3. Results

3.1. Solubility of ATRA in water

The solubility of ATRA in water was determined to be 4.53± 0.30 μg/ml at 37 °C (n=3).

3.2. Physicochemical properties of ATRA incorporated in liposomes

DOTAP/cholesterol or DSPC/cholesterol liposomes were prepared with or without ATRA. The zeta potential and particle size of ATRA incorporated in DOTAP/cholesterol liposomes were about +50 mV and 125 nm, respectively. On the other hand, the zeta potential and particle size of ATRA incorporated in DSPC/cholesterol liposomes were about −3 mV and 110 nm. When ATRA was incorporated in DOTAP/cholesterol liposomes, the zeta potential and particle size of the DOTAP/cholesterol liposomes remained unchanged.

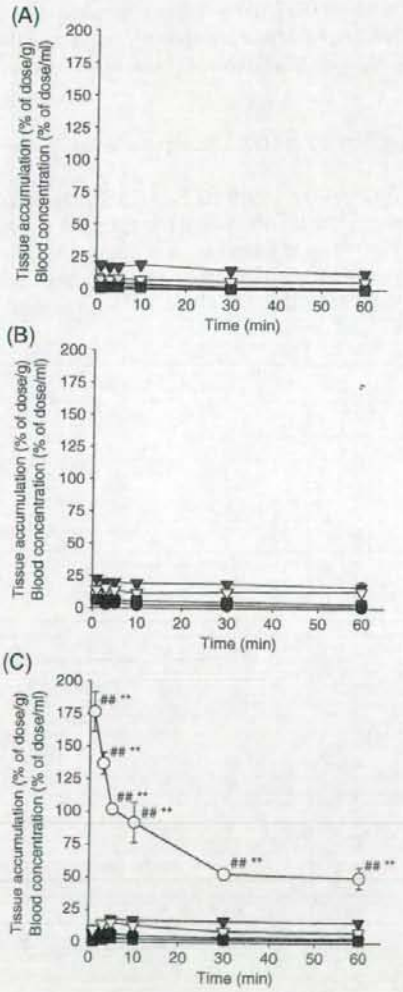

Fig. 1. Blood concentration and tissue accumulation time-courses of [^{3}H]ATRA dissolved in serum (A), [^{3}H]ATRA incorporated in DSPC/cholesterol liposomes (B), and [^{3}H]ATRA incorporated in DOTAP/cholesterol liposomes (C) after intravenous injection of the formulation at ATRA dose of 0.585 mg/kg and liposomal lipid dose of 120 mg/kg. Radioactivity was determined in the blood (●), lung (○), liver (▼) kidney (▽), and spleen (■). ATRA was injected at a dose of 0.585 mg/kg weight. Values are means±S.D., n=3– 5. Significant difference ##P<0.01 versus [^{3}H]ATRA dissolved in serum, **P<0.01 versus [^{3}H]ATRA incorporated in DSPC/cholesterol liposomes.

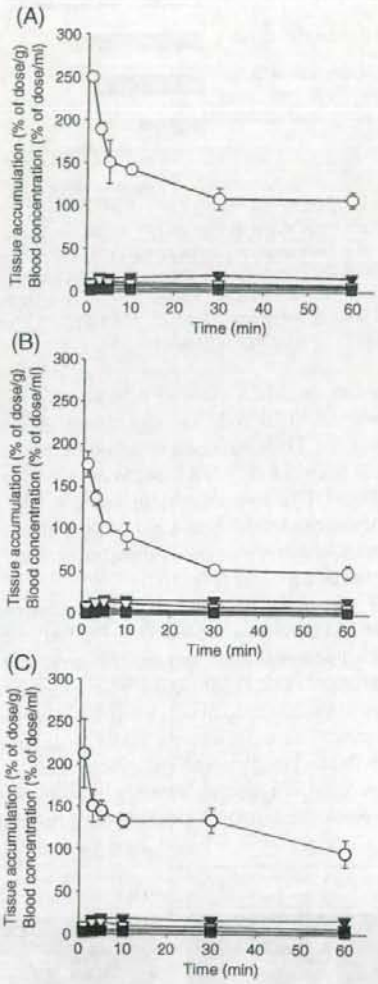

Fig. 2. Blood concentration and tissue accumulation time-courses of [^{3}H]ATRA incorporated in DOTAP/cholesterol liposomes after intravenous injection at an ATRA dose of 0.117 (A), 0.585 (B), and 2.93 mg/kg (C). DOTAP/cholesterol liposomes were fixed at a lipid dose of 120 mg/kg. Radioactivity was determined in the blood (●), lung (○), liver (▼) kidney (▽), and spleen (■). ATRA was injected at a dose of 0.585 mg/kg weight. Values are means±S.D., n=3.

