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A new 85 kDa, breast tumour associated antigen — A potential diagnostic and prognostic agent

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Abstract. In an attempt to develop measures for early diagnosis and prognosis of the disease and to explore association of murine mammary tumour virus (MuMTV) or related virus in breast cancer, we purified a breast tumour associated antigen (BTAA) from the breast tumour tissues of untreated female cancer patients. The BTAA purified by DEAE discontinuous column chromatography followed by SE-HPLC was an 85 kDa glycoprotein. A high level of circulating antibodies against this antigen was observed, using ELISA, in all the untreated female breast cancer patients. The BTAA was not immunologically related to MuMTV antigens but strongly resembled an 83 kDa glycoprotein tumour associated antigen, purified from MuMTV induced mouse mammary tumour. In patients after surgical removal of the breast tumour, the circulating antibodies to the BTAA decreased gradually, but reappeared in the patients with secondary metastasis. In healthy age matched women or in female patients with carcinoma of tissues other than breast, no significant titre of the BTAA antibodies was observed.

Keywords. Breast cancer; breast tumour associated antigen; murine mammary tumour virus; anti-tumour antibody.

1. Introduction

Carcinoma of the breast is one of the major cause of cancer mortality among women in India. Though various risk factors for breast cancer have been identified, no single etiological agent is known. The search for a viral etiology of human breast cancer has, till now, yielded debatable results. Presence of murine mammary tumour virus (MuMTV) structural antigens, mainly the major envelop glycoprotein gp52 (Mesa-Tejada *et al* 1978, 1982) and circulating antibodies to MuMTV (Holder and Wells 1983) have been indicated in human breast cancer patients, though the specificity of the antigens and antibodies and their role as diagnostic and prognostic marker of the disease are yet to be established (Dion *et al* 1987; Kovarik *et al* 1989).

We have reported the purification of an 85 kDa, breast tumour associated glyco protein antigen, BTAA, from human breast tumour tissues (Pal *et al* 1995). In this report, we show that the BTAA closely resembles an 83 kDa murine mammary tumour associated antigen MTAA, purified from MuMTV induced spontaneous mammary tumour of C_3H/Jax mice (Chattopadhyay *et al* 1984a) and the BTAA has the potentiality to be developed as a diagnostic and prognostic agent for breast cancer.

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2. Materials and methods

2.1 *Tumour tissues*

Human malignant breast tumors (MBT) were obtained from patients undergoing surgery in Chittaranjan National Cancer Institute (CNCI) Hospital, Calcutta. Mouse mammary tumours (MMT) were surgically removed from female C_3H/Jax mice with MuMTV induced spontaneously arising mammary tumour.

2.2 *Serum samples*

Sera were collected from pre-operated and post-operated breast cancer patients, patients suffering from benign breast diseases and carcinoma of organs other than breast and normal healthy individuals. Aliquots of the sera were absorbed repeatedly with normal human breast tissue (NHB) pellets and stored at —20°C until used.

2.3 *Antisera*

Anti-MMT sera were raised in Belgian rabbits, decomplemented, and were absorbed exhaustively with tissue pellets of pooled organs of tumour-free C_3H/Jax mice. Anti-MuMTV, the goat polyvalent antiserum to Triton-disrupted MuMTV, was obtained from NCI, Bethesda, MD, USA, as a generous gift.

2.4 *Purification of BTAA and MTAA*

The BTAA and MTAA were partially purified from MBT and MMT respectively, by DEAE discontinuous gradient column chromatography. Three DEAE-cellulose chromatography fractions of MBT-HF_1, HF_2, HF_3 and of MMT- MF_1, MF_2, MF_3 were eluted with 0·15M, 0·5M and 1·0M NaCl in phosphate buffered saline (PBS) of pH 7·2. MTAA was purified from MF_1 by SDS-PAGE (Chattopadhyay *et al* 1984a). Purification of the BTAA was achieved by subjecting HF_1 to size-exclusion high performance liquid chromatography (SE-HPLC) (Pal *et al* 1995).

2.5 *Enzyme-linked immunosorbent assay*

The presence of circulating antibodies in the test sera against the DEAE fractions of MBT and MMT were determined by enzyme-linked immunosorbent assay (ELISA) (Pal *et al* 1995).

2.6 *Western blot analysis*

The specificity of reaction between the reactive antigenic component of HF_1 and the circulating antibodies in the sera of breast cancer patients was determined using Western blot analysis (Pal *et al* 1995).

3. Results

3.1 *Circulating antibodies in the sera of breast cancer patients against the BTAA*

The presence of circulating antibodies to 50 pre- or post-operated breast cancer patients was examined by ELISA against the three DEAE fractions, HF_1, HF_2 and HF_3 of MBT. As shown in table 1, the reaction of sera of pre- and post-operated breast cancer patients with HF1 was significantly higher ($P < 0{\cdot}001$) than that with HF_2 and HF_3. The sera of healthy women, patients with benign breast diseases and patients with carcinoma of other organs reacted weakly with all the antigens (table 1).

