Int. J. Cancer: **119,** 1383–1388 (2006)

UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 6 (ppGalNAc-T6) mRNA as a potential new marker for detection of bone marrow-disseminated breast cancer cells

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The evaluation of disseminated epithelial tumor cells in patients with early stages of breast cancer has generated considerable interest because of its potential association with poor clinical outcome. Considering that O-glycosylation pathways are frequently altered in breast cancer, we performed this work to evaluate the potential usefulness of UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts) (a family of glycosyltransferases which catalyze the first key step of mucin-type O-glycosylation) to detect disseminated cells in bone marrow samples from patients with operable breast cancer. Using RT-PCR assays, we studied the gene expression of 9 enzymes (ppGalNAc-T1–T9). Among the ppGalNAc-Ts expressed by breast tumors (-T1, -T2, -T3, -T6 and -T7), the best specificity (negative results on all PBMN cell samples from healthy donors) was shown for ppGalNAc-T6. Thus, we selected this enzyme as a target gene for further evaluation. ppGalNAc-T6 mRNA was found in 22/25 (95%) breast cancer samples, in all 3 human breast cancer cell lines evaluated (MCF-7, ZR75-1 and T47D), in 1/30 (3%) PBMN cells and 0/19 bone marrow samples obtained from patients without cancer. Using this method, 22/61 (36%) patients with breast cancer, who underwent curative surgery, showed positive ppGalNAc-T6 mRNA in bone marrow aspirates obtained prior to surgery, including 11/34 patients with stage-I or -II, without histopathological lymph node involvement. In a preliminary follow-up evaluation, 19/61 patients experienced recurrence of the disease. ppGalNAc-T6 was positive in 11/19 (57.9%) of these patients. Interestingly, in the group of patients without lymph node involvement, disease recurrence was observed in 54.5% of patients who showed ppGalNAc-T6 mRNA-positive bone marrow aspirates and only in 4.3% of patients when ppGalNAc-T6 was negative ($p = 0.014$). These results indicate that ppGalNAc-T6 mRNA could be a specific marker applicable to the molecular diagnosis of breast cancer cells dissemination.

Key words: ppGalNAc-transferases; disseminated cancer cells; RT-PCR; breast cancer

Early detection of breast cancer clearly improves the management of patients. A substantial proportion of breast cancer patients have cancer cells present in their blood at time of diagnosis.[1] These cells seem to first disseminate from the early, primary lesions and then acquire additional genetic defects.[2] Many investigators have conceptually accepted that breast cancer is a systemic disease.[3] There is now increasing evidence that the detection of disseminated malignant cells in bone marrow aspirates of patients with early stages of breast cancer is of prognostic relevance.[4–7] In addition, evidence of persistent disseminated tumor cells (DTC) in bone marrow of these patients indicates an increased risk for subsequent recurrence.[8] Therefore, the identification of occult metastases could influence future strategies of adjuvant systemic therapy. Several molecular methods have been employed for searching epithelial cell-specific protein expression in nonepithelial tissues. The application of monoclonal antibodies directed against surface antigens or cytokeratins has led to improvements in the ability to detect breast cancer cells by different immunolabelling techniques.[9] The use of RT-PCR is a highly sensitive tool widely assayed for detection of isolated breast cancer cells amplifying several transcripts, *e.g.*, CK19,[10] CEA,[11] mammaglobin,[12] telomerase[13] and MUC5B.[14] Unfortunately, until now, none of the mRNA markers investigated have proven to be specific and sensitive enough to gain access to the clinical practice. Even if an immunocytochemically detectable CK expression in ''normal'' bone marrow cells is a rare event, by using more sensitive techniques such as RT-PCR, several publications report false-positive results. The ideal mRNA tumor marker, expressed at high levels in breast cancer cells and not at all in hematological samples, remains to be identified.

