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Chapter 9

LYMPHATIC SPREADING PROPENSITY AND ABERRANT MUC1 BEARING TN/TN-LIKE CARBOHYDRATE OF AGGRESSIVE BREAST CANCER CELLS

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ABSTRACT

The spread of breast cancer in the human body comprise a unique metastatic cascade, in which ①"pleura↔lymph node↔liver↔adrenal gland" and ②"skin↔lung↔liver↔bone/bone marrow" cross at the liver. This suggests that breast cancer cells utilize either lymphatic or hematogenous system, or both systems for their metastatic spread in human body. Since metastasis is intimately associated with the interaction of cancer cells and host tissues especially with respect to cell surface adhesion molecules including carbohydrates, we examined the relationship between carbohydrate expression of cancer cells in primary lesions and lymph node metastasis of breast cancer. Since the relationship is not as strong as expected, we examined it through analysis of the relationship between carbohydrate expression as recognized by two kinds of lectins and/or MAbs and lymph node metastasis status. We found that 31 combinations of two lectins and/or MAbs correlate significantly with lymph node metastasis. These combinations formed a completely interrelated linkage (or network), when all lectins and MAbs in the combinations are connected with one another. The network includes linkages among anti-Tn monoclonal antibody (MAb: HB-Tn1: Tn), *Vicia villosa* agglutinin (VVA-B_4: VVA), anti-blood- group H MAb (H), *Anguilla anguilla* agglutinin (AAA), and anti-Lewis ^{X} MAb (LEX) in the center. VVA occupied the central core because it is the only reagent among them significantly related to lymphatic invasion (ly factor). Under denaturing and reducing conditions the major VVA-binding proteins had molecular sizes of >200 kDa, ~75 kDa, ~50 kDa, ~33 kDa, and ~26 kDa. The >200 kDa, ~75 kDa, ~50 kDa, and ~26 kDa proteins were identified as MUC1 mucin, serotransferrin, IgG heavy chain, and IgG light chain, respectively. Expression of the ~33 kDa protein was most relevant to lymph node metastasis; this protein may be MUC1. VVA-binding carbohydrate was considered to be non-clustered Tn antigen and/or clustered Tn antigen, and the noncluster form of Tn antigen is implicated in aggressive growth of primary breast cancer cells, particularly in lymphatic metastasis. We conclude that atypical MUC1 bearing the noncluster form of Tn antigen is implicated in the aggressive growth of primary breast cancer cells, particular in lymphatic metastasis. Recently, we developed an experimental model using rat hepatoma with high lymph node metastasis propensity and discovered 30∼40 kDa atypical MUC1 bearing Tn antigen. In this system, intercellular adhesion molecule-1 (ICAM-1) appeared in the vascular walls around the tumors. It is likely that VVA-binding carbohydrates are also an important molecular target in other cancer cells as well, because VVA-binding carbohydrate expression is related to the malignant phenotype of various human cancers including

lung cancer, uterine cervical cancer, colon cancer, pancreatic cancer, urinary bladder cancer, and malignant lymphoma.

INTRODUCTION

Breast cancer is the most important cause of cancer-related deaths among Japanese women. Currently, about 70% of breast cancer patients can be cured by surgery alone or surgery with additional therapies [1], but the remaining （approximately 30%） have incurable disease. In general, aggressive cancer growth properties are significantly related to the invasion and metastatic abilities of cancer cells in patients. Invasion of cancer cells is prerequisite for metastasis and generally it is divided into four types; direct invasion to tissues including parenchyma cells, invasion into lymphatic vessels, invasion into blood vessels, and invasion into neural tissues, in which body cavities are included in lymphatic vessels. The significance of every type on spread in the body is different from cancers, and in the case of breast cancer, invasion to lymphatic vessels (ly factor) and blood vessels (v factor) are especially important. This is seen in the facts that the frequencies of cancer cell embolisms in lymphatic vessels and blood-vessels are prognostic factors of breast cancer [2, 3].

We are interested in the carbohydrate expression of cancer cells as they are related to aggressive cancer cells, especially for their cellular properties of invasion and metastasis. This is because carbohydrates fundamentally bear the functions of transduction systems of cellular signals in the body, and altered glycosylation appears to be a universal feature of cancer cells. Here, we briefly describe mainly our own studies concerning altered glycosylation of cancer cells in aggressive breast cancer as well as colorectal cancer, gastric cancer, lung cancer and others [5-22]. The basic style of this paper is that of a review of our own studies; however, it should be noted that some parts require detailed description with several references because some data are still unpublished and would be difficult to understand as a review.

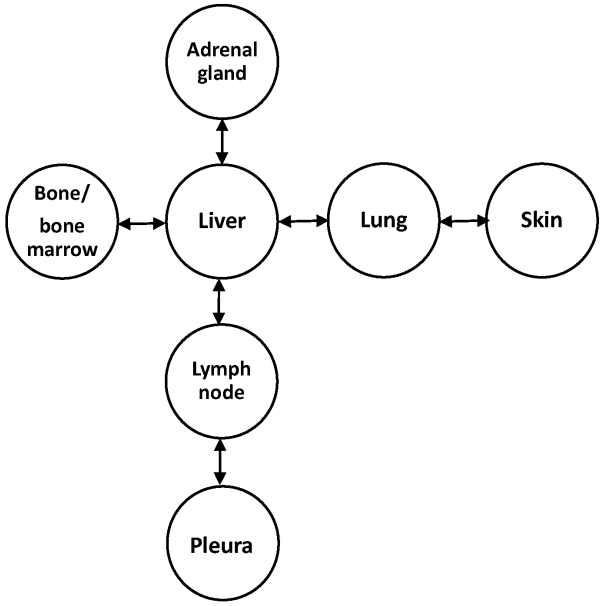

Chart 1. Correlation of organ metastasis in human breast cancer, which was found in 97 autopsy cases. Pearson's correlation coefficient was obtained between two organs having metastasis with frequencies higher than 20% in all cases. Organs are connected by a line if there was a significant ($p<0.05$) relationship between them [5].

I. Expression of *Vicia Villosa* Agglutinin (VVA)-Binding Carbohydrate of Breast Cancer Cells in Relation to The Lymphatic Spread

Since the study of Furmanski et al. in 1981 [23], many researchers have investigated the relationship between aggressive growth and carbohydrate antigen expression of breast cancer cells. These studies indicate a possible relationship between lymph node metastasis status and carbohydrate expression of cancer cells, but the relationship is not understood to the extent that would justify the use of cellular carbohydrate expression as a diagnostic indicator.

