Different Expression of Thymidylate Synthase in Primary Tumour and Metastatic Nodes in Breast Cancer Patients

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Abstract. *Background: To date an accurate evaluation of predictive markers in breast cancer is mainly conducted at the primary site, although the main goal of the adjuvant therapy is the control of micrometastases. Adjuvant therapy drugs need a high proliferative cell rate to be effective. The proliferating activity can be evaluated by the Ki-67 marker and even by thymidylate synthase (TS), a cell cycle enzyme present in proliferating cells. In this study the TS levels in primary tumours were compared to those of their metastases. Patients and Methods: The TS expression and Ki-67 were evaluated by means of immunohistochemistry in 80 primary breast tumours (PTs) and in their matched axillary metastatic lymph-nodes (ALNs). Results: In 16% of patients, malignant cells of involved nodes showed a lower TS expression than the PTs. In the same group, we also found a lower number of Ki-67 immunoreactive cells in lymph node metastases when compared with primary tumours. Conclusion: The group of patients with lower TS and Ki-67 expression in lymph node metastatic cells may be less sensitive to 5-fluorouracil and high dose methotrexate requiring them to be treated with other drug combinations.*

Breast cancer is the most frequent neoplasm in women of Western countries and is the first cause of oncological morbidity and mortality. This tumour is characterized by strong variability, so therapeutically predictive biological markers need to be identified. For breast cancer, many prognostic markers that initially appeared promising have failed to maintain their clinical predictive value. Few reports have analysed the consistency over time of biological and clinical information provided by biomarkers (1). In an analysis of many individual parameters such as differentiation, histotype and nuclear pleomorphism, it has been shown that the presence of proliferative activity is the strongest predictor of reduced survival and response to treatment (2). There are many methods to assess proliferative activity such as flow cytometric analysis of the cells in the S-phase, the thymidine labeling index and immunohistochemical analysis of proliferation antigens, of which Ki-67 is the most frequently used. Thymidylate synthase (TS) could also be considered a marker of proliferation status since it is related to the percentage of cells in the S-phase of the cell cycle (3). TS is the most important target of 5-fluorouracil (5-FU), a drug which is frequently used in chemotherapeutic combinations such as for the treatment of breast cancer FAC, FEC, CMF. Although much of the recent prognostic emphasis in breast cancer patients has been related to node negative status (4), it may be important to study the clinical, prognostic and predictive features of axillary metastases. Even though nodal status is the single most important predictor of distant metastases and overall survival, this factor is not always sufficient to allow a treatment decision to be made; in fact for those patients who present axillary lymph node (ALN) involvement, therapeutic options range from tamoxifene alone to several anti neoplastic drug combination-based protocols (5). To date, in breast cancer, both proliferation index and the other immunohistochemical prognostic factors (estrogen and progesterone receptors, C-Erb2, Mib-1, p53) are exclusively assessed on the primary tumour (PT) (6) while the main goal is the control of micrometastases. In our opinion these features even in metastatic lymph nodes could influence the clinician's therapeutic choice.

The aim of this study was to evaluate as a therapeutic and/or prognostic marker the proliferation status of the PTs

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Table I. *Clinicopathological features of patients included in the study.*

Patients		N
Age (years)	<35	25
	>35	55
Stage	II (UICC-TNM)	80
Grading	G1	17
	G2	30
	G3	33
Histotype	Ductal infiltrating carcinoma	43
	Lobular infiltrating carcinoma	26
	Lobular-ductal infiltrating carcinoma	4
	Tubulo-lobular carcinoma	7

and of the ALNs in 80 breast cancer node-positive patients using the TS expression. The TS levels evaluated both in PTs and in metastases could give important information not only about the proliferation status of the tumours but could also be predictive of the response to fluoropyrimidine drugs. In order to confirm the predictive value of TS for proliferation, the same group of patients already evaluated for Ki-67 expression was chosen in which we had observed that a small group of patients (16%) showed lower Ki-67 positivity in matched lymph nodes when compared with PTs (7).

Patients and Methods

The clinical records of 80 patients with axillary positive nodes, who had undergone surgery for previously untreated breast adenocarcinoma, were reviewed. In a previous study the Ki-67 expression in these samples had been evaluated (7).

The cases comprised 43 ductal infiltrating carcinomas (CDI) (31 CDI, 8 mucinous carcinomas, 4 apocrine carcinomas), 26 lobular infiltrating carcinomas, 4 lobular and ductal infiltrating carcinomas and 7 tubulo-lobular carcinomas. All cases were in stage II according to the UICC-TNM staging system (Table I).

Before starting the study, the patients' consent was obtained.