3.3. Distribution characteristics of ATRA or ATRA incorporated in liposomes

Fig. 1 shows the tissue distribution profiles after intravenous injection of [^{3}H]ATRA dissolved in serum, [^{3}H]ATRA incorporated in DSPC/cholesterol liposomes or DOTAP/cholesterol

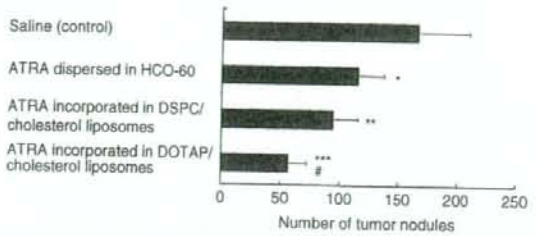

Fig. 3. Therapeutic effects in a pulmonary metastasis model after intravenous injection of ATRA dispersed in HCO-60, or ATRA incorporated in DSPC/ cholesterol or DOTAP/cholesterol liposomes. ATRA was injected at a dose of 0.585 mg/kg weight. Values are means±S.D., n=5–6. Significant difference compared with saline groups (*P<0.05, **P<0.01, ***P<0.001) or ATRA dispersed in HCO-60 groups (#P<0.05).

liposomes at an ATRA dose of 0.585 mg/kg. The pulmonary distribution of ATRA following injection of ATRA dissolved in serum and the DSPC/cholesterol liposome formulation ranged from about 6.8 to 1.0 (Fig. 1(A)) and from 11 to 3.5% of the dose/g tissue (Fig. 1(B)), respectively. In contrast, the DOTAP/cholesterol liposome formulation improved the pulmonary distribution of ATRA with a pulmonary distribution from about 175 to 50% of the dose/g tissue (Fig. 1(C)).

Fig. 2 shows the distribution profiles after intravenous injection of [^{3}H]ATRA incorporated in DOTAP/cholesterol liposomes at a liposomal lipid dose of 120 mg/kg that provides an ATRA dose of 0.117, 0.585, and 2.93 mg/kg when ATRA and liposomes at a molar ratio of 0.2, 1 and 5:100, respectively, were administered. The lung was the major distribution organ for ATRA in DOTAP/cholesterol liposomes regardless of the dose of ATRA used, suggesting that the distribution profiles of ATRA incorporated in DOTAP/cholesterol liposomes were not affected by the dose of ATRA but by the characteristics of the cationic liposomes.

3.4. Anti-cancer effect on the mouse pulmonary metastasis model by ATRA or ATRA incorporated in liposomes

The number of cancer nodules in mice given ATRA incorporated in DOTAP/cholesterol liposomes indicated a greater anti-cancer activity, but this was not observed following the administration of ATRA (dispersed by HCO-60) or ATRA incorporated in DSPC/cholesterol liposomes at an ATRA dose of 0.585 mg/kg (Figs. 3 and 4). The number of cancer nodules in mice given bare DOTAP/cholesterol liposomes was the same as in the control group (data not shown), suggesting that DOTAP/cholesterol liposomes themselves are not cytotoxic to the cancer.

3.5. Liver toxicity of ATRA incorporated in liposomes

Fig. 5 shows the serum ALT and AST concentrations after intravenous injection of ATRA incorporated in DOTAP/cholesterol liposomes at a lipid dose of 120 mg/kg. The ALT and AST levels were equal to those of the control injected with saline, suggesting that there was no hepatic toxicity at this liposomal lipid dose.

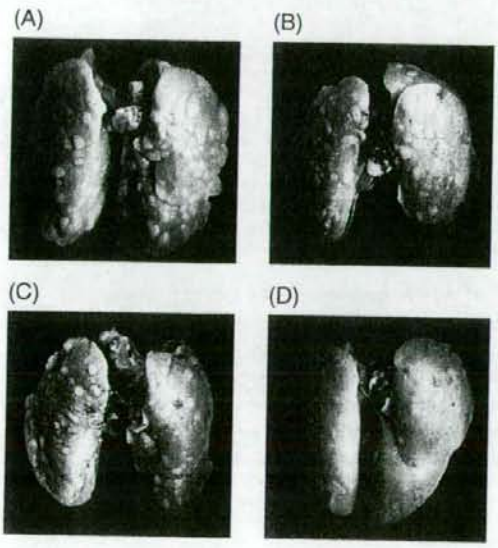

Fig. 4. Typical morphology of lung treated with saline (A), ATRA dispersed in HCO-60 (B), ATRA incorporated in DSPC/cholesterol liposomes (C), or DOTAP/cholesterol liposomes (D). ATRA was injected at a dose of 0.585 mg/kg weight.