Table 1. Metastatic distribution of breast cancer in autopsy

Organ or tissue	%
Brain	15.5
Hypophysis	6.2
Leptmeninges	3.1
Dura mater	12.4
Thyroid gland	14.4
Heart	4.1
Pericardium	13.4
Lung	80.4
Pleura	39.2
Liver	70.1
Spleen	12.4
Pancreas	12.4
Kidney	16.5
Adrenal gland	46.4
Stomach	0.0
Intestine	6.2
Urinary bladder	1.0
Ovary	13.4
Uterus	11.3
Peritoneum	7.2
Bone/bone marrow	67.0
Lymph node	76.3
Skin	34.0

% : percentage of metastasis cases in autopsy cases (total 98 cases).

We think that the possible relationship between carbohydrate antigen expression and lymph node metastasis status may have serious implications, and therefore should be explored in detail. Putative carbohydrate indicator(s) may be inconspicuous in nature when only individual carbohydrates are considered, because such indicators may consist of several kinds of carbohydrates forming networks. This is supported by a recent report suggesting that oligosaccharides may be packed tightly together, generating a "clustered saccharide patch" for specific recognition [24].

In order to confirm this hypothesis, we examined the association between lymph node metastasis status of breast cancer and the carbohydrate phenotypes

of cancer cells in primary lesions, from the viewpoint of synergistic and/or antagonistic effects of carbohydrates recognized by 2 kinds of lectins and/or monoclonal antibodies (MAbs) as well as those recognized by individual lectins or MAbs. Here we describe the studies somewhat in detail, as we have not previously published an original English paper in any journals, except for a review article [18].

Table 2. Some clinicopathological characteristics of patients.

Characteristics	No. of case	(%)
Age (years old)		
51≥	145	(51.1)
51<	139	(48.9)
ABO blood group		
A	126	(44.4)
B	67	(23.6)
AB	14	(4.9)
O	77	(27.1)
T factor		
T1 (≤ 2cm)	91	(32.2)
T2 (2.1 cm> ~ 5 cm≤)	154	(54.2)
T3 (>5 cm)	24	(5.3)
T4 (Any size with direct extension to chest wall or skin)	15	(5.3)
Lymphatic vessel invasion		
Negative	181	(71.8)
Positive	71	(28.2)
Venous vessel invasion		
Negative	196	(76.6)
Positive	60	(23.4)
Axillary lymph node metastasis		
Negative	145	(48.9)
Positive	139	(51.1)

I-1. Materials and Methods for Carbohydrate Network Analysis

A total of 284 female cases of breast cancer were obtained from the files at the Department of Pathology, Fukushima Medical College, Fukushima City, Japan, between February 5, 1985 and December 10, 1992. All clinicopathological items were dealt with according to the classification of the Japanese Breast Cancer Society, which is fundamentally the same as that of the WHO as described in detail by Sakamoto [25]. Some clinicopathological characteristics of the patients are shown in Table 2. Lectins and MAbs used and carbohydrate-specificities of every lectins and MAbs are shown in Table 3. The extent of staining was divided into 2 categories: more or less than 50% for lectins and more or less than 10% for MAbs. Positive or negative staining was determined from the staining of cancer cell cytoplasm and cell surface membrane. The percentage of total cancer cells that were stained was obtained by scanning entire fields of the sections. In general, these values were able to be determined easily without counting the cells. In equivocal cases the scores were determined by counting the number of stained cells in sections with more than 200 cells in at least 3 regions of the field.

Association between staining by 2 kinds of lectins and/or MAbs and lymph node metastasis was determined as described in Table 4. Statistical analysis of differences between groups was performed using the χ^2 test with Yates's correction for continuity. A p value < 0.05 was considered to be statistically significant.

I-2. Frequency of Positive Staining of all the Cases and Changes by T Factor and Histological Subtype

Jacalin, MPA, VVA, RCA-I, ABA, PHA-L, WGA, ConA, Tn, and H showed a frequency of more than 30% positive staining, whereas the frequencies of staining of T, STn, A, and B were less than 10%. The remaining reagents GS-I-B_4, HPA, SBA, DBA, RCA-II, PNA, ECA, Lotus, AAA, UEA-I, LCA, and LEX, showed frequencies of positive staining between 11% and 29%. The influence of T stage on the frequency of positive staining was studied statistically. No statistically significant difference was found for any of the reagents except for VVA and ConA. The frequency of positive staining of VVA in T2 cancers was greater than that for T1 cancers ($p < 0.01$), whereas the frequency of positive staining with ConA in T2 cancers was lower than that for T1 or T3 + T4 tumors.

We examined staining characteristics among the histological subtypes by comparing the incidence of positive staining cases. We found that the frequency of positive staining with GS-I-B_4 and PNA were statistically different among all histological subtypes.

Table 3. Concentrations of lectins and MAbs and carbohydrate specificities recognized by these reagents.

Lectins	Common name	Concentration	Major carbohydrate epitope(s) recognized
Griffonia simplicifolia IB-4	GS-IB4	1:400	Galα1-3Gal; Galα1-3(fucα1-2)Galβ1
Artocarpus integrifolia	Jacalin	1:1,000	Gal
Vicia villosa agglutinin	VVA	1:500	DGalNAcα1-3DGal; Cad antigen; Sd; GalNAcα1-3GalNAc (Forssman antigen); GalNAcα1-3β1-3GlucNAc; GalNAc-Ser/Thr (Tn antigen);
Helix pomatia agglutinin	HPA	1:500	GalNAcα1-3(fuc1-2)Galβ1- ; GalNAcα1-3GalNAc (Forssman antigen)
Macura pomifera agglutinin	MPA	1:500	Melibose
Dolichos biflorus agglutinin	DBA	1:500	GalNAcα1-3Gal;
Glycine max agglutinin	SBA	1:500	GalNAc-Ser(Thr)
Ricinus communis agglutinin I	RCAI	1:1,000	Galβ1-4GlcNAcβ1
Ricinus communis agglutinin II	RCAII	1:1,000	Galβ1-4GlcNAcβ1
Erythrina cristagalli agglutinin	ECA	1:100	β-Gal
Agaricus bisporus	ABA	1:400	β-Gal
Peanut agglutinin	PNA	1:500	Core disaccharide:Galβ(1-3) GalNAc

Table 3.(Continued

Ulex europeus agglutinin	UEA1	1:100	LFucα1-2Galβ1-4GlcNAcβ-6
Lens culnaris	LCA	1:500	L-Fucose
Anguilla anguilla agglutinin	AAA	1:100	L-fucose
Phaseolus vulgaris-L	PHA-L	1:500	GlcNAcβ1-6Manα1-6Man
Concanavalia ensiformis	ConA	1:2,000	Mannose
Triticum vulgaris agglutinin	WGA	1:2,000	Bisecting
HB-T1	T	1:200	Galβ1-3GalNAcα1-0-Ser/Thr
HB-Tn1	Tn	1:400	GalNAcα1-O-Ser/Thr
HB-STn1	STn	1:200	NeuAcα2-6GalNAcα1-O-Ser/Thr
anti-blood group A	A	1:400	Blood group substance A
anti-blood group B	B	1:400	Blood group substance B
anti-blood group H	H	1:1,500	Blood group substance H type 2
LEX-2	LEX	1:100	Lewis X
FH-6	SLEX	1:2,000	sialyl dimeric Lewis^{X-i}

From manufacture's data sheet.

