 2A is associated with high levels of topo I expression or ‘haploinsufficiency’ [183, 186]. Interestingly, Her2 amplified, TOP 2A amplified cells were shown to be highly sensitive to anthracyclines, while HER2 amplified, TOP 2A deleted cells displayed a poor degree of sensitivity to these drugs [183]. Determination of topo II by IHC utilizing two different monoclonal antibodies was compared with other detection methods such as polymerase chain reaction-aided transcript titration assay, enzyme activity assay and western blotting and was competitive and easier to perform [187]. Topo II expression was also analyzed by mRNA in situ hybridization using synthetic oligonucleotide probes, and a close correlation with IHC was obtained. Discordance between topo II amplification and overexpression by IHC has been reported [188–190], suggesting that the protein expression can be regulated at different levels, including post-transcription and post-translation steps. While in one of these studies a correlation between TOP2A amplification and Her2 amplification was observed [188], in the other this correlation was not found [190]. TOP2A amplification and Her2 amplification both assayed by chromogenic in situ hybridisation (CISH) were significantly correlated in a small study in locally advanced BC patients [191]. In two studies, increased expression of topo II was associated with Her2 amplification [192] or with HER2 overexpression [193], while no association between the overexpression of the two proteins was reported by others [188, 194, 195].

Prognostic role

Only three retrospective adjuvant studies evaluating overexpression of topo II by IHC were selected through the criteria chosen (Table 9) [192, 193, 196]. A positive association with HER2 overexpression or amplification was observed in all of them, as well as with tumor size or stage, and in two studies also with high Ki-67 or MIB 1 [192, 193]. Overexpression of topo II predicted a shorter DFS, specific survival (SS) and/or OS in multivariate analysis only in two studies [193, 196]. Large prospective studies using standardized methods are needed to better define the potential clinical relevance of topo II as a prognostic factor.

Predictive role

Some retrospective studies have investigated the role of either the TOP 2A gene or the topo II expression as predictive factors of response to anthracycline-based therapy in all stages of disease, but for this review we could find only one ‘eligible’ study. Response to primary CT utilizing an anthracycline-based regimen was retrospectively evaluated in patients with operable metastasis-free BC larger than 3 cm. A high percentage of topo II expression measured by IHC was correlated to high Ki-67 and to HR-negativity, but not to HER2 overexpression, and it was predictive of response to CT, in multivariate analysis, with tumor size <40 mm and ER-negativity [194]. In this paper, HER2 did not seem to be directly involved in the prediction of anthracycline efficacy, in contrast to what has been reported by other authors [188, 191, 197]. The predictive value of TOP 2A amplification when it does not translate into protein overexpression remains to be determined, but it could be hypothesized that both the

Table 9. Studies evaluating topoisomerase II as prognostic marker

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Number of patients</th>
<th>Median FU</th>
<th>Stage</th>
<th>Prognostic value in multivariate analysis</th>
<th>Analysis type</th>
<th>Evidence level</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>184</td>
<td>61 months</td>
<td>I–IV</td>
<td>No</td>
<td>R</td>
<td>IV</td>
<td>[192]</td>
</tr>
<tr>
<td>IHC</td>
<td>356</td>
<td>99 months</td>
<td>N–</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[193]</td>
</tr>
<tr>
<td>IHC</td>
<td>863</td>
<td>149 months</td>
<td>N– N+</td>
<td>Yes</td>
<td>R</td>
<td>III</td>
<td>[196]</td>
</tr>
</tbody>
</table>

IHC: immunohistochemistry; N–, node-negative; N+, node-positive, R, retrospective analysis.
TOP 2A gene and the topo IIα protein might be useful tools to define the tumor profile with regard to sensitivity to anthracyclines. An ongoing meta-analysis of four studies comparing CMF to anthracycline-based regimens in the adjuvant setting and involving 4600 patients will likely clarify the predictive value of topo IIα and HER2 status for response to anthracyclines (Di Leo A, personal communication).