Tumor development is usually associated to alterations in cell-surface carbohydrates. Glycosylation changes arising as a consequence of malignant transformation influence cell growth as well as differentiation, adhesiveness and immunogenicity of cancer cells.[15] Moreover, it has been shown that carbohydrate structures on the tumor cell surface are associated to the metastatic potential of tumor cells, and even the prognosis of cancer patients.[16] Glycosyltransferases constitute a large group of enzymes that are involved in the biosynthesis of oligosaccharides and polysaccharides.[17] One of the reasons why tumor-associated carbohydrate antigens are produced is a deregulation of glycosyltransferases, resulting in changes in enzyme activity and specificity for specific substrates. It has been shown that several glycosyltransferases are useful tumor markers, as for example: (*i*) core 2 β1,6-*N*-acetylglucosaminyltransferase mRNA is a good marker for lung carcinomas,[18] (*ii*) expression of β1,6-*N*-acetylglucosaminyltransferase V (GnTV)[19] and sialyltransferase CMP-sialic acid:Galβ1,3GalNAc-R α6-sialyltransferase[20] correlate with a poor prognosis and low patient survival in human colorectal carcinomas, (*iii*) the gene encoding for mannosyl (α-1,3-)-glycoprotein β-1,4-*N*-acetyl-glucosaminyl-transferase (GnT-IV) is significantly upregulated in colon adenocarcinomas associated to high metastatic potential,[21] (*iv*) it was reported that the detection of mRNAs of α1,4-*N*-acetylglucosaminyltransferase[22] and the ganglioside GD2 synthetase (β1,4-*N*-acetylgalactosaminyltransferase)[23] are useful procedures to detect circulating cells from gastric cancer and neuroblastoma, respectively.

It is widely known that the O-glycosylation pathways are altered in malignant transformation.[24] An incomplete elongation of *O*-glycan saccharide chains in mucins has been associated to malignant breast transformation.[25] Mucin-type linkages (GalNAc-*O*-Ser/Thr) are initiated by a family of glycosyltransferases called UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosami-

Grant sponsor: Comisión Honoraria de Lucha Contra el Cáncer (Uruguay).
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Received 16 July 2005; Accepted 7 February 2006
DOI 10.1002/ijc.21959
Published online 4 April 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – PRIMERS SEQUENCES

Gene	Sequences 5′–3′	Size of PCR product
ppGalNAc-T1	AAAAGCCTCATGAAGGTCCTGG ATCCAGAACGTTGTTCCATTCG	410 pb
ppGalNAc-T2	CCGCAACAAGTTCAACCAGGTG GCATGAGGCCTTCTCGTCGATC	316 pb
ppGalNAc-T3	GGGGAGCTAAACACTGCTTT TCCTAGCAACCGAGCAGTGATC	362 pb
ppGalNAc-T4	ATGGCGGTGAGGTGGACTTG GGAGCAAAGTCGACCAGGCTTC	457 pb
ppGalNAc-T5	CCTGATAAAGGAGCCGTAAGGC TATGGCTTCACTGGGTCACAGG	200 pb
ppGalNAc-T6	TCCAAATCAGGGCTCCAGAAG CACCTGCAGCTGCTTCACGTAC	499 pb
ppGalNAc-T7	AGCACCATGCTGGAGGAGATTC CTTCACTAGGCCATTCCACAGC	524 pb
ppGalNAc-T8	GTGGAGCTTAGCCTGAGGGTGT CTGGCCTCTGCAATCAGTTGTC	510 pb
ppGalNAc-T9	TACCGGCCCAGAAAGTGCAG ACCGCTTGTTGACGTACTGGTC	223 pb
β-Actine	TCTACAATGAGCTGCGTG CTCCTTCTGCATCCTGTC	679 pb