I-3. Relationship between Lymph Node Metastasis and Reactivity with Lectin or Monoclonal Antibodies

To characterize cancers with or without axillary lymph node metastasis [n (+) and n (−)] by carbohydrate expression of cancer cells in primary lesions, we examined the difference in frequencies of positive staining with every lectin and MAb between the n (+) and n (−) groups. No correlation was found in any of the cases. Next, we examined the correlation between the presence of lymph node metastasis and the frequencies of positive staining according to T stage. The results revealed that LEX and H staining were correlated with lymph node metastasis in T1 and T2 cancers, respectively, and that H and VVA staining of cancers were correlated with

lymph node metastasis in papillotubular and solid-tubular subtypes, respectively.

I-4. Carbohydrate Network Associated with Lymph Node Metastasis

Synergistic and antagonistic relationships between staining by 2 kinds of lectins/MAbs and the nodal status were estimated as shown in Table 4. A total of 783 combinations were examined among all the cases and also according to T stage and histological subtypes. The total number of 29 combinations showed a statistically significant correlation among the 783 combinations (3.7%) for all the cases. Fifteen of the 29 combinations were synergistic and the remaining 14 were antagonistic. When the combinations among lectins and MAbs were connected with each other by synergistic or antagonistic symbols, a completely closed linkage (network) was formed with all the cases. Synergistic linkages were found among Tn, VVA, anti-H, AAA, and LEX in the center of the network and all of them were related positively to the lymph node-positive status (Chart 2). These lectins and MAbs were linked like satellites to other lectins/MAbs in a synergistic or antagonistic manner. ECA and ConA were 2 major lectins that showed antagonistic linkages to the node-positive status. ECA linked to VVA, Tn and Jacalin (data, not shown), and ConA linked to VVA, AAA, STn, B (data, not shown), and H. With regard to T1 cancer, 15 of the 783 combinations (1.9%) showed a statistically significant correlation. Six of the 15 combinations (40%) were synergistic and 9 (60%) were antagonistic. These formed a completely closed network that involved both the synergistic and antagonistic linkages. LEX and Tn were situated in the center of the synergistic linkages, whereas PHA-L occupied the center of the antagonistic linkages. With regard to T2 cancers, 21 combinations (2.7%) showed significant correlation. All of them formed a completely closed linkage, where synergistic linkages among H, AAA, VVA, and Tn were in the center and DBA may be also included in it (Data, not shown). It was noted that PHA-L, which was situated in the center of the antagonistic linkage within T1 cancers, exhibited synergistic linkages to H and AAA. Papillotubular, solid-tubular and scirrhous type carcinomas showed significant correlation in 3 (0.4%), 16 (2.0%), and 4 (0.5%) of the 783 combinations, respectively. In papillotubular type carcinoma, H was related synergistically to LEX, and antagonistically to Lotus and ConA. In solid-tubular carcinoma, VVA, accompanying Tn was situated in the center of a

completely closed network that had 11 combinations of synergistic linkages. Since lectins/MAbs were related to VVA and Tn and did not link to each other, the network looked like a wheel (Chart 3). An antagonistic relationship was found only between AAA and UEA-I. In contrast to papillotubular and solid-tubular carcinomas, scirrhous carcinoma had 2 groups of relationships. One was a synergistic negative correlation between HPA and RCA-1 or ConA along with an antagonistic relationship between HPA and UEA-I. The other was a synergistic relationship between AAA and Tn.

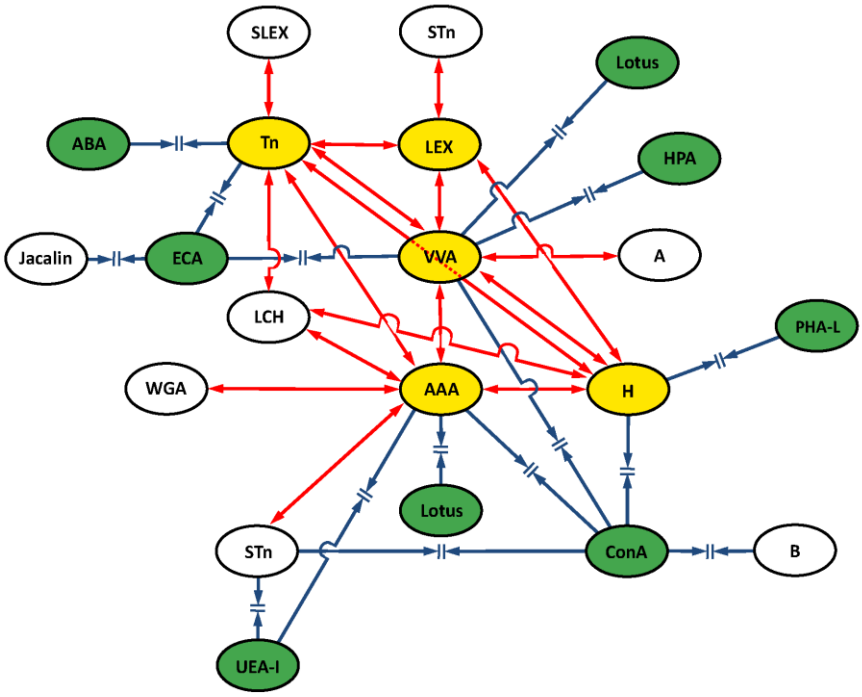

Chart 2. Lymph node metastasis-related carbohydrate network for breast cancer. Combination analysis revealed that 31 combinations had a significant correlation with lymph node metastasis. These combinations formed a completely closed linkage (or network) when lectins and MAbs were connected by lines that indicated a significant ($p<0.05$) relationship between them. The network included among HBTn-1, VVA, anti-H, AAA, and LEX-2 in the center. VVA occupied the central core. VVA was the reagent whose staining was related to ly factor (lymphatic invasion), which suggests that lymph node metastasis occurs when VVA-reactive carbohydrates develop [Kawaguchi T. et al. Basic Invest. Breast Carcinoma, 7, 1998, 59-63. (in Japanese).

