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RESEARCH

Near-Diploid Hyperploidy in Early Breast Cancer (T1a,b) is Associated with Higher Risk of Lymph Node Involvement

Joško Bezić · Ivana Šamija-Projić · Petar Projić · Jelena Ljubković · Sandra Tomaš-Zekić · Maja Marinović-Guić · Snježana Tomić

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Abstract Due to the worldwide implementation of the mammographic screening program early breast cancer (T1a,b) has become more prevalent form of breast cancer. Although T1a,b breast cancers are generally associated with excellent prognosis, some of them, particularly those with lymph node involvement, has unfavourable outcome. Searching for additional prognostic factors, we investigated DNA content of 163 T1a,b cancers measured by DNA flow cytometry, and correlated it with regional lymph node status. T1a,b cancers were divided into four ploidy classes based on their DNA index (DI): hypodiploid (DI<0.95), diploid (DI 0.95–1.05), low-hyperploid (DI 1.06–1.3), and high-hyperploid (DI>1.3). Diploid T1a,b cancers were associated with negative lymph node status (p=0.003). Among aneuploid cancers only low-hyperploid tumors were associated with positive lymph node status (p=0.03). The histopathological features of low-hyperploid group of T1a,b cancers did not differ from the other three ploidy groups of cancers, except for lower S-phase fraction of tumor cells in low-hyperploid group compared to high-hyperploid group (p=0.01). Our data showed that near-diploid hyperploid T1a,b cancers are associated with higher risk of lymph node involvement despite similar clinicopathological features shared with other ploidy classes of T1a,b tumors.

Keywords Early breast cancer · Nodal metastases · DNA flow cytometry · DNA index

Introduction

The prognosis of patients with breast cancer depends on earlier detection of tumors in their preinvasive or early invasive stages. The mortality of non-invasive cancers is extremely low, while smaller size of invasive cancers is associated with better chance of survival [1, 2]. The detection of early breast cancers (non-invasive and small invasive cancers) has become the main goal in preventive public health strategies, with screening mammography as primary screening tool [3].

The implementation of screening mammography has increased the detection rate of small invasive breast cancers (breast cancer with maximum diameter ≤1cm, T1a,b). The prognosis of T1a,b breast cancers is favorable, with 10-year relapse-free survival rates higher than 90 % for the lymph node negative tumors (T1a,bN0), even without adjuvant systemic therapy [4, 5]. However, there is also prognostically unfavorable subset of cancers within this group, particularly those with lymph node involvement (T1a,bN1) [6, 7]. The adverse prognostic factors for the group of T1a,bN0 cancers are high tumor grade, present lymphovascular invasion, high proliferation index and younger patient's age [5]. The identification of other possible unfavorable prognostic factors whitin the group of T1a,b breast cancers remains an important goal of basic and clinical research.

In this study we tried to explore possible prognostic role of tumor DNA content expressed by DNA index (DI), measured by DNA flow cytometry in T1a,b breast cancers. Because of the overall excellent prognosis of this group of tumors, and insufficient follow-up we used axillary node

J. Bezić (✉) · I. Šamija-Projić · P. Projić · J. Ljubković · S. Tomaš-Zekić · S. Tomić
Institute of Pathology, Forensic Medicine and Cytology, Clinical Hospital Center Split, Spinčićeva 1, 21 000 Split, Croatia
e-mail: jbezic@mefst.hr

M. Marinović-Guić
Split University School of Medicine, Split, Croatia

status as the second-best surrogate of final outcome for these patients. We tried to identify possible significant difference in DI values between T1a,bN0 and T1a,bN1 tumors.

Patients and Methods

Patients

A series of 163 T1a,b breast carcinomas operated on at Clinical Hospital Center Split in the period from 1997 to 2010 were retrospectively analyzed. The analysis included 51 T1a,bN1 cancers, and 112 T1a,bN0 cancers, surgically treated with total mastectomy or segmental excision followed by subsequent irradiation. All patients had at least level I and level II axillary dissection. The major part of the series (40 T1a,bN1 cancers and 112 T1a,bN0 cancers) was been used in the previous study focused on the identification of a group of T1a,b breast cancers with low risk of axillary nodal involvement [8]. Pathohistological features were retrieved from the database of the Institute of Pathology and Cytology, Clinical Hospital Center Split, Croatia. The following features were analyzed: patient's age at diagnosis, tumor size, histological type, Nottingham histological grade, peritumoral lymphovascular invasion, hormonal receptor expression, HER-2/neu status, axillary lymph node status, DI, and S-phase fraction (SPF). According to the age the patients were divided into two groups: ≤50 years and > 50 years.