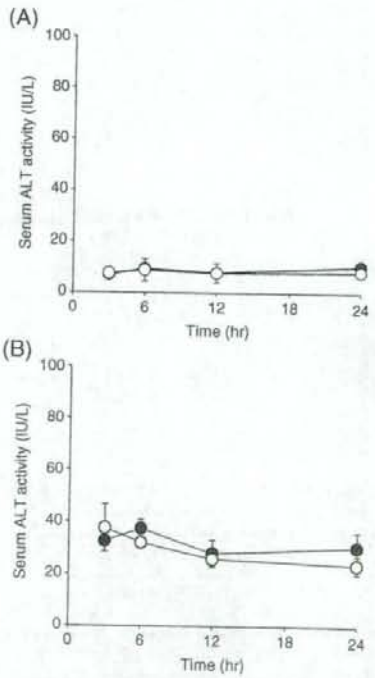

Fig. 5. Serum ALT (A) and AST (B) levels after intravenous administration of ATRA incorporated in DOTAP/cholesterol liposomes. ATRA and lipid was injected at a dose of 0.585 and 120 mg/kg weight. Saline (●) or ATRA incorporated in DOTAP/cholesterol liposomes (○) were injected. Values are means±S.D., n=3.

4. Discussion

In mammary carcinoma cell lines, ATRA has been shown to induce growth inhibition by triggering either cell cycle arrest or apoptosis or both [12–14]. Recently, we demonstrated the inhibition of murine liver metastasis by ATRA incorporated in O/W emulsions, which could accumulate in the liver [15]. This observation provides evidence that targeting of ATRA by a drug carrier could improve the efficacy of cancer differentiation therapy. To date, there are few published reports on cancer differentiation therapy in the lung. Since ATRA is expected to be selectively cytotoxic to cancer cells, the passive targeting of the organ (lung) is enough for the therapy of lung metastasis without producing cytotoxic effects in normal lung cells. This manuscript summarizes our initial efforts to investigate whether cationic liposomes offer a significant advantage as an alternative carrier of ATRA for the treatment of pulmonary metastasis of CT-26 cells following intravenous injection.

The liposomes composed of DOTAP/cholesterol (50:50 M ratios) and DSPC/cholesterol (60:40 M ratios) were selected as the ATRA carriers in this study. DSPC/cholesterol liposomes for drug delivery were prepared as reported previously [16–18]. Since water solubility of ATRA is very low, it located within this lipid membrane of the liposomes due to its lipophilicity. Moreover, our previous study reported that ATRA was almost completely incorporated in the liposomes (>95%) [6]. The ATRA incorporated liposomes exhibited small particle size and positive charge in DOTAP/cholesterol liposomes preparation or neutral in DSPC/cholesterol liposomes preparation. The ATRA liposomes remained stably over 1 month after preparation at 4 °C.

To clarify the effect of the liposomal incorporation of ATRA, the inherent distribution of ATRA must be examined. Because the water solubility of ATRA is too low, solubilizing agents are needed for intravenous injection. Some organic solvents, e.g. propylene glycol or ethanol, are sometimes used, but this has the disadvantage that precipitation can occur following dilution in the blood. In order to avoid such a problem, mouse serum was selected as a dissolving agent to investigate the inherent distribution of hydrophobic drugs after intravenous injection [19]. The pulmonary distribution of ATRA by injection of ATRA dissolved in serum and a DSPC/cholesterol liposome formulation was much lower than that of a DOTAP/cholesterol liposome formulation (Fig. 1). In addition, this high pulmonary accumulation of ATRA by DOTAP/cholesterol liposomes was observed at ATRA doses of 0.117, 0.585, and 2.93 mg/kg (Fig. 2). These results show that DOTAP/cholesterol liposomes are able to deliver ATRA to the lung selectively at these ATRA doses.