Table 4. Statistical assessment of synergistic and antagonistic relationships between staining by two kinds of lectins/MAbs and axillary nodal status

Staining of two kinds of lectins or MAbs[1)]		No. of case of n (−) and n (+)		Node-positive rate (%)	Statistics[2)]
A	B	n (−)	n (+)		
−	−	a	b	[b/a + b]×100	①
+	−	c	d	[d/c + d]×100	②
−	+	e	f	[f/e + f]×100	③
+	+	g	h	[h/g + h]×100	④

1) + and − indicate positive staining and negative staining by lectins or MAbs. Positive and negative were determined as described in Materials and Methods.

2) If p values of ①versus ④ and ② versus ③ were smaller than 0.05, the relationships between A and B were synergistic and antagonistic, respectively.

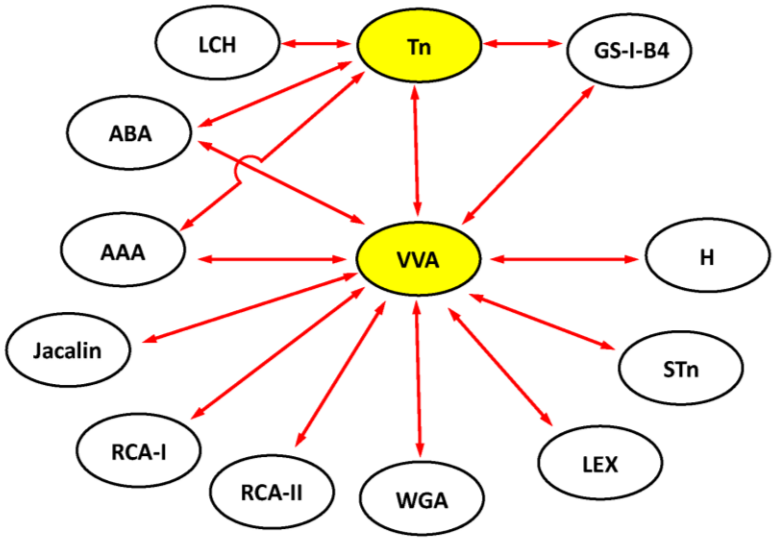

Chart 3. Lymph node metastasis-related carbohydrate network for breast cancer according to histological subtype. This shows a sample of solid-tubular carcinoma of invasive ductal carcinoma (poorly differentiated adenocarcinoma). In this case, VVA, accompanying Tn was situated in the center of a completely closed ne twork.

I-5. Correlation Between Lymphatic Vessel Invasion or Vein Invasion and Carbohydrate Expression of Cancer Cells

To clarify the significance of the carbohydrate network of cancer cells in lymph node metastasis, we examined the correlation between ly or v factors and carbohydrate expression profiles of cancer cells. We found a positive correlation between ly factor and HPA, VVA, and ConA staining, but there was no correlation between v factor and staining by any of the lectins and MAbs.

I-6. Significance of Combination Analysis of Carbohydrate Antigen Expression on Lymph Node Metastasis Status of Primary Breast Cancer Cells

The present study clearly demonstrated a relationship between axillary lymph node metastasis status of invasive ductal carcinoma of the breast and carbohydrate expression phenotypes of cancer cells in the primary lesion. The relationship was found clearly by examination of individual lectins or MAbs (individual analysis), but was more evident by combinations with 2 kinds of lectins/MAbs (combination analysis). The concept of synergistic and/or antagonistic relationships is well known; but as far as we know, the present study is a first attempt to examine this concept in the field of cancer metastasis research. At the present time, we cannot give a clear reason why the analysis revealed a relationship between carbohydrate expression of cancer cells and lymph node metastasis. It may be that oligosaccharides are packed together, generating a clustered saccharide patch for specific recognition. It may also be likely that breast cancer cells need different kinds of carbohydrates in order to accomplish the process involved in lymph node metastasis. Nonetheless, the results of our study indicate that VVA-binding carbohydrate(s) can play a major role in lymphatic spreading of aggressive breast cancer, while being especially marked in T2 staged, solid tubular carcinoma (poorly differentiated adenocarcinoma).

II. VVA-BINDING CARBOHYDRATE(S) ASSOCIATED WITH AGGRESSIVE GROWTH OF BREAST CANCERS: PARTIAL MOLECULAR CHARACTERIZATION AND IDENTIFICATION

Several studies, including our own, have demonstrated that VVA-binding carbohydrate expression in cancer cells is related to the aggressive behavior of various malignancies. These studies prompted us to consider the glycoprotein(s) bearing VVA-binding carbohydrates as potential drug targets. However, molecular characterization of glycoproteins in cancer cells has received little attention, although details of exclusive VVA-binding to the Tn antigen (epitope) [*N*-acetylgalactosamine (GalNAc) 1α-*O*-serine (Ser)/threonine (Thr)] have been well known.

One purpose of our study was to determine whether VVA-binding carbohydrate differs from HB-Tn1-binding carbohydrate. Terasawa et al. [26] and Avichezer and Arnon [27] initially posed this question in 1996. Terasawa et al. found that the expression of VVA-binding carbohydrates in uterine cervical cancer was different from that of HB-Tn1-binding carbohydrates with respect to their relationship to clinicopathological parameters. *In vitro* and *in vivo* studies by Avichezer and Arnon revealed discrepancies in reactivity of breast cancer to VVA and anti-Tn antibody. In addition, few clinicopathological reports support the concept proposed by Springer et al. [28], that Tn antigen expression is crucial for aggressive growth of cancer cells.

II-1. Localization of Carbohydrate Antigen by Staining With VVA and HB-Tn1 Mab in Primary Breast Cancer

We first investigated the localization of carbohydrate(s) stained with VVA and HB-Tn1 (Tn) in primary cancer tissues. VVA staining appeared almost exclusively in cancer cells. Although the cytoplasm was often stained, intense staining was typically found in the cell periphery or on cell membranes at the luminal and/or lateral surfaces (Figures 1a, b). In some cases, VVA-positive substances in the cytoplasm or around the cancer cells appeared to be secretory in nature (Figure 1c). With regard to invasion and metastasis, dilated lymphatic vessels contained floating cancer cells, showing VVA-positive staining, which was usually seen on outer cell surfaces (Figure 1d). Normal