Pathology

Tumor size was defined microscopically as the greatest dimension of the invasive component. The tumors with the greatest dimension measuring ≤ 0.5 cm were classified as T1a cancers, while those with the greatest dimension measuring 0.6–1 cm were classified as T1b cancers [9]. The cancers were histologically classified according to the World Health Organization Classification of breast tumors [9]. The group of cancers with a favorable prognosis was composed of tubular, mucinous, cribriform, and tubulolobular histological types [10]. The group of cancers designated as "others" was composed of mixed ductal and lobular, medullary, papillary, and micropapillary cancers. Tumor grade was assessed according to Elston and Ellis (Nottingham histological grade) [11]. The mitotic index (MI), the integral part of Nottingham histological grade, was assessed by counting definite mitoses in 10 consecutive high power fields [11]. The mean value of MI for all tumors of 7 ± 9 (median 4, range 0–56) was used to divide cancers into subsets with low or high mitotic activity. The peritumoral lymphovascular invasion was evaluated by routine H&E preparation as the presence of tumor cells in vascular lumina and confirmed by immunohistochemistry with CD34 antibody (clone 1A6, 1:50 dilution, Dako, Glostrup, Denmark). Lymph node status was determined by standard histological sectioning and H&E staining of isolated nodes, with no serial sectioning or immunohistochemistry preformed. The patients with one or more tumor-positive lymph nodes were considered node-positive (N1).

Hormonal receptor status was determined by immunohistochemistry with ER (clone 1D5, 1:300 dilution, Dako, Glostrup, Denmark) and PgR (clone 1A6, 1:600 dilution, Dako, Glostrup, Denmark) antibodies. The cancers were considered hormonal receptor-positive if there was nuclear staining in ≥ 1 % of neoplastic cells for at least one hormonal receptor [12].

HER2/neu status was determined immunohistochemically using the HercepTest™ (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. The score of 3+ was considered true-positive, while the scores of 0 and 1+ were considered negative. Indeterminate scores of 2+ were additionally evaluated with chromogenic in situ hybridization (CISH).

All immunoassays were performed on DAKO autostainer (DakoCytomation) using the standard avidin-biotin complex method.

CISH was done on all 2+ HercepTest scores using commercially available SPOT-Light® HER2 CISH Kit (Invitrogen/Zymed, Camarillo, USA) according to the manufacturer's instructions. HER-2/neu amplification was recorded when the nuclei of >50 % cancer cells contained >10 dots or large clusters, or a mixture of multiple dots and large clusters of HER-2/neu gene (high amplification); or when the nuclei of >50 % cancer cells contained 5–10 dots or small clusters, or a mixture of multiple dots and small clusters of HER-2/neu gene (low amplification) [13]. There were five 2+ HercepTest scores with four T1a,b tumors without HER-2/neu amplification and one T1a,b tumor with low amplification of HER-2/neu gene revealed by CISH.

DNA Flow Cytometry

Ploidy and S-phase fractions (SPF) were determined by DNA flow cytometry (FACScan flow cytometer, Becton-Dickinson, San Jose, USA) on paraffin-embedded tumor tissue using the standardized method [14]. DNA histograms were evaluated using Modfit 3.0 (Verity Software House, Inc.) computerized software program. Ploidy was expressed by DNA index (DI), which is a ratio of the mean channel number of the G0/G1 peak of the tumor cell population to the mean channel number of the diploid reference peak. The reference diploid cells were non-malignant cells represented in the analyzed tumor sample. The tumors were classified as diploid if they showed a single G0/G1 peak (DI =0.95–1.05), whereas they were classified as aneuploid if a separate, identifiable G0/G1 peak (with more than 10 % of events) was present (DI <0.95 or >1.05). The samples with three separate peaks were classified as multiploid, where the clone with the highest DI value was taken for further group stratification according to the DI values. The coefficient of variation (CV, standard deviation relative to mean value) was calculated on the G0/G1 peak with the maximum acceptable value of 8 %. The mean CV was 4.25 ± 1 (median 4.17; range 2.27–6.67). The mean value of SPF for all tumors of 2.71 ± 2.57 (median 1.79 %; range 0.2–18.44 %) was used to divide cancers into subsets with low or high SPF.