To investigate the effects on pulmonary metastasis of colon carcinoma cells in mice, the number of tumor nodules was counted. The therapeutic effect on pulmonary tumor nodules produced by ATRA incorporated in DOTAP/cholesterol liposomes is in general agreement with the distribution results. As shown in Figs. 3 and 4, the pulmonary metastasis of colon carcinoma cells was efficiently inhibited by the intravenous injection of ATRA incorporated in DOTAP/cholesterol liposomes. Although the detailed mechanism is not yet clear, these results provide evidence that ATRA incorporated in DOTAP/cholesterol liposomes efficiently improves the suppression of lung cancer metastasis by targeting ATRA to the lung.

Recently, Choi et al. reported the inhibition of tumor growth following the subcutaneous injection of ATRA containing biodegradable microspheres, which could achieve the sustained release of ATRA in a human head-and-neck cancer xenograft by continuous exposure to ATRA [20]. However, the disposition characteristics of ATRA after release were not controlled in this microsphere system. Recently, Shimizu et al. and we ourselves reported that targeted delivery of ATRA to liver by liposomes and emulsions could enhance the inhibition of liver metastasis at an ATRA dose of 0.585 mg/kg [11,15]. In this study, we demonstrated that the inhibition of pulmonary metastasis was enhanced by the targeting of ATRA to the lung (target site) by cationic liposomes. These observations have convinced us that targeting formulations of ATRA can enhance the therapeutic potential of ATRA under *in vivo* conditions.

Over 100 different genes have been introduced into the cells in human gene therapy trials. Around 60% of the genes transferred are cytokine genes [21]. Since pDNA complexed with cationic liposomes (i.e. lipoplexes) accumulates in the lung immediately after intravenous injection, this leads to a high gene expression in that tissue [22–24]. Among the cationic liposomes investigated, DOTAP/cholesterol liposomes are often used because of their high transfection efficacy [25]. We previously reported that intravenously injected interferon encoding plasmid DNA complexed with cationic liposomes prevents lung metastases in a mouse lung CT-26 metastasis model [10]. Recently, Dow et al. reported that repeated intravenous infusion of liposome-DNA complexes in cancer-bearing dogs is safe and well tolerated at low doses and may be capable of eliciting antitumor activity in some animals with advanced tumor metastases [26]. However, selective cytotoxicity against cancer cells is required for more efficient systems. Since ATRA is expected to induce selective cytotoxicity against cancer cells, combined therapy with the cytokine gene therapy system could more efficient than its use alone. Therefore, in the near future, the combination of cytokine therapy and differentiation therapy could be used to improve the treatment of non-small cell lung carcinoma and/or pulmonary cancer metastasis based on cancer immunotherapy.

It has been reported that intravenous injection of lipoplexes induces hepatic toxicity [27,28] but not lung toxicity [29]. However, it is suggested that hepatic toxicity is induced by proinflammatory cytokines secreted from macrophages by the recognition of the CpG motif in plasmid DNA [30]. In fact, no hepatic toxicity was observed after intravenous injection of lipid at a dose of 120 mg/kg (2.16–3.00 mg/mouse) (Fig. 5). This finding about hepatic toxicity agrees in part with the report of Tan et al. who showed that the intravenous injection of DOTAP/cholesterol liposomes at a dose of 900 nmol/mouse (about 0.488 mg/mouse) did not induce hepatic toxicity [31]. Although we selected DOPAP/cholesterol (1:1) liposomes because they are used for *in vivo* transfection to the lung, they were optimized for drug targeting to the lung: i) to reduce the DOTAP molar

ratio, and/or ii) to select a safer cationic lipid for clinical application in lung cancer.

In summary, we have demonstrated that ATRA incorporated in DOTAP/cholesterol liposomes preferentially accumulates in lung after intravenous injection. In the pulmonary metastasis of CT-26 cells in mice, ATRA incorporated in DOTAP/cholesterol liposomes was particularly effective in suppressing tumor metastasis to the lung. Although many additional studies of relevant tumor models are required to improve the efficacy of ATRA incorporated in cationic liposomes, the results in this study warrant the further development of cationic liposomes to investigate their use in clinical differentiation therapy.

Acknowledgements

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Preclinical study

Expression of *Vicia villosa* agglutinin (VVA)-binding glycoprotein in primary breast cancer cells in relation to lymphatic metastasis: is atypical MUC1 bearing Tn antigen a receptor of VVA?