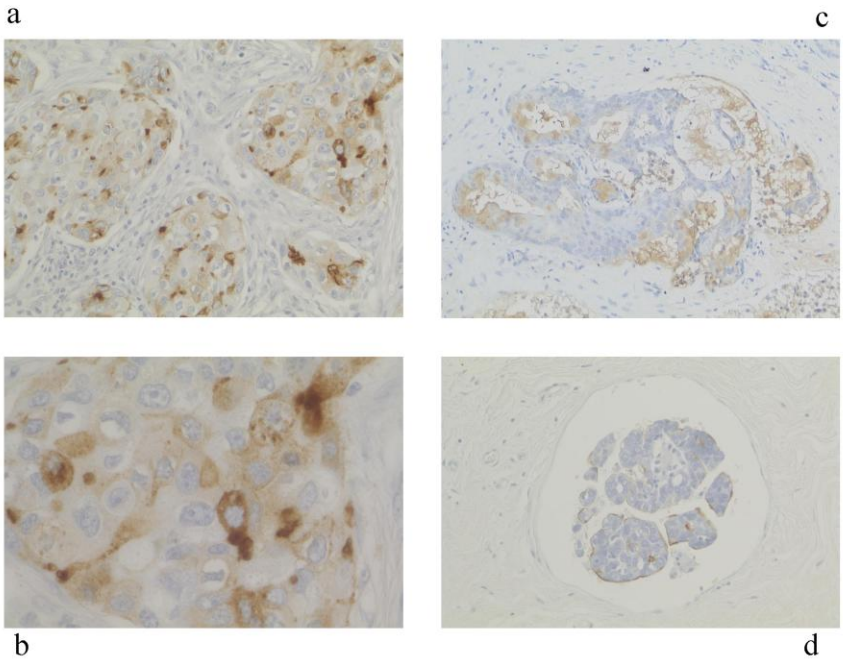

Figure 1. VVA staining in primary breast cancer. (a) Strong positive staining was found mainly on cancer cell surface. Original magnification × 200. (b) In some cases, the cytoplasm of the cancer cells stained intensely. Original magnification × 400. (c) Some positive VVA staining seen in the cytoplasm and/or around the cancer cells had a secretory, mucin lake-like appearance. Original magnification ×100. (d) Cancer cells in dilated lymphatic vessels usually showed linear VVA staining in cell surface membranes. Original magnification × 100 [20].

mammary ducts also showed positive VVA staining but the staining was weak and usually restricted to apical duct surfaces. A few lymphocytes showed VVA staining, while the staining was rarely found in blood vessel and lymphatic vessels and other normal counterparts. Staining features with Tn resembled those of VVA. On serial thin sections, VVA-stained features demonstrated staining that was directly correlated with that of Tn, but Tn only stained parts of the VVA area.

II-2. Correlation of Breast Cancer Aggressiveness with Expression of VVA-Binding Protein, HB-Tn1-Binding Protein, and MUC1

Next, we investigated the relationship between VVA-binding carbohydrate expression and aggressive growth of breast cancers by using formalin-fixed and paraffin-embedded surgical samples. We then used frozen samples to explore the possible carbohydrate epitopes of VVA and its carrier proteins, with a focus on the Tn antigen[20].

Medical records of 322 consecutive cases of female breast cancer were obtained from the files of the Department of Pathology, Fukushima Medical University School of Medicine, Fukushima City Japan during the period between February 5, 1985 and December 10, 1994. Descriptions of clinicopathological findings usually followed the general rules for studies of breast cancer in surgery and pathology. Clinicopathological variables and expressions of major molecules that are related to aggressive breast cancers were examined, as shown in Table 5. Frozen samples (39 cases) taken at operations, along with corresponding formalin-fixed, paraffin-embedded samples, were obtained. Immunohistochemistry was performed essentially according to previously described methods[19,20]. Immunohistochemical methods were used to obtain the percentage of stained cells. The results were classified as positive on the basis of clear staining of cancer cells (cell membrane and cytoplasm; see Figure 1), with the degree of staining divided into 2 categories: the cases with more or less than 50% for VVA and the cases with more than 5% stained cells for Tn and MUC1 regarded as positive.

Table 5 summarizes the correlations between VVA-positive staining and several clinicopathological parameters related to aggressiveness of breast cancer growth. VVA-positive staining was significantly correlated with tumor stage, lymphatic invasion, and lymph node metastasis. VVA-positive staining and disease-free survival or 5-year survival rates showed no significant correlation. Also, no significant correlations were found between VVA-positive staining and estrogen receptor status, progesterone receptor status, p53 expression, blood group A status or type H2 expression, or the ploidy pattern of cancer cells. However, strong correlations were again found for VVA-positive staining and staining for Tn- staining ($p<0.0001$).

Table 5. Relationships between VVA-positive staining, HB-Tn1-positive staining, or VU-3C6 anti-MUC1 MAb staining and clinicopathological variables.

Variable	VVA-positive case % (*p* value)	Tn-positive case % (*p* value)	VU-3C6-positive case % (*p* value)
All cases	43.5	46.9	75.1
Age (years)	(0.0601)	(0.7639)	(0.700)
50≦	51.2	47.7	70.9
51≧	34.7	46.0	73.3
Tumor stage(TNM)	(0.0385)	(0.1048)	(0.636)
I	48.3	40.0	77.4
II	39.0	51.3	76.8
III	39.5	60.5	77.8
IV	76.9	61.5	80.0
Lymphatic invasion	(‹ 0.0010)	(0.3635)	(0.837)
Negative	38.0	45.5	23.9
Positive	58.6	51.7	25.0
Venous invasion	(0.074)	(0.5849)	(0.391)
Negative	41.0	46.4	76.3
Positive	52.6	50.0	71.4
Lymph node metastasis	(0.043)	(0.1466)	(0.278)
Negative	37.9	42.9	72.4
Positive	49.0	49.1	77.8
Estrogen receptor	(0.2672)	(0.4108)	(0.599)
Negative	39.7	49.2	73.3
Positive	46.2	44.3	76.1
Progesterone receptor	(0.3464)	(0.1669)	(0.387)
Negative	43.5	51.3	72.6

Table 5.(*Continued*)

Variable	VVA-positive case % (*p* value)	Tn-positive case % (*p* value)	VU-3C6-positive case % (*p* value)
Positive	43.0	43.0	77.2
p53	(0.3567)	(0.0318)	(0.327)
Negative	40.2	41.7	74.6
Positive	45.3	62.8	80.5
Blood group type A	(0.3383)	(0.5347)	
Negative	44.0	48.4	
Positive	54.5	54.1	
Blood group type H2	(0.8037)	(0.2393)	
Negative	44.4	50.6	
Positive	46.3	53.7	
Ploidy	(0.7972)	(0.2946)	(0.669)
Diploid	47.4	59.6	74.5
Aneuploid	45.0	54.1	70.9
VVA		(‹0.0001)	(0.2609)
Negative		28.6	38.0
Positive		70.7	37.8
Tn	(‹0.0001)		(0.0006)
Negative	26.4		65.3
Positive	65.6		82.9
MUC1	(0.2609)	(0.0006)	
Negative	38.0	65.3	
Positive	37.8	82.9	

[Ref.19, 20]

Relationships between Tn-positive staining and the clinicopathological variables mentioned above are shown in Table 5. These results showed marked differences from the results for VVA-positive staining. For example, no relationships were found for Tn-positive staining and tumor stage, lymphatic invasion, and lymph node metastasis. However, there was a marginal relationship between Tn-positive staining and 5-year survival rate ($p = 0.061$). Tn-positive staining was strongly related to staining for p53 ($p = 0.0318$), VVA ($p < 0.0001$), and MUC1 ($p < 0.0006$).