Our study was performed using formalin-fixed paraffin-embedded primary tumour samples, retrieved from archival material. For each case, four 3-mm sections that were dewaxed and rehydrated were used. Antigen retrieval was carried out using Dako (Glostrup, Denmark) antigen retrieval fluid and microwaving at full power for 30 min, divided into 6 periods. The sections were then treated with hydrogen peroxidase for 5 min, followed by TS 106 antibody at a dilution of 1:10 overnight at 4˚C. Visualization was obtained with a Dako visualization system. For metastatic localization, as a positive internal control the follicle germinal centres of lymph nodes were used, while for primary tumours a sample of colorectal carcinoma was used as a positive external control. The TS staining was evaluated as being positive when the intensity of staining of the samples (cytoplasmatic and/or nuclear) was greater than the intensity of staining of the respective control. Finally, negative controls, lacking the primary antibody, were included in each run. The TS staining positivity was evaluated in 10 microscopic high power fields (HPFs) in which the neoplastic epithelial area was more than 70% of the whole field. The TS staining positivity was evaluated semi-quantitatively as strong positivity (++) if more than 50% of the neoplastic areas were positive, mild positivity (+) if 10-50% of neoplastic areas were positive and negativity (–) if less than 10% of the neoplastic areas were positive.

Table II. *TS expression: ++, strong positive; +, mild positive; –, negative in primary tumours (PTs) and in matched axillary lymph-nodes (ALNs).*

	PT – / ALN + PT + / ALN ++	PT ++ / ALN ++ PT + / ALN +	PT ++ / ALN + PT + / ALN –
Category A	47	0	0
Category B	0	20	0
Category C	0	0	13

Results

On the basis of our results, the patients were divided into three groups. Category A; This consisted of 47 cases (59%) with the following histological types: 29 infiltrating ductal carcinomas, 2 mucinous carcinomas, 1 apocrine carcinoma, 13 infiltrating lobular carcinomas, 1 lobular – ductal infiltrating carcinoma and 1 tubulo-lobular carcinoma. All these cases showed an increased TS expression in the ALNs when compared with PTs. Category B; This consisted of 20 cases (25%) with the following histological types: 5 infiltrating ductal carcinomas NOS, 1 mucinous carcinoma, 1 apocrine carcinoma, 10 infiltrating lobular carcinomas, 1 lobular–ductal infiltrating carcinoma and 2 tubulo-lobular carcinomas. All these cases showed the same TS expression in both the ALNs and the PTs. Category C; This consisted of 13 cases (16%) showing the following histological types: 6 infiltrating ductal carcinomas NOS, 1 mucinous carcinoma, 1 apocrine carcinoma, 3 infiltrating lobular carcinomas, 1 lobular–ductal infiltrating carcinoma and 1 tubulo-lobular carcinoma. In this category, the cases showed a decreased TS expression in ALNs when compared with the PTs.

The decrease of TS expression in the ALNs of category C proved to be independent of the histotype and the histological grade of the tumour. These data were concordant with the Ki-67 findings (Figure 1).

Discussion

The search for prognostic factors for breast cancer is largely centred on node-negative disease, where about the 25% of node-negative patients are destined to relapse despite the treatment (4), and the goal of these studies has been to direct treatment to the minority of patients who would