Statistical Analysis

For the purpose of statistical analysis the tumors were divided into four groups according to the their DI values: group 1, tumors with DI <0.95 (hypodiploid tumors); group 2, tumors with DI 0.95–1.05 (diploid tumors); group 3, tumors with DI 1.06–1.3 (low-hyperploid tumors); and group 4, tumors with DI >1.3 (high-hyperploid tumors). These four groups were compared for the frequency of axillary lymph node involvement as a main outcome measure, and for the other usual tumors' clinicopathological features. The comparison was made using χ^2 test. The significance of the differences was calculated as p value, with probabilities lower than 5 % ($p<0.05$) considered significant. Statistical analysis was performed with the Statistical Package for Social Sciences, version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Clinical, pathohistological and DNA flow cytometric features of analyzed T1a,b cancers are shown in Table 1. The majority of the women were postmenopausal (75.5 %). T1b tumors predominated over T1a tumors (87.7 % vs. 12.3 %), with infiltrating ductal carcinoma as the most common histological type (69.3 %). The other tumors' pathohistological characteristics were prognostically favorable with predominance of grade 1 cancers with low mitotic index (56.4 % and 68.1 %, respectively), high percentage of hormonal receptor positivity (88.3 %), and low incidence of lymphovascular invasion (11.7 %) and HER2/neu expression (6.1 %). According to the distribution of the DI the majority of the tumors were diploid (47.2 %), followed by high-hyperploid (28.8 %), low-hyperploid (14.1 %), and hypodiploid (9.8 %) tumors. The approximately two thirds of cancers (68.7 %) showed low proliferative activity measured by SPF.

The comparison between different DI groups and nodal status is shown in Table 2. The diploid tumors showed

Table 1 Clinical, pathohistological and DNA flow cytometry features of T1a,b cancers

Feature	Number (%)
Age (years)	
≤50	33(20.2)
>50	123(75.5)
unknown	7(4.3)
Tumor size	
T1a	20(12.3)
T1b	143(87.7)
Histology	
Ductal	113(69.3)
Lobular	10(6.1)
Favorable subtypes	21(12.9)
Others	19(11.7)
Histological grade	
1	92(56.4)
2	51(31.3)
3	20(12.3)
Mitotic index	
Low (<7)	111(68.1)
High (≥7)	52(31.9)
Lymphovascular invasion	
Present	19(11.7)
Absent	144(88.3)
Hormonal receptor	
Positive	144(88.3)
Negative	19(11.7)
HER2/neu	
Positive	10(6.1)
Negative	153(93.9)
DI	
<0.95 (Hypodiploid)	16(9.8)
0.95–1.05 (Diploid)	77(47.2)
1.06–1.3 (Low-hyperploid)	23(14.1)
>1.3 (High-hyperploid)	47(28.8)
S-phase fraction	
Low (<2.7 %)	112(68.7)
High (≥2.7 %)	51(31.3)

Table 2 Relationship between DI and nodal status

DI	Node negative/N(%)	Node positive/N(%)	P value[a]
<0.95	8(7.1 %)	8(15.7 %)	0.13
0.95–1.05	62(55.4 %)	15(29.4 %)	**0.003**
1.06–1.3	11(9.8 %)	12(23.5 %)	**0.03**
>1.3	31(27.7 %)	16(31.4 %)	0.68

[a] Chi-squared test

significantly lower incidence of lymph node metastases (p=0.003). Among aneuploid tumors only low-hyperploid group of cancers was with significantly higher incidence of lymph node metastases (p=0.03).

The comparison between different DI groups and clinicopathological features of T1a,b cancers is shown in Table 3. As low-hyperploid group of cancers has emerged as the group of interest because of its association with higher incidence of lymph node metastases, we compared clinicopathological features of this particular group of T1a,b cancers to clinicopathological features of each DI group separately. There were no significant differences between the tested clinicopathological features of low-hyperploid group of tumors and those of the remaining three DI groups, except in SPF between low-hyperploid and high-hyperploid DI group (26.1 % low-hyperploid cancers with high SPF versus 61.7 % high-hyperploid cancers with high SPF, p=0.01).