nyltransferase (ppGalNAc-Ts, EC 2.4.2.41).[26] Considering that ppGalNAc-Ts isoforms display tissue-specific expression in adult mammals, we performed this study in order to evaluate the potential utility of ppGalNAc-T mRNA expression to detect disseminated human breast cancer cell in bone marrow samples. We have analyzed first, the expression of 9 ppGalNAc-T genes (ppGalNAc-T1 to -T9) in human breast cancer extracts and normal peripheral blood mononuclear (PBMN) cells using RT-PCR assays. The best results of sensitivity and specificity were obtained with the ppGalNAc-T6 assay. Thus, we assessed the expression of this gene in bone marrow aspirates obtained from 61 patients with operable breast cancer. The results obtained here strongly suggest that the ppGalNAc-T6 RT-PCR assay is a potential tool applicable to disseminated cells' diagnosis in breast cancer patients.

Material and methods

Patients and bone marrow aspirates

Bone marrow aspirates were obtained from 61 patients with histological diagnosis of operable breast cancer at different stages. Our study was examined and approved by the Ethical Review Board of Hospital de Clínicas, School of Medicine, Montevideo, Uruguay. Informed consent was obtained from all patients entered onto the protocol. All patients were screened for metastases by conventional staging (chest x-ray, liver ultrasound, blood tests and bone scan in patients with stage II or III). Five-milliliter samples of bone marrow aspirates were collected in EDTA anticoagulant, by sternal and iliac crest punctures, under anesthesia before surgery, and they were immediately sent to the laboratory. Additionally, samples of peripheral blood cells were taken, under the same conditions, from healthy donors. Samples were first centrifuged at 1,500*g* for 5 min and the buffy coats removed into fresh tubes. The remaining red blood cells were eliminated by lysis buffer (154 mM ammonium chloride, 12 mM sodium bicarbonate and 0.1 mM EDTA) in gentle agitation for 10 min. The mononuclear cells were recovered by centrifugation at 1,500 *g* for 5 min, washed in PBS, recentrifuged and resuspended directly into Tri-Reagent (Sigma, St. Louis, MO). Tumor samples were pulverized in liquid nitrogen and then resuspended in Tri-Reagent.

Reverse transcriptase-polymerase chain reactions

Total RNA was extracted from breast cancer cell lines, PBMN cells, bone marrow samples, as well as from malignant breast tissues, with Tri-Reagent, according to the manufacturer's instructions. First-strand cDNA was synthesized by using MMLV reverse transcriptase (Amersham, Piscataway, NJ), as previously reported.[27] Briefly, 4 μg of RNA was added to 200 units of enzyme in the presence of 2 μl 20 mM of each deoxynucleotide triphosphate (dNTPs) and 1 μl 250 μg random hexamers in a 20 μl total reaction volume. After incubation at 37°C for 1 hr, the mixture was heated to 96°C, snap-cooled and stored at −20°C. Different RT-PCR reactions, with the respective negative controls, were optimized in order to amplify fragments of ppGalNAc T1 to T9. Primer sequences are shown in Table I. Amplification of the actine gene was carried out in the same PCR reaction tube in order to verify cDNA quality. cDNA (1 μl) was added to a final volume of 25 μl of a PCR mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 300 nM each primer and 1 unit of Taq DNA polymerase (Life Technologies, Carlsbad, CA). Amplification was performed under the following conditions for 35 cycles: 1 min at 95°C, 1 min at 57°C and 2 min at 72°C. PCR products (15 μl) were analyzed by electrophoresis on 2% agarose gels by direct visualization after ethidium bromide staining.

Statistical methods

Disease free survival time (defined as the time from surgery until diagnosis of recurrent disease) and overall survival (defined as the time from surgery until the date of death or last follow-up) were used as follow-up end points. Survival curves were determined using a Kaplan-Meier product-limit method.[28] Statistical significance between groups was assessed using the log-rank test. Statistical analyses were performed by SAS software (SAS Institute Inc.)