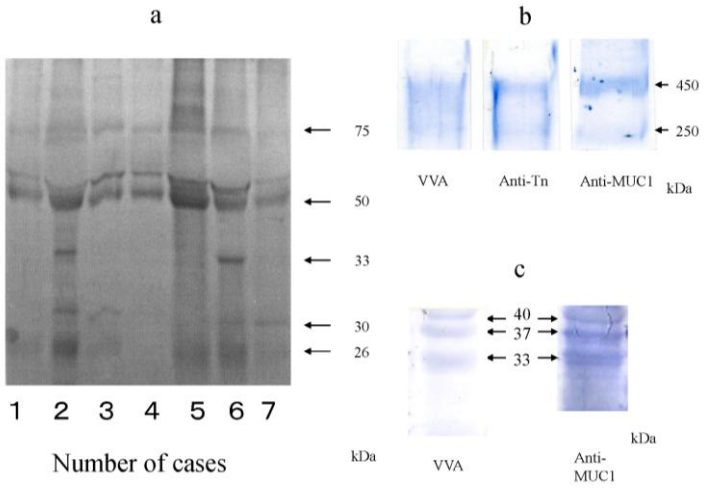

Figure 2. (a) Expression of VVA-binding proteins of human breast cancers demonstrated by Western blotting. Results for proteins of relatively small low molecular size from seven cases of primary cancer are shown. Among the proteins, the one of ~33 kDa was most relevant to lymph node metastasis. (b) VVA-, HB-Tn1, and VU-3C6-positive molecules of > 200 kDa. One case served as the source of samples used in all staining evaluation. VVA-positive bands sometimes appeared smeared, and therefore the precise molecular size was difficult to estimate. Staining-positive bands for HB-Tn1 (anti-Tn) and VU-3C6 (anti-MUC1) appeared located at ~250 and 450 kDa, respectively. (c) VU-3C6-positive molecules of ~33, 37, and 40 kDa, but not ~ 30 kDa, were found in this breast cancer case.

We analyzed expression of VVA- and Tn-binding proteins in a stock of frozen breast cancer samples (39 cases total) by SDS-PAGE followed by Western blotting. VVA primarily bound relatively small molecules (< 100 kDa) and showed distinct positive bands at approximately 26, 30, 33, 50, and 75 kDa under reducing and denaturing conditions (Figure 2a). These cases showed no distinct differences in VVA reactivity for the ~26-, 50-, and 75-kDa bands but did demonstrate marked differences for the ~30- kDa and 33-kDa bands (Figure 2a). Although we were able to perform this analysis for 8 cases, we did not examine in detail the VVA reactivities for >200- kDa molecules due to a lack of sample. Of the 39 cases examined, 11 (28.2%) and 23 (60.0%) showed expressions of the ~30- and ~33-kDa bands, respectively. Statistically significant differences were not found for cases with expression of the ~30 -kDa VVA-positive band, compared with cases without this expression, with regard to the ly factor, v factor, skin invasion and lymphatic metastasis. About half the cases (6 of 11) with the ~30 - kDa band had

aggressive cancers. In contrast, expression of the ~33-kDa VVA-positive band was related to cancer aggressiveness, especially in lymphatic invasion. Cases with the ~33-kDa band more often had lymphatic invasion (ly factor) than cases without the ~33-kDa band ($p = 0.0076$). Of the 8 cases that were evaluated for expressions of >200 kDa VVA-binding molecules, almost all these cases had a VVA-positive band (Figure 2b). No distinct tendency for cancer aggressiveness was found, however. We also examined 29 cases for expressions of Tn-binding proteins and found 2 and 7 cases with Tn-positive bands at ~30 and 33 kDa, respectively. Seven of the 8 cases with >200-kDa VVA-binding molecules had Tn−positive bands (Figure 2b). Expressions of Tn−binding proteins were not correlated with cancer aggressiveness.

In the present study, with regard to clinicopathological parameters, we demonstrated that the expression of VVA-binding molecules in primary breast cancer cells were different from those of HB-Tn1−binding molecules. Furthermore, we defined some VVA-binding molecules at the Western blotting level. We discovered a ~33- kDa VVA- binding molecule whose expression directly corresponded to lymphatic vessel invasion. Due to the insufficient number of cases examined, we could not definitely demonstrate that expressions of VVA-binding molecules of ~30 kDa and >200 kDa are related to breast cancer aggressiveness. However, this possibility remains.

II-3. Partial Characterization of VVA-Binding Carbohydrates in Relation to Tn Antigen

VVA is 1 of the 2 lectins found in *Vicia villosa* (hairy vetch) seeds: GalNAc-specific lectin and mannose-specific lectin. VVA-B_4 is predominant and reportedly binds preferentially to Tn antigen. Many researchers have used purified GalNAc-specific VVA to detect the Tn antigen on cancer cells. However, VVA is not the same as the immune determinant recognized by anti-Tn MAb (Tn) as described before. We were therefore interested in the carbohydrate epitope recognized by VVA, that was related to breast cancer aggressiveness.

Hapten inhibition tests were carried out on tissue section in order to discriminate the carbohydrate(s) that were recognized by VVA and HBTn1. VVA and HB-Tn1 were incubated with 1, 10, 50, and 100 mM *N*-acetylgalactosamine (Sigma), 0.12 µg *N*-acetylgalactosamine-α-1-O-*p*-Aminophenyl (PAP)-human serum albumin (HSA ; IsoSep AB, Tullinge,

Sweden) or Tn epitope (TRC, Toronto, Canada) for 1 h at room temperature, followed by incubation at 4° C overnight. The solutions were then centrifuged at a speed of 12,000 rpm for 1 h at 4° C. The resulting supernatants were used for lectin histochemistry or immunohistochemistry by the same procedures described above. The solutions that did not contain hapten sugars were used as controls.

A hapten inhibition test revealed that VVA staining and Tn staining on thin sections were completely abolished by preincubation of VVA/HB-Tn1 with 10 and 100 mM of GalNAc. This test was also applied to molecules obtained from breast cancer tissues and blotted on PVDF membranes. VVA binding to the ~30- and 33-kDa molecules was completely absorbed by preincubation with either 10 and 100 mM GalNAc or 1 mM Tn antigen. VVA binding to >250-kDa molecules was not examined. VVA samples from EY Laboratories, Vector, and Sigma bound strongly to either GalNAc-α-I-*O*-PAP-HSA or GalNAc-β-*O*-PAP-HSA that were transferred to PVDF membranes. In contrast, HB-Tn1 showed no binding to GalNAc-α-1-*O*-PAP-HSA or GalNAc-β-1-*O*-PAP-HSA.