To examine the specificity of the reaction of HF_1 with the antibodies in breast cancer patients, aliquots of individual serum samples of 12 pre-operated and 10 post-operated patients, 8 patients with carcinoma of other organs and 5 healthy women were absorbed repeatedly and exhaustively with the tissue pellets of NHB. As shown in table 2, all the absorbed pre-operated and 8 out of 10 post-operated breast cancer patients' sera were strongly reactive with HF_1. The absorbed sera of all the control groups reacted poorly with HF_1. By repeated screening, an optical density (OD) of less than 0.1 was observed in all the negative controls and therefore, an OD value of more than 0.1 was considered to indicate the presence of tumour specific antibodies. The high OD values obtained with HF_1, reacting specifically with the absorbed sera of pre- and post-operated breast cancer patients suggested presence of a tumour associated antigen in this fraction. The tumour associated antigen (BTAA) present in HF_1, was purified by SE-HPLC as reported by Pal *et al* (1995).

Table 1. Circulating antibodies in the sera of breast cancer patients against the DEAE fractions of MBT.

	Optical density (Mean ± SD)		
	DEAE fractions		
Unabsorbed sera	HF_1	HF_2	HF_3
Pre-operated breast cancer(30)[a]	0·352* ± 0·039	0·163 ± 0·028	0·116 ± 0·025
Post-operated breast cancer(20)	0·316** ± 0·030	0·142 ± 0·027	0·106 ± 0·031
Healthy women(10)	0·126 ± 0·026	0·118 ± 0·027	0·069 ± 0·042
Other cancers(12)	0·150 ± 0·026	0·146 ± 0·037	0·078 ± 0·030
Benign breast diseases (5)	0·158 ± 0·022	0·152 ± 0·028	0·091 ± 0·025

[a]The number of sera tested. All the sera were diluted 100-fold.
* Significantly higher ($P < 0{\cdot}001$) than all other values except the corresponding value of post-operated breast cancer sera.
**Significantly lower ($P < 0{\cdot}01$) than the corresponding value of pre-operated breast cancer sera, but significantly higher ($P < 0{\cdot}001$) than the remaining values.

Table 2. Specificits of the HF_1 antigens and HF_1-reactive antibodies in breast cancer patients.

Absorbed sera	Optical density (Mean ± SD)
Pre-operated breast cancer (12)[a]	0·322* ± 0·038
Post-operated breast cancer (10)	0·268 ± 0·048
Healthy women (5)	0·063 ± 0·021
Other cancers (8)	0·103 ± 0·017

[a] The number of sera tested. All the sera were diluted 100-fold.
* Significantly higher than the corresponding value of operated breast cancer sera ($P < 0·01$) and the values of healthy women and other cancers ($P < 0·001$).

The specificity of the BTAA and the circulating antibodies present in breast cancer patients' sera was determined by Western blot analysis. The BTAA was electrophoresed and transferred onto nitrocellulose paper. The blot was treated both with the absorbed serum of a pre-operated breast cancer patient and a normal healthy individual. A strong reaction band was observed only with the serum of cancer patient (figure 1). The mobility and moelcular weight (MW) of this band was same as that of the BTAA (MW 85 kDa).

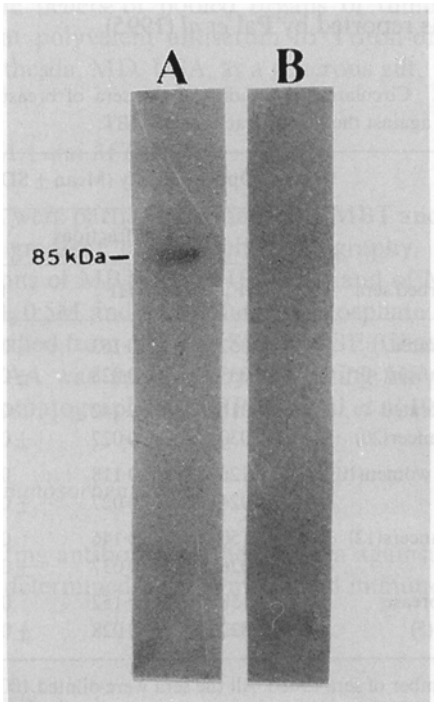

Figure 1. Western blot analysis of BTAA.
The DEAE fraction, HF1 was electrophoresed in 10% SDS-PAGE and transferred electrophoretically to nitro-cellulose paper at 70 V. The blot was probed with the absorbed serum of a pre-operated breast cancer patient (lane A) and a normal healthy individual (lane B).