Results

ppGalNAc-transferase gene expression in breast cancer tissues and normal PBMN cells

To evaluate the potential usefulness of ppGalNAc-T genes mRNA detection in the identification of disseminated breast cancer cells in bone marrow, we have first studied the ppGalNAc-Ts expression in breast cancer tissue samples and PBMN cells. Therefore, we developed a RT-PCR assay for each ppGalNAc-T (from -T1 to -T9), which was co-amplified with β-actine transcripts (to control both RNA preparation and cDNA synthesis) (Fig. 1*a*). Several ppGalNAc-Ts found in breast cancer tissue samples (ppGalNAc-T1, -T2, -T3, -T7 and -T8), were also found in PBMN normal cells (Table II). On the contrary, ppGalNAc-T4, -T5 and -T9 were not found either in breast tumors, or in PBMN control cells. Interestingly, ppGalNAc-T6 was found in the majority of the studied breast cancer samples (5/6) and was absent in

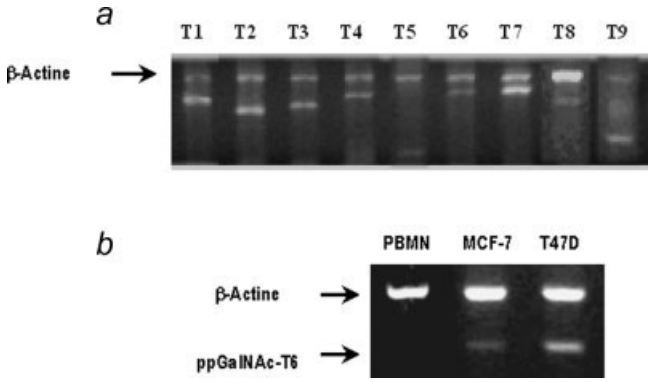

FIGURE 1 – Representative results of ppGalNAc-Ts mRNA detection by RT-PCR. (*a*) RT-PCR products for each ppGalNAc-T mRNA evaluated in appropriate control cell extracts. (*b*) RT-PCR for ppGalNAc-T6 mRNA evaluated in MCF-7 and T47D human breast cancer cell lines and in PBMN cells.

PBMN normal cells (0/5). Thus, this encouraged us to study the mRNA expression of this ppGalNAc-T in a larger number of breast cancer samples and evaluate their potential usefulness to detect disseminated breast cancer cells.

ppGalNAc-T6 mRNA expression in human breast cancer cell lines and tissues

We have detected ppGalNAc-T6 mRNA in all 3 human breast cancer cell lines evaluated (MCF-7, ZR75-1 and T47D) (Fig. 1*b*) and in 22 of 25 (95%) human primary breast tumors, while the test was positive in only 1/30 (3%) PBMN cell samples of healthy donors. To determine the specificity of ppGalNAc-T6 RT-PCR assay in normal bone marrow cells, we tested bone marrow mRNA from 19 patients without cancer undergoing orthopedic surgery (Banco Nacional de Prótesis, Montevideo). All samples were negative (data not shown). The sensitivity of ppGalNAc-T6 RT-PCR assay for breast tumor cell detection was evaluated by preparing serial dilutions of MCF-7 cell mRNA seeded into different tubes containing constant PBMN cell mRNA. The assay was able to identify one breast cancer cell among 10^4 PBMN cells. This assay was achieved 2-folds in triplicate, showing a good intra- and interassay reproducibility (data not shown).

Analysis of bone marrow samples

We evaluated ppGalNAc-T6 mRNA expression in bone marrow aspirates from 61 patients undergoing primary tumor resection. ppGalNAc-T6 was positive in 22 of them (36%) (Table III). Correlations between the ppGalNAc-T6 RT-PCR assay in bone marrow and various clinical–pathological parameters (stage, tumor size and nodal status) are shown in Table IV. No correlation was found between ppGalNAc-T6 expression and tumor size or lymph node involvement. Interestingly, the test was positive in 11/34 (32.3%) patients with stage I or II, without histopathological lymph node involvement. Stage I patients were less frequently positive (4/20; 20%) than stage IIa (11/24; 45.8%), and stage IIb (6/15; 40%) ones, but differences were not statistically significant.