Conclusions

One hundred and thirty-five papers evaluating the prognostic and predictive roles of both some 'old' and some 'new' proliferative markers have been reviewed. A total of 159,516 BC patients have been examined in these studies, exemplifying the tremendous efforts made in an attempt to classify these patients better according to risk and to select their optimal treatment. While as many as 102 studies were 'positive', not one of these markers has been included among the standard ones. The results obtained for some markers related to cell cycle control are summarized in Figure 1. The criteria required to define a clinically useful prognostic/predictive factor are quite complex, and all must be fulfilled [198]. For some markers, such as SPF, Ki 67 and p27, we selected several studies that reported a significant correlation of the examined marker with clinical outcome on multivariate analysis, but the majority had a level of evidence III or IV and, therefore, their prognostic or predictive value still remains undefined. For other markers such as TLI, the predictive value shown in prospective trials reached level 1 evidence, but the practical difficulties linked to the determination method precluded its adoption in clinical practice.

Among the reasons for these ambiguous results are: (1) the retrospective nature of the great majority of studies; (2) the utilization of archived materials handled and preserved in different conditions; (3) the relatively small numbers of patients analyzed and the fact that the group of patients included in the translational research study is always a subset of the clinical trial population, hence inducing bias; (4) the heterogeneity of the population of patients evaluated; (5) the different methods used to assess the expression of biological and molecular markers, as well as the threshold values to define their expression; (6) the lack of standardization and quality control evaluation of different methods; and, sometimes (7) the relatively short duration of the median follow-up. Nevertheless, important messages can be derived from these studies, even if a publication selection bias cannot be ruled out. First, proliferative activity seems to have an important role in defining the behavior of BC, with theoretically possible implications for the selection of treatments, such as dose dense CT. Secondly, a standardization of the evaluation methods with intra- and interlaboratory quality controls is the first requisite to evaluate a marker. Thirdly, it is crucial to properly design large prospective trials trying to include more than one proliferative marker in order to select the best ones or the best combinations with prognostic/predictive value. International collaboration and close interaction between the basic and clinical research worlds (through translational research) are essential to avoid the duplication of efforts and fragmentation of research, and to lead to the needed level 1 evidence. In the era of high throughputs technologies, such as microarray gene profiling and proteomics, there is a greater chance to develop multimarker models that might turn out to be powerful prognostic or predictive tools. Microarray studies focusing on re-classification of BC have consistently shown the existence of a special subtype, called basal-like, which is characterized among other things, by a high level of proliferative markers such as topoisomerase IIα.

**MARKERS RELATED TO CELL CYCLE CONTROL**

<table>
<thead>
<tr>
<th>PROGNOSTIC ?</th>
<th>CYCLIN D1</th>
<th>PREDICTIVE ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes, in 2/7 studies: N=1509</td>
<td>(Southern, Northern, IHC)</td>
<td>Yes, in I/1 study: N=167</td>
</tr>
<tr>
<td>Yes, in 8/12 studies: N=3224</td>
<td>p27</td>
<td>Yes, in 2/2 studies: N=973</td>
</tr>
<tr>
<td>Yes, in 4/11 studies: N=3034</td>
<td>p21</td>
<td>↑ Tam benefit (low)</td>
</tr>
<tr>
<td>Yes, in 7/10 studies: N=2368</td>
<td>CYCLIN E</td>
<td>Yes, in I/1 study: N=107</td>
</tr>
<tr>
<td>Yes, in 15/15 studies: N=5137</td>
<td>(Western, IHC, RT-pCR)</td>
<td>↓ CMF benefit (high)</td>
</tr>
<tr>
<td>Total = 15272</td>
<td></td>
<td>↑ Tam benefit (high)</td>
</tr>
<tr>
<td>36/55 studies: level 3 or 4 evidence of independent prognostic value</td>
<td>KI67</td>
<td>Yes, in 2/5 studies: N=945</td>
</tr>
<tr>
<td></td>
<td>(IHC)</td>
<td>↑ Chemohazard (high risk under therapy)</td>
</tr>
<tr>
<td>Total = 2300</td>
<td></td>
<td>↓ Signature of optimal treatment benefit</td>
</tr>
</tbody>
</table>

**Figure 1.** The prognostic and predictive value of the proliferative markers related to cell cycle control in breast cancer: summary of results; IHC, immunohistochemistry; Tam, tamoxifen; chemo, chemotherapy; CMF, cyclophosphamide, methotrexate; 5-fluorouracil.
References


166. Giles KM, Daly JM, Beveridge DJ et al. The 3′-untranslated region of p21WAF1 mRNA is a composite cis-acting sequence bound by RNA-binding proteins from breast cancer cells, including HuR and poly(C)-binding protein. J Biol Chem 2003; 278: 2937–2946.


184. Caffo O, Doglioni C, Veronese S et al. Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immuno-