3.2 *The serum level of BTAA-antibody in breast cancer patients during post-surgical disease free period*

A lowering of the reactive antibody titer against BTAA was observed in the sera of post-operated breast cancer patients (tables 1 and 2). The correlation of prolongation of the post-surgical disease free period and the level of circulating anti-BTAA antibodies was studied. The post-operative patients were divided into four groups according to the length of disease free period after surgical removal of their tumour. The level of anti-BTAA antibodies in their sera, absorbed with NHB tissue pellets, were measured by ELISA. It was observed that the anti-BTAA antibody titer declined progressively with increased interval following surgery (table 3). All the sera were highly reactive with the BTAA up to 10 months after removal of the tumour and between 15-24 months, the antibody titer decreased significantly. Two patients examined after 24 and 48 months of surgical removal of the tumour, respectively, did not have any BTAA reactive circulating antibody.

Table 3. Correlation of the level of circulating anti-BTAA antibodies with the length of disease free post-surgical period in breast cancer patients.

Post-surgical period (in months)	Optical density (Mean ± SD)
1–2 (7)[a]	0.390 ± 0·064
6–10 (5)	0·302 ± 0·076
15–24 (5)	0·185* ± 0·027
28–48 (2)	0·089* ± 0·010

[a] The number of sera tested. All the sera were diluted 100-fold.
*Significantly lower (P <0·001) than other two values.

3.3 *Immunological relatedness of human BTAA with murine MTAA and their association with MuMTV*

We have earlier reported the purification of an 83 kDa, MTAA from MuMTV induced mammary tumour of C3H/Jax mice (Chattopadhyay *et al* 1984a). To assess the immunological relatedness between the BTAA and MTAA, the BTAA was allowed to react against the absorbed anti-MMT sera. In a parallel set of experi ments, ELISA were carried out using MTAA and the absorbed sera of pre-operated breast cancer patients. A strong cross-reaction was absorved between the BTAA and absorbed anti-MMT as well as between the MTAA and absorbed breast cancer sera (data not shown), indicating an immunological relatedness between the BTAA and MTAA.

Since the MTAA was purified from the MuMTV induced mammary tumour, the relatedness of this antigen and BTAA with the structural antigens of MuMTV was determined by testing their reactions in ELISA, against a highly purified goat anti-MuMTV serum. Both the antigens failed to react with the anti-MuMTV serum.

4. Discussion

The presence of circulating antibodies against a DEAE fraction of MBT (HF_1) in pre- or post-operated breast cancer patients but not in patients with carcinoma of other organs, benign breast diseases and normal healthy women (table 1) suggested a specific immune response of the breast cancer patients against a breast tumour associated antigen. The observations that the HF_1 reacted with the breast cancer sera even after their exhaustive absorption with NHB tissue pellets (table 2) but not with the absorbed sera of healthy women or patients with cancer of other organs, suggested the specificity of reaction between the circulating antibodies and the HF_1. The weak reactions of HF_1, observed with the unabsorbed sera of control groups of cancer patients and healthy women might be due to the presence of autoantibodies against normal breast epithelial antigens (Ronai and Sultizeanu 1986). Western blot analysis using BTAA, purified from HF_1, confirmed the presence of circulating antibodies in breast cancer sera but not in normal sera against the 85 kDa, glycoprotein BTAA (figure 1). The observed specificity of the circulating antibody against the BTAA, suggested that the antigen can potentially be used as a diagnostic agent.

The gradual lowering of the anti-BTAA antibody titer in the post-operative breast cancer patients with increase in disease free interval after surgery (table 3) also signified the importance of the antigen as a prognostic marker of the disease. The complete absence of the anti-BTAA antibody in two post-operative patients after an interval of 28 and 48 months following surgery (table 3) reflected a loss of immune response of the patients against the BTAA due to the absence of antigenic stimulus. Anti-BTAA antibody was observed in a post-operated patient with secondary metastasis, after 24 months of surgery (data not shown).

We have earlier reported purification of a tumour associated antigen (MTAA) from MuMTV induced mammary tumours of C_3H/Jax mice (Chattopadhyay *et al* 1984a). This 83 kDa, glycoprotein molecule was a membrane associated antigen and we observed circulating antibodies against MTAA in mammary tumour bearing mice and breast cancer patients (Chattopadhyay *et al* 1984b), which suggested antigen sharing between the mice mammary and human breast tumours. An immunological relatedness between the BTAA and MTAA was also indicated in the observed cross-reaction of the BTAA and MTAA with absorbed anti-MMT and absorbed breast cancer sera respectively. The BTAA also resembled the MTAA in MW, thermostability and susceptibility to proteolytic enzymes and neuraminidase (Chattopadhyay *et al* 1984a; Pal *et al* 1995). The close resemblance between the BTAA and MTAA led us to examine whether these antigens were the MuMTV structural antigens. Absence of any reaction of either of the antigens with anti-MuMTV serum suggested that they were not MuMTV antigens. MuMTV as an etiological agent of human breast cancer is still a debatable issue (Dion *et al* 1987; Kovarik *et al* 1989). The data presented in this report do not provide any direct evidence in support of MuMTV as the etiological agent of breast cancer. However, the close similarity between the BTAA and the MTAA also does not allow us to deny any possible association of MuMTV or a related virus with human breast cancer.

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