In a follow-up evaluation of the 61 patients included in our study (median 68 months, minimum 10 months, maximum 106 months), 19 patients (31.1%) experienced recurrence of the disease and 15 of them (24.6%) died from metastatic disease. Of the 19 patients that recurred, 11 (57.9%) showed ppGalNAc-T6 mRNA-positive bone marrow aspirates (Table III). Interestingly, the ppGalNAc-T6 assay was positive in 2 of 3 stage I patients who showed recurrence of the disease. In a preliminary evaluation of the patient's clinical outcome and ppGalNAc-T6 expression in bone marrow, a correlation with bad evolution was seen between disease free survival and ppGalNAc-T6-positive patients (Fig. 2*a*), although no statistically significant differences were observed. Nodal status and bone marrow ppGalNAc-T6 expression were relevant covariates for determining free survival time distributions in both univariate and multivariate analysis. Figure 2*b* shows that nodal status determines poor free-survival probabilities regardless of marker status ($p = 0.954$), whereas in the group of patients without lymph node involvement, disease recurrence was observed in 6/11 (54.5%) patients who showed ppGalNAc-T6 mRNA-positive bone marrow aspirates, and only in 1/23 (4.3%) patients when ppGalNAc-T6 was negative (difference statistically significant, $p = 0.014$).

TABLE II – ppGalNAc-T EXPRESSION IN HUMAN BREAST TUMORS AND PBMN CELLS

	T1	T2	T3	T4	T5	T6	T7	T8	T9
Breast tumors	4/4	6/6	5/6	0/4	0/4	5/6	3/3	1/4	0/3
PBMN cells	4/4	6/6	1/5	0/3	0/6	0/5	2/4	1/4	0/3

Discussion

In this work, we report for the first time, that ppGalNAc-T6, a glycosyltransferase which catalyzes the first key step of mucin-type O-glycosylation, is expressed by human breast cancer cells. We detected ppGalNAc-T6 mRNA in 22/25 (88%) primary tumors and in all 3 breast cancer cell lines evaluated. Human ppGalNAc-T6 gene, located on chromosome 12q13, contains 10 exons of 1,869 base pairs which encode a type-II transmembrane protein, expressed in a restricted pattern, mainly in normal placenta, trachea, brain, pancreas and fibroblast cells.[29] Although it has been detected in a pancreatic adenocarcinoma cell line and in squamous carcinomas of the oral cavity,[29] there is no available data regarding its expression in breast cancer. Our results are strongly supported by protein expression analyses performed in our laboratory, where ppGalNAc-T6 was found in most breast carcinomas (60/74, 81%), whereas this enzyme was not detected in normal breast tissues obtained from healthy women.[30] ppGalNAc-T6 is, thus, the second member of the ppGalNAc-T family reported to be overexpressed in breast cancer. Previously, Nomoto *et al.*[31] showed that ppGalNAc-T3 mRNA is expressed in cell lines derived from mammary gland adenocarcinomas.

The presence of DTC in bone marrow would serve for 3 purposes in clinical oncology: (*i*) as evidence for an early occult spread of tumor cells; (*ii*) as a relevant risk factor for subsequent metastasis; and (*iii*) as a marker for monitoring treatment susceptibility.[32] These DTC are believed to be rare components among the cellular population of primary tumor cells.[2] Genome and transcriptome analyses of single DTC demonstrated that the majority of DTCs are cells with genetic aberrations compatible with malignancy,[33,34] which possess unique gene expression signatures.[35] Among the ppGalNAc-Ts studied, ppGalNAc-T6 mRNA was detected by RT-PCR in a high percentage of breast cancers, whereas it was absent in normal breast, in bone marrow samples obtained from patients without cancer, and was identified only in 1/30 PBMN cell samples. Therefore, we selected, in our study, the ppGalNAc-T6 gene as a target to detect disseminated breast tumor cells in bone marrow. Using this method, 22 of 61 patients who underwent curative surgery showed positive ppGalNAc-T6 mRNA in bone marrow aspirates obtained prior to surgery. We did not find a clinical–pathological correlation between the presence of the ppGalNAc-T6 in bone marrow and the tumor size, lymph node involvement or stage. In our preliminary data on 61 patients' follow-up, the presence of ppGalNAc-T6-positive cells