Discussion

The proportion of detected T1a,b cancers has raised from 10 to 20 % of all detected cancers in prescreening era to 30–60 % of all detected cancers in the countries with implemented mammography screening program [15, 16]. Although generally coupled with excellent prognosis, a small proportion of women with T1a,b breast cancers will relapse and eventually die of the disease. The identification of such prognostically unfavorable cases depends on the evaluation of standard patohistological factors, which in some cases

Table 3 Relationship between DI and clinicopathological features

Parameter	DI groups / N(%)				P value[a]		
	A <0.95	B 0.95–1.05	C 1.06–1.3	D >1.3	C vs A	C vs B	C vs D
Age (years)							
≤50	2(12.5)	19(24.7)	2(8.7)	10(21.3)	0.95	0.15	0.29
>50	11(68.6)	56(72.7)	21(91.3)	35(74.5)			
unknown	3(18.8)	2(2.6)	0	2(4.3)			
Tumor size							
T1a	1(6.3)	13(16.9)	2(8.7)	4(8.5)	0.74	0.52	0.66
T1b	15(93.7)	64(83.1)	21(91.3)	43(91.5)			
Histology							
Ductal	12(75)	49(63.6)	16(69.6)	36(76.6)	0.16	0.17	0.08
Lobular	0	5(6.5)	3(13)	2(4.2)			
Favorable	2(12.5)	12(15.6)	4(17.4)	3(6.4)			
Others	2(12.5)	11(14.3)	0	6(12.8)			
Histological grade							
1	12(75)	47(61)	14(60.9)	19(40.4)	0.65	0.96	0.23
2	3(18.7)	22(28.6)	7(30.4)	19(40.4)			
3	1(6.3)	8(10.4)	2(8.7)	9(19.2)			
Mitotic index							
Low (<7)	12(75)	56(72.7)	15(65.2)	28(59.6)	0.76	0.66	0.84
High (≥7)	4(25)	21(27.3)	8(34.8)	19(40.4)			
Lymphovascular invasion							
Present	0	6(7.8)	2(8.7)	11(23.4)	0.63	0.76	0.24
Absent	16(100)	71(92.2)	21(91.3)	36(76.6)			
Hormonal receptor							
Positive	15(93.7)	67(87.1)	21(91.3)	41(87.2)	0.10	0.84	0.91
Negative	1(6.3)	10(12.9)	2(8.7)	6(12.8)			
HER2/neu							
Positive	1(6.3)	3(3.9)	3(13)	3(6.4)	0.87	0.26	0.63
Negative	15(93.7)	74(96.1)	20(87)	44(93.6)			
S-phase fraction							
Low (<2.7 %)	14(87.5)	63(81.8)	17(73.9)	18(38.3)	0.52	0.59	**0.01**
High (≥2.7 %)	2(12.5)	14(18.2)	6(26.1)	29(61.7)			

[a] Chi-squared test

fail to predict patient's outcome. Searching for the additional prognostic information we investigated DNA content measured by DNA flow cytometry in the group of 163 T1a,b breast cancers, with particular interest on the relationship between different ploidy classes and regional lymph node status. DNA flow cytometry may be used as relatively simple and inexpensive tool for measuring genetic deviation in early breast cancer, with some limitations concerning the low sensitivity and specificity of the method.

We found that 52.8 % of T1a,b cancers in this work had an abnormal DNA content which indicates that the DNA aneuploidy may be an early event in the breast cancer evolution. Ottesen et al. found an abnormal DNA content in 49 % of the cases of screened invasive breast cancers with maximum diameter ≤15 mm [17]. Ewers et al. reported the frequency of aneuploid clones of 43 % in the invasive cancers with mean diameter of 9 mm, while Blegen et al. in their study reported that 47 % of T1b breast cancers were aneuploid [18, 19]. The majority of the aneuploid T1a,b cancers in our work were high-hyperploid with DI >1.3 (28.8 %), followed by the cancers with low-hyperploid (DI=1.06–1.3) and hypodiploid (DI <0.95) clones (14.1 % and 9.8 %, respectively). In the work of Ottesen et al., which was the single work in the literature with presented DI distribution of early breast cancers, the majority of aneuploid cancers were low-hyperploid, followed by high-hyperploid tumors [17].

When we correlated the DNA content of Ta,b cancers with axillary lymph node status as a main outcome measure, we found significantly lower incidence of nodal metastases in diploid group of cancers, and significantly higher incidence of nodal metastases in low-hyperploid group of T1a,b cancers. Only a few works, our previous work included, reported statistically significant correlation between ploidy/SPF and nodal involvement in the group of early breast cancers [8, 17, 20, 21]. However, among them only Ottesen et al. correlated different DI classes with lymph node status. They found significant association between node positive tumors and DNA hypertetraploidy, confirming the results presented in the other works in which the poorest prognosis of the breast cancers was associated with hypertetraploid clones, irrespective of tumor size [17, 22, 23]. In our group of T1a,b cancers we couldn't confirm the association between hypertetraploidy and nodal involvement, due to the small number of hypertetraploid tumors (3.7 %), here included in high-hyperploid group. However, in this work, another aneuploid class of T1a,b cancers, slightly hyperdiploid tumors, has emerged as a group of early breast cancers associated with lymph node involvement.