Although the precise nature of the molecule must await further studies, we believe that one of the VVA-binding carbohydrate epitopes of the ~30- and 33- kDa molecules is the Tn antigen, because VVA binding completely disappeared after pre-incubation of VVA with Tn epitope. Although both VVA and Tn bind specially to Tn antigen, the reason why the binding carbohydrate of VVA was not consistent with that of HB-Tn1 needs to be evaluated. This issue would be resolved if VVA could bind both single (or free) and clustered Tn antigens and HB-Tn1 (Tn) could only bind to Tn antigen clusters. In fact, this possibility is likely as Nakada et al. [29] and Oppezzo et al [30] demonstrated that several anti-Tn MAbs could not bind to single (free) Tn antigens but they could bind to clusters of Tn antigen. Therefore, we believe that VVA may bind to molecules of ~30, 33 and more than >200 kDa sizes, which expose both single Tn antigens and their clusters, whereas Tn MAb(HB-Tn1) may bind to molecules that expose only Tn antigen clusters.

It may also be important that the Tn antigen is one of the epitopes with which VVA reacts. As demonstrated by our research and those of others, VVA bound not only to terminal α-linked GalNAc but also to terminal β-linked GalNAc. In addition, VVA can bind to various kinds of GalNAc residues such as the Cad antigen, Forssman antigen, and GalNAcα→3Gal (data, not shown). More importantly, the present study demonstrated that VVA did not bind to

mannose residues of serotransferrin and that this binding was modulated by formalin fixation.

II-4. MUC1 as a Possible Carrier Protein of VVA-Binding Carbohydrate(S) and HB-Tn1−Reactive Tn Antigen

Other important findings from our recent studies concern the molecules that carry the VVA-binding carbohydrates and Tn antigen. This information was derived from our studies as follows.

(1) Relationship between MUC1 expression of breast cancer cells and clinicopathological parameters

We are interested in MUC1 for a number of reasons. MUC1 is a transmembrane protein characterized by repeated sequences of 20 amino acids (tandem repeats: TAPPAHGVTSAPDTRPAPGS). The exact functions of MUC1 in vivo are still unclear, although this protein may be associated with the aggressive growth of cancer because it affects the integrity of cells and tissue and plays a role in signal transduction systems via ras and β catenin. In addition, MUC1 interferes with the lysis of metastatic cancer cells mediated by natural killer cells and killer T cells. Also of interest is that MUC1 is a carrier of a large amount of carbohydrates, mainly of *O*-glycosylated carbohydrates. Human breast cancer cells, particularly metastasizing cancer cells, express a large amount of MUC1 in the cytoplasm, in contrast with the low levels of expression in cells at apical sites in healthy, undifferentiated (nonlactating) mammary glands.

Our results using VU-3C6 anti-MUC1 MAb are shown in Table 5. MUC1-positive results (staining of 5% or more) were found in 75.1% (232 among 309) of our samples. No statistically significant relationships were found between MUC1 expression and age (more or less than 50-years old), TNM staging, lymph node metastasis, or invasion of lymphatic vessels and blood vessels. There was also no statistically significant correlation between MUC1 expression and the cumulative survival rate. In addition, MUC1 expression and the expressions of estrogen receptor, progesterone receptor, p53, and the ploidy pattern were not significantly related. We attempted to correlate MUC1 expression and the expressions of Tn antigen, sialyl-Tn (STn), and Thomsen-Friedenreich (T) antigens, because MUC1 is believed to carry *O*-glycosylated core carbohydrates. In fact, we found a strong correlation

between MUC1 expression and Tn antigen expression ($p < 0.0006$). However, the expressions of MUC1 and those of STn and T antigens were not correlated.

(2) Demonstration of small-sized atypical MUC1 and breast cancer aggressiveness

A MUC1-positive band was detectable by Western blotting in 32 of 47 cases (68.1%). Twenty-five of 32 cases (78.1%) showed 1 MUC1-positive band, 6 cases (18.8%) showed 2 MUC1-positive bands, and 1 case (3.1%) showed 3 MUC1-positive bands. Two MUC1-positive bands between 250 and 450 kDa were found under denaturing and reducing conditions, as shown in Figure 2. The largest MUC1-positive band, calculated as ~650 kDa, was found in three MUC1-positive bands (Figure 2).

We investigated MUC1-positive bands in the lower molecular size range under various gel conditions. These examinations revealed 1 case with a strong MUC1-positive bands at ~30 kDa and a weak band at ~44 kDa, in addition to 1 MUC1-postive band at ~250 kDa. A Tn (HB-Tn1)-positive band was found at ~44 kDa at nearly the same position as the weak MUC1-positive band, but not at the ~30 kDa position [19]. When MUC1 expression by Western blotting studies was examined for relationships to the aggressiveness of breast cancer, no statistical correlations were found for samples with MUC1-positive bands and venous invasion, skin invasion, or lymph node metastasis. However, lymphatic invasion was found more often in samples with 1 MUC1-positive band than in those with 2 or 3 MUC1-positive bands ($p=0.014$). In addition, samples with 1 MUC1-positive band had statistically significant higher rates of lymph node metastasis than did those with more than 2 MUC1-positive bands ($p<0.043$). A case that showed MUC1-positive bands at ~30 and ~44 kDa had axillary lymph node metastasis.

The molecular size of MUC1 varies between 200 and 450 kDa under reducing and denaturing conditions according to the number of tandem repeats, as it is well known. However, we and other researchers have discovered a very small MUC1. We are interested in small MUC1 forms and their relation to the metastatic potential of breast cancer cells in lymph nodes. Direct evidence for the manner in which these atypical MUC1 molecules are generated is not currently available. However, the following options are possibilities.

One is reduced or dysfunctional MUC1 glycation, but this possibility is unlikely because the size of the mucin core is about 68 kDa. Second is fragmentation of MUC1 by a proteolytic enzyme, which may yield several small MUC1 fragments. Third is the possibility of splice variants of MUC1:

several workers recently discovered MUC/Y, which include small sizes of MUC1s such as ~30- and 33- kDa ones. The ~30- and 33- kDa molecules were positive for VU-3C6 MUC1 staining (Figure 2C). We attempted to isolate these molecules by using VVA affinity chromatography with samples from 1 case. Although the separation was not successful, we isolated the ~30- kDa molecule by PNA affinity chromatography. We used PNA affinity chromatography to separate this protein because MUC1 is known to have many PNA-binding carbohydrates. The ~30- kDa protein isolated by PNA affinity chromatography was positively stained with by VVA, Tn (HB-Tn1), PNA, and VU-3C6. VVA-binding molecules of >200 kDa were detected in samples from several cases. These samples were also stained for Tn and VU-3C6 anti-MUC1 MAb (Figure 2). VVA-binding molecules of ~30, 33, and > 200 kDa appeared to be reactive with the VU-3C6 anti-MUC1 MAb, which suggests that these molecules were either MUC1 or MUC1 fragments containing MUC1 tandem repeats.