TABLE III – ppGalNAc-T6 mRNA EXPRESSION IN BONE MARROW SAMPLES OF OPERABLE BREAST CANCER PATIENTS

Patient No.	Tumor size	Nodal status	UICC Stage	ppGalNAc-T6	Follow-up (month)
1	1	N0	I	Negative	No relapse (96)
2	1	Positive	II a	Positive	No relapse (93)
3	2	N0	II a	Positive	Relapse (28), death (31)
4	2	N0	II a	Positive	No relapse (103)
5	2	N0	II a	Negative	No relapse (103)
6	2	N0	II a	Positive	No relapse (93)
7	1	Positive	II a	Positive	No relapse (92)
8	1	N0	I	Negative	No relapse (77)
9	2	N0	II a	Positive	Relapse (62), death (104)
10	2	N0	II a	Negative	No relapse (64)
11	1	N0	I	Negative	No relapse (106)
12	2	N0	II a	Positive	No relapse (97)
13	2	Positive	II b	Positive	Relapse (76), death (83)
14	2	Positive	II b	Positive	Relapse (39), death (65)
15	2	Positive	II b	Negative	Relapse (25), death (51)
16	1	Positive	II a	Negative	Relapse (96), alive (96)
17	2	Positive	II b	Negative	Relapse (23), death (82)
18	1	N0	I	Negative	No relapse (100)
19	1	Positive	II a	Positive	Relapse (31), death (49)
20	1	N0	I	Positive	Relapse (52), death (71)
21	3	Positive	III a	Positive	No relapse (95)
22	1	Positive	II a	Negative	No relapse (97)
23	2	Positive	II b	Positive	No relapse (49)
24	1	N0	I	Negative	Relapse (6), death (10)
25	1	N0	I	Negative	No relapse (77)
26	1	N0	I	Negative	No relapse (94)
27	1	N0	I	Positive	Relapse (44), alive (91)
28	2	Positive	II b	Negative	No relapse (79)
29	1	N0	I	Negative	No relapse (82)
30	1	Positive	IIa	Negative	Relapse (21), death (71)
31	2	N0	II a	Negative	No relapse (82)
32	1	N0	I	Negative	No relapse (36)
33	2	Positive	II b	Negative	Relapse (60), death (85)
34	2	Positive	II b	Positive	Relapse (20), death (23)
35	1	N0	I	Positive	No relapse (81)
36	2	Positive	II a	Negative	Relapse (38), death (42)
37	2	Positive	II b	Negative	No relapse (64)
38	1	N0	I	Negative	No relapse (51)
39	1	N0	I	Negative	No relapse (48)
40	1	N0	I	Negative	No relapse (72)
41	2	Positive	II b	Negative	Relapse (14), death (25)
42	2	N0	II a	Negative	No relapse (70)
43	1	N0	I	Negative	No relapse (71)
44	2	N0	II a	Negative	No relapse (42)
45	2	Positive	II b	Negative	No relapse (33)
46	1	N0	I	Negative	No relapse (76)
47	1	Positive	II a	Positive	No relapse (72)
48	2	N0	II a	Positive	Relapse (19), death (59)
49	2	Positive	II b	Positive	Relapse (20), alive (73)
50	1	Positive	II a	Negative	No relapse (62)
51	1	Positive	II a	Negative	No relapse (56)
52	2	Positive	II b	Negative	No relapse (67)
53	3	Positive	III a	Negative	No relapse (27)
54	2	N0	II a	Negative	No relapse (70)
55	2	N0	II a	Negative	No relapse (67)
56	1	N0	I	Negative	No relapse (65)
57	1	N0	I	Negative	No relapse (46)
58	2	Positive	II b	Positive	No relapse (62)
59	1	N0	I	Positive	No relapse (60)
60	2	Positive	II b	Negative	No relapse (26)
61	2	N0	II a	Positive	Relapse (25), alive (41)