When we correlated the classic clinicopathologic features of low-hyperploid group of T1a,b cancers with the features of other three DI groups of tumors (hypodiploid, diploid, and high-hyperploid) separately, we couldn't find any significant difference between them, except higher proliferation index measured by SPF in high-hyperploid group of tumors. This finding indicates that using the classic clincopathologic characteristics, slightly hyperdiploid T1a,b breast cancers which might have particular propensity for lymph node metastases, remain "invisible" among different ploidy groups of the early breast cancers.

Cytogenetically, near-diploid hyperploidy in breast cancers may be the final result of two different evolutionary pathways. In the majority of breast cancers structural chromosome rearrangements occur, followed by endoreduplication that transitionally increases DNA content close to tetraploidy. Subsequent chromosomal losses and unbalanced rearrangements lead to hypotetraploidy and even to near-diploid hyperploidy in the most advanced stages [24]. Alternatively, as Molist at al. recently described, in some breast cancers gain of whole chromosomes as a result of mitotic malsegregations prevails, which in association with few chromosomal rearrangements directly lead to low-hyperploidy [25]. Such evolutionary scenario in which aneuploidy is a consequence of numerical, not structural, chromosome aberrations has also been described in tumors associated with microsatelite instability, like in colorectal carcinoma of the proximal colon [26].

Low-hyperploid early breast cancers were only mentioned in the litterature by Ottesen et al., who described the predominance of DNA low-hyperploid clones in the different groups of early breast cancers, clinically and screening detected invasive breast cancers with maximum diameter ≤15 mm included. Those low-hyperploid clones were particularly present in highly differentiated small cancers, as well as in the group of invasive cancers with predominance of DCIS, which led the authors to conclude that near-diploid hyperploidy may be related to invasion [17]. Some older works suggested that prognosis in patients with near-diploid hyperploid breast cancers is similar or even better to that in patients with diploid cancers [27, 28]. In their recent work, Elie et al. investigated clinicopathological features and outcome of 134 near-diploid hyperploid tumors (DI=1.1–1.3) in the group of 1771 patients with T1-T4 ductal breast carcinomas. Having compared that slightly hyperdiploid group of cancers with diploid/hypodiploid group they found that their clinicopathological features were similar, except for the higher proliferative activity in hyperdiploid tumors. Overall survival in these two groups of tumors was also similar, but near-diploid hyperploid tumors were significantly related to poorer metastases free survival. There were no significant differences in overall survival and metastases free survival between near-diploid hyperploid and other hyperploid cancers in this study. The authors concluded that near-diploid hyperploid cancers might represent a particular subset of near-diploid tumors with poorer metastases free survival compared to diploid/

hypodiploid tumors, possibly related to their increased SPF [29]. The mean SPF of tumors presented in this work was low (2.7 %), which is related to small tumors' size (T1a,b), and to the predominance of low grade, hormonal receptor positive tumors. All aforementioned tumor characteristics correlate with low SPF values [30]. The percentage of T1a,b tumors with high SPF values in this work progressively increased from hypodiploid to high-hyperploid tumors, which also mirrored previous findings about positive correlation between SPF and DNA ploidy [30]. Contrary to Elie et al., we didn't find significant difference in SPF values between hypodiploid/diploid tumors and lymph node involvement prone low-hyperploid T1a,b tumors [29]. Moreover, high-hyperploid tumors in our work had significantly higher SPF values than low-hyperploid tumors, indicating that high proliferative activity is not related to the lymph node metastatic potential in the group of early breast cancers. The equally distributed MI between the correlated ploidy classes in this work also confirms this finding.

Herein, we have confirmed the metastatic potential of low-hyperploid T1a,b cancers which had significantly higher rate of lymph node metastases than all other aneuploid groups of early breast cancers. We were not able to perform cytogenetical analysis of low-hyperploid clones presented in this work, in order to conclude which cytogenetical pathway dominated in their generation, but we can postulate that in small breast cancers near-diploid hyperploidy is genetically an early event, and that it is probably generated mainly by tumor clones with whole chromosome gains. In such scenario, low-hyperploid T1a,b cancers acquire immediate metastatic potential which is somehow related to this specific type of chromosome alteration. This finding indicates that the group of near-diploid tumors might really represent prognostically distinct subset of breast cancers even in the earliest phase of their development.

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