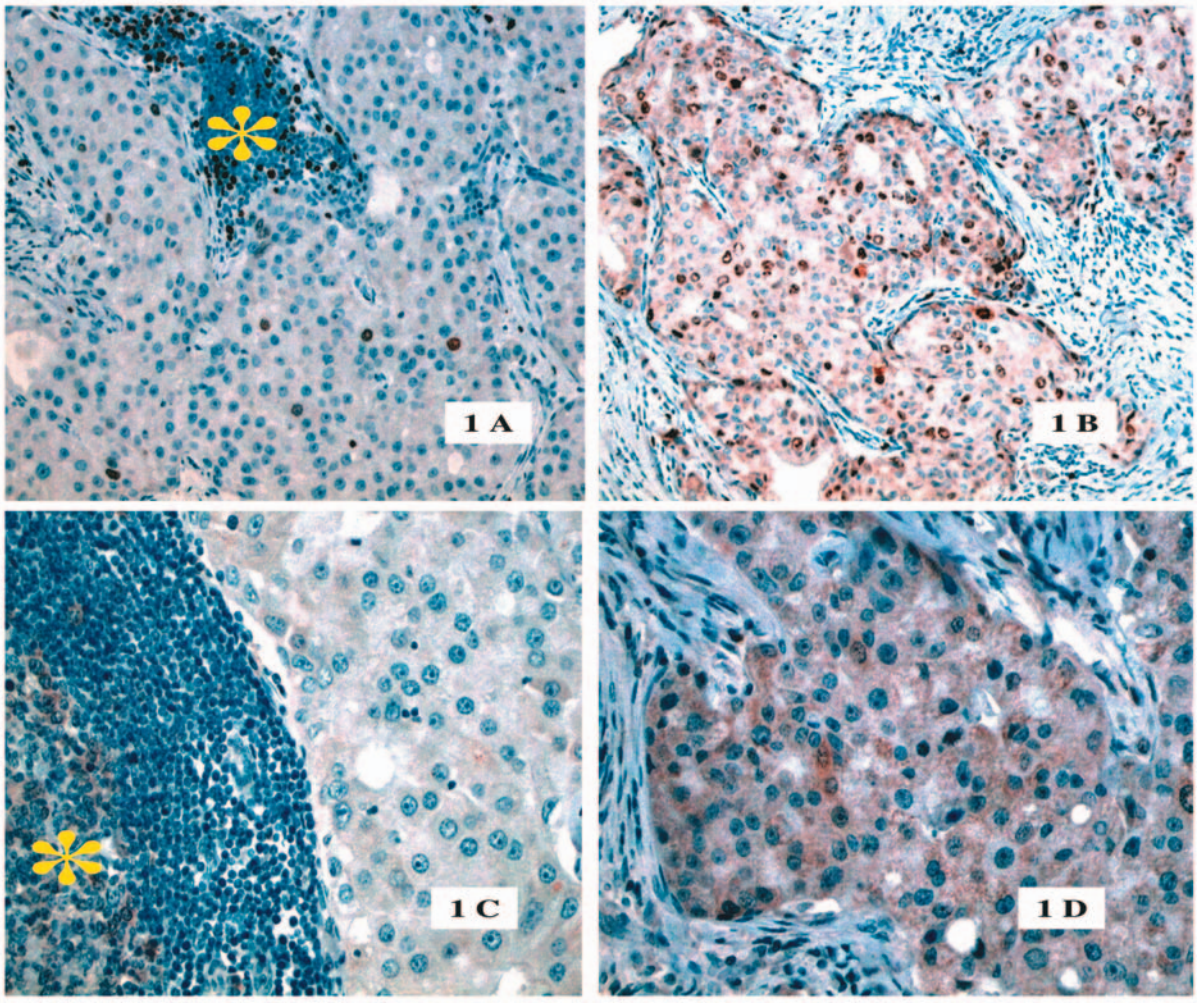

Figure 1. *1A-1B) Nuclear positive immunostaining for Ki-67 antibody is very rare in metastatic lymph nodes (1A) and very frequent in primary tumour (1B) (x200). 1C-1D) Cytoplasmic positive immunostaining for TS antibody is absent in metastatic lymph node and present in primary tumor (x400). Note for positive control lymphoid germinal centre (*), positive for both antibodies.*

derive benefit from adjuvant chemotherapy. In contrast, the vast majority of patients with node-positive breast cancer can expect to receive adjuvant chemotherapy routinely. Prognostic factors in this group have received less attention, in large part because of the definite benefit derived from adjuvant chemotherapy and the belief that additional measures would have little impact on the prognosis predicted by the presence of positive axillary lymph nodes.

TS converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is essential for DNA biosynthesis and is a critical target for the fluoropyrimidines and high dose methotrexate (HDMTX). Both 5-FU and fluorodeoxyuridine are converted in tumour cells to FdUMP which inactivates TS by the formation of a ternary covalent complex in the presence of the folates cofactor 5,10-methylenetetrahydrofolate; while MTX usually inhibits dihydrofolate reductase abolishing the tetrahydrofolate synthesis that usually transfers one carbon group to d-uridine-monophosphate (d-UMP). In a key metabolic event, d-UMP is converted to thymidylate, an essential component of DNA, by TS. Expression of TS has been shown to be associated with response to 5-FU in human colorectal, gastric and breast cancer, in fact it is widely accepted that high TS levels are associated with a worse prognosis (8). However, some studies have shown that some histological types of colorectal tumours, with the worst prognosis such as the signet ring cell histotype, had low TS levels (9) so patients with low TS expression also fail to respond to treatment (6) (10). Our study showed that in some patients (group C), breast primary carcinomas have a high TS expression, while their matched ALNs have a lower TS expression.

Our data showed the same relationship between TS positivity and cell proliferation index as that assessed by the Ki-67 levels. The low TS and Ki-67 values in some lymph nodes, suggesting a low proliferative activity, was unexpected due to the well known aggressiveness of metastases, thus mechanisms other than proliferation could be responsible for the escape of these cells. De la Haba *et al.* reported that in 60% of cases, the immunohistochemical expression of some proteins such as estrogen, progesterone, Ki-67, p53, and HER-2, was modified during tumour development and dissemination, and led to immunophenotypical differences between PTs and ALNs (11). They hypothesized that this fact could be related to the intrinsic hetereogeneity of the neoplastic cells. As different clones are present in primary breast carcinomas, it is possible that only a small sub-population of the PT cells metastasize. Buxant *et al.* found a significantly higher proliferation index in ALNs than in PTs, suggesting an increased aggressiveness of metastatic neoplastic cells as compared to their PT counterparts. They concluded that those cells with "the most aggressive potential (like a high proliferative index) are the most likely to escape from the primary tumour", or that "cells that escape from primary tumour, lose down-regulator factors or suppressor genes and become more aggressive" (12). On this basis, we can hypothesize that metastatic node cells with low Ki-67 and TS contents could be less sensitive to 5-FU, because its target is lacking. If this hypothesis is confirmed by other clinical and experimental studies, the value of performing imunohistochemical assessment of these markers not only in PTs, but also in the respective metastases, should be considered.

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