TABLE IV – ppGalNAc-T6 EXPRESSION IN BONE MARROW SAMPLES OF OPERABLE BREAST CANCER PATIENTS: CORRELATION WITH TUMOR SIZE, LYMPH NODE STATUS AND STAGE OF DISEASE

	Tumor size			Lymph node status		UICC stage			
	T1	T2	T3	Negative	Positive	I	IIa	IIb	III
ppGalNAc-T6 expression	8/29 (27.5%)	13/30 (43.3%)	1/2 (50%)	11/34 (32.3%)	11/27 (40.7%)	4/20 (20%)	11/24 (45.8%)	6/15 (40%)	1/2 (50%)

was observed in 11/19 patients who showed a worse clinical outcome, including those with early disease (a positive test was observed in 2/3 stage-I patients who relapsed). The most interesting result was observed when ppGalNAc-T6 mRNA expression in bone marrow aspirates was correlated with patient nodal status. In node-positive patients, similar clinical outcome was

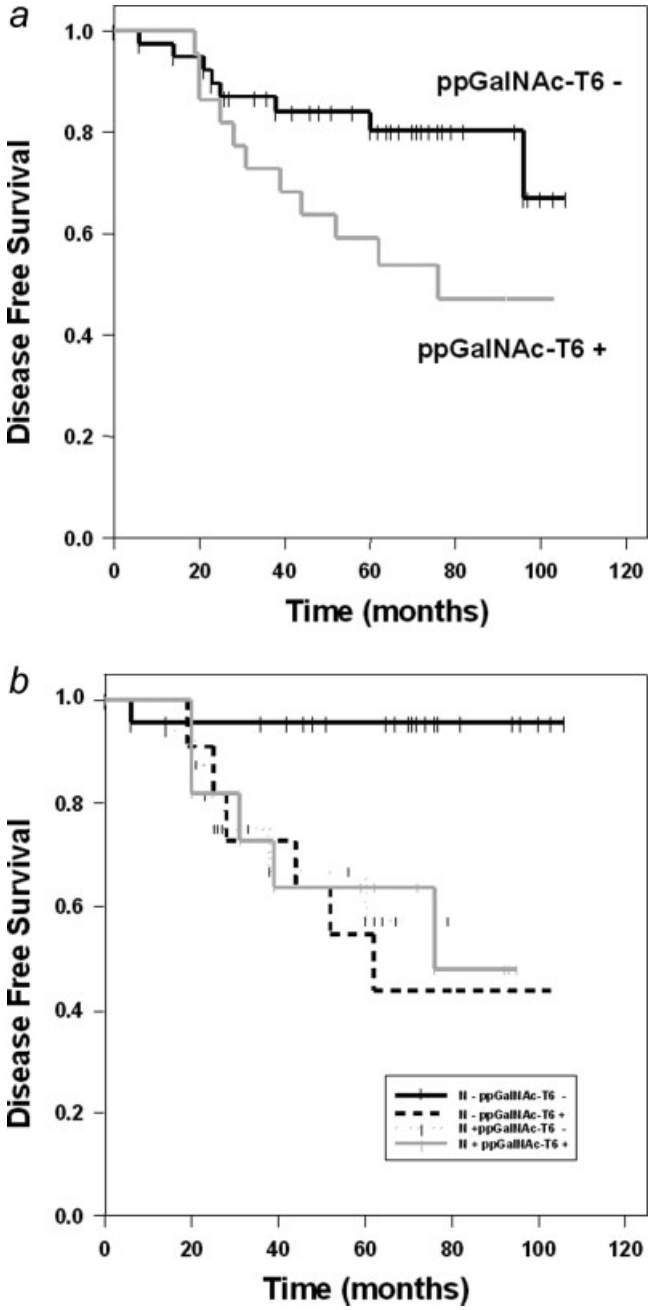

FIGURE 2 – Correlation between clinical outcome and ppGalNAc-T6 expression in bone marrow. (*a*) Disease-free survival in patients with ppGalNAc-T6 positive *vs*. ppGalNAc-T6 negative. (*b*) Disease-free survival in patients classified by lymph node status ($N_{(+)} = 27$, $N_{(-)} = 34$).

observed in spite of ppGalNAc-T6 expression in bone marrow. By contrast, in the group of patients stage I or II without lymph node involvement, a statistically significant higher rate of disease recurrence was observed in patients who showed ppGalNAc-T6 mRNA-positive bone marrow aspirates (54.5%), compared with ppGalNAc-T6-negative patients (only 1/23, 4.3%) ($p = 0.014$). These results suggest that ppGalNAc-T6 mRNA evaluated by RT-PCR could be a new marker applicable to the molecular diagnosis of breast cancer cell dissemination. Recent findings show the prognostic significance of circulating tumor cell levels in a prospective trial of 177 metastatic breast cancer patients.[36] The number of circulating tumor cells before treatment was an independent predictor of progression-free survival and overall survival in patients with metastatic breast cancer.[36] The application of real-time RT-PCR techniques, which allow both detection and quantification of the transcript levels, could open new horizons for ppGalNAc-T6 evaluation in cancer diagnosis.