These atypical MUC1 molecules with VVA-binding carbohydrate, which is presumably the Tn antigen, may be used for adhesion of cancer cells to lymphatic vessels, as expression of the ~33- kDa molecule in breast cancer cells was related to lymphatic invasion. Adhesion of cancer cells to lymphatic endothelial cells is necessary in order for them to pass from a primary lesion into a lymph node. In fact, cancer cells that strongly expressed MUC1 frequently embolize in lymphatic vessels. MUC1 along with the Tn antigen may interact with intercellular adhesion molecule-1 (ICAM-1) on endothelial cells as suggested by Rhan et al. [31].

In the present study, expression of the VVA-binding carbohydrate in cancer cells was independent of several important aggressiveness-related factors. However, we also found that the VVA staining of some kinds of glycoproteins was strongly modulated by formalin fixation. Therefore, additional investigations are required for precise characterization of the relationships between VVA staining (e.g. non-clustered Tn antigen) and clinicopathological parameters, including cancer cell aggressiveness and prognosis.

III. Aberrant Muc1 Bearing Tn/Tn-Like Antigen in Rat Ascites Hepatoma Cells With Strong Lymph Node Metastasis Propensity

Recently, we investigated aberrant MUC1 bearing Tn antigen of rat ascites hepatoma AH109A cells (poorly differentiated hepatocellular carcinoma) with a strong lymphatic metastasis propensity [22]. These cells metastasized to lymph nodes 2 to 3 weeks after the inoculation into subcutaneous tissue of the abdominal wall when ICAM-1 appeared in the vascular walls around the tumors. AH109A cells contained a considerable amount of substances, mainly in their cytoplasm, which were reactive to VVA. These cells also contained substances, which were reactive to anti-MUC1 antibody which was raised against amino acids 961-1255 that map the C-terminus of MUC1 of human antigen. SDS-PAGE analyses followed by Western blotting demonstrated that primary tumor cells in the abdominal walls and metastatic tumor cells in the lymph nodes possessed mainly MUC1 protein of 30—40 kDa, as purified via affinity chromatography with a VVA column or a PNA column (See Figure 3).

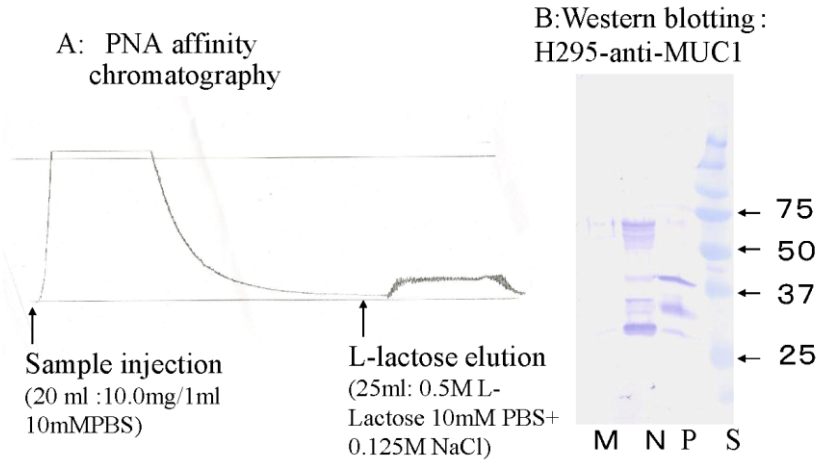

Figure 3. MUC1 purified by peanut agglutinin (PNA) affinity chromatography. A. PNA affinity chromatography. B. Western blotting profiles of P (primary sc tumor), N (lymph node metastasis), and M (submaxillary mucin) in denaturing and reducing conditions. H-295-positive bands were detected by using alkaline phosphatase. S is standard molecules.

VVA-reactivity of the 30－40 kDa proteins from primary tumors was completely absorbed after pre-incubation with 1 mM Tn antigen, but VVA-reactivity of the proteins from metastatic tumors was not absorbed after this treatment. Antibodies raised against the N-terminus and C-terminus did not react with these MUC1 proteins of 30－40 kDa bearing the Tn antigen. We concluded that rat ascites hepatoma AH109A cells express an aberrant type of MUC1 proteins of 30－40 kDa bearing the Tn antigen. These proteins appear to be derived from the extracellular sequence of the C-terminal subunit and transmembrane domain of MUC1.

Therefore, we hypothesize that aberrant MUC1 protein bearing the Tn antigen of rat ascites hepatoma cells may participate in lymphatic metastasis via the release of tumor cells from primary tumors and intravasation of these released cells into lymphatic vessels in primary tumors. Lack of Tn antigen in the aberrant MUC1 proteins from the metastatic cancer cells may be related to the escape of tumor cells from lymphocyte attack.

CONCLUSION

We investigated aggressive growth of breast cancer, especially from the viewpoints of expression of carbohydrates in cancer cells. The major results were as follows.

1. The aggressive growth of primary breast cancer depends on the capacity of cancer cells to intravasation into lymphatic vessels and/or blood vessels. The unique metastatic distribution of breast cancer is well explained by the cascade theory, in which cancer cells are disseminated via either lymphatic vessels or blood vessels, or both.
2. We explored the adhesion molecule(s) including carbohydrate(s) of aggressive breast cancer cells and discovered the candidate molecules of 30－40 kDa aberrant MUC1 bearing Tn/Tn-like antigen (~33 kDa aberrant $MUC1^{Tn}$), whose expression is closely related to the lymphatic spread of aggressive breast cancer. The possible ligand of lymphatic vessel for the aberrant $MUC1^{Tn}$ may be ICAM-1.
3. At present, we have no information on the adhesion molecules including carbohydrates associated with the blood-borne cancer cells. The selective adhesion of cancer cells via Lewis ^{X} and/or sialyl Lewis ^{X} to the selectin of the endothelial cells of blood vessels appears to be

insufficient to explain the hematogenous spread of human aggressive breast cancer cells. We are looking for another candidate molecule(s).

We believe that the acquisition of aberrant adhesion molecules including carbohydrates is a determinant by which benign tumor cells covert to malignant aggressive tumor cells, e.g. cancer cells in breast carcinogenesis.

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