Different mucin-type cancer associated structures (such as Tn, TF and sialyl-Tn antigens) are associated to a more aggressive phenotype.[16,37] It is unknown whether ppGalNAc-T6 expression could be related to events of breast cancer biology, but the fact that this enzyme is not expressed in normal breast suggests a different regulation of this glycosyltransferase in breast cancer.[30] The ppGalNAc-T6 activity could be related with the expression of simple mucin-type cancer associated antigens, especially Tn, since ppGalNAc-T6 expression changes may induce shifts in O-glycosylation and cell-surface carbohydrates in carcinoma. For example, Marcos *et al*.[38] observed that ppGalNAc-Ts expression was associated with different glycosylation forms of the MUC1 tandem repeat in gastric carcinoma cell lines. The ppGalNAc-T3 gene, highly similar to ppGalNAc-T6 one, was associated to tumor aggressiveness and prognosis of adenocarcinoma of pancreas,[39] colorectal,[40] and gallbladder carcinoma.[41] ppGalNAc-T6 expression could be responsible for a phenotype related to a worse outcome in breast cancer. A detailed characterization of this glycosyltransferase expression is in progress in order to determine its role in breast cancer biology.

In conclusion, this study revealed the expression of ppGalNAc-T6 gene transcripts in breast tumors and in human breast cancer cell lines, using a nested RT-PCR assay. ppGalNAc-T6 mRNA appears to be a good target for detection of disseminated breast cancer cells, because it was identified in bone marrow aspirates obtained from patients with operable breast cancer and not in bone marrow samples obtained from patients without cancer. Further studies are necessary to determine the clinical implications of ppGalNAc-T6 RT-PCR for disseminated breast cancer cell detection and to clarify the biological role of its abnormal expression in breast cancer.

Acknowledgements

We thank Ivana Facini for her helpful contribution, and Dr. Bernardo Aizen, Dr. Roberto Estrugo and Dr. Justino Menendez, who provided clinical samples.

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