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Key words: Breast cancer, lymphatic metastasis, MUC1, Tn antigen, *Vicia villosa* lectin

Summary

Aberrant carbohydrate expression frequently occurs in breast cancer and may endow cells with metastatic potential. Here we first studied the relationship between expression of *Vicia villosa* agglutinin (lectin) (VVA)-binding carbohydrates and aggressive breast cancer. We then investigated the molecular characteristics of these glycoproteins and compared them with those of glycoproteins recognized by the mouse anti-Tn monoclonal antibody (MAb) HB-Tn1. Histochemical studies of samples from 322 cases of invasive ductal carcinoma demonstrated that VVA-binding carbohydrate expression correlated with tumor stage, lymphatic invasion, and lymph node metastasis ($p = 0.0385$, $p = 0.0019$, and $p = 0.0430$. respectively). Western blotting analysis of frozen materials from 39 cases, under denaturing and reducing conditions, revealed that the major cancer cell-specific VVA-binding proteins were molecules of about 30, 33, and >200 kDa. Cases expressing the ~33 kDa molecule had significant lymphatic invasion more frequently than did cases not expressing this molecule ($p = 0.0076$). Binding of VVA to the ~30 and ~33 kDa molecules was completely lost by preincubation of VVA with 1 mM Tn antigen (*N*-acetylgalactosamine α1-O-serine). The VVA-binding molecules appeared to react with VU-3C6 anti-MUC1 MAb. Expression of HB-Tn1 in breast cancer cells showed significant correlation with expression of VVA-binding carbohydrate(s) ($p < 0.0001$) but HB-Tn1 reactivity was not clearly related to breast cancer aggressiveness. Because anti-Tn MAbs bound to Tn antigen clusters, we concluded that atypical MUC1 bearing the noncluster form of Tn antigen is implicated in aggressive growth of primary breast cancer cells, particularly in lymphatic metastasis.

Introduction

Altered glycosylation is a universal feature of cancer cells [1–4]. Because glycoproteins and glycolipids play a crucial role in a cell's biological functions, including invasion and metastasis, certain types of glycan modifications likely have direct relationships to processes involved in breast cancer aggressiveness, such as progression, invasion, and metastasis [5–7]. Several studies, including our own demonstrated that expression of *Vicia villosa* agglutinin (lectin) (VVA)-binding carbohydrates in cancer cells is related to the aggressive behavior of various malignancies such as cancers of the urinary bladder, uterine cervix, colon and rectum, lung, pancreas, and breast and malignant lymphoma [8–17]. These studies prompted us to consider that glycoproteins bearing VVA-binding carbohydrates may become an important drug target. 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 published [18–23].

VVA is one of the two lectins found in *Vicia villosa* (hairy vetch) seeds: GalNAc-specific lectin and mannose-specific lectin [20,21]. The GalNAc-specific lectin consists of several isolectins: VVA-B_4, VVA-A_4, and VVA-A_2B_2. VVA-A_4 binds human blood group type A erythrocytes. VVA-B_4 is predominant and reportedly binds preferentially to Tn antigen. VVA-A_2B_2 has characteristics intermediate between those of VVA-A_4 and VVA-B_4. A lectin showing specific

binding to mannose was separated by Qian et al. and defined as VVL_m [24]. It consists of at least four isolectins.

Many researchers have used GalNAc-specific purified VVA to detect Tn antigen on cancer cells [9–13]. However, the carbohydrate epitope (or epitopes) recognized by VVA is not the same as the immunodeterminant recognized by anti-Tn monoclonal antibody (MAb) that is related to cancer aggressiveness. For example, Terasawa et al., who studied the relationship between expression of HB-Tn1 (anti-Tn MAb)-binding carbohydrate and clinicopathological parameters including the prognosis of uterine cervical cancer, claimed that the carbohydrate recognized by VVA differed from the Tn antigen that was recognized by HB-Tn1 [14]. We also discovered that the VVA-binding carbohydrate was quite similar to the carbohydrate recognized by HB-Tn1 but not the same as that recognized by HB-Tn1 with respect to the relationship to lymph node metastasis in breast cancer [7].

We were therefore interested in the carbohydrate epitope recognized by VVA, which is related to breast cancer aggressiveness. In the present study, we first investigated the relationship between VVA-binding carbohydrate expression and aggressive growth of breast cancers by using formalin-fixed and paraffin-embedded surgical materials. We then used frozen materials to explore the possible carbohydrate epitope of VVA and its carrier proteins, with a focus on Tn antigen.

Materials and methods

Patients and specimens

Medical records of 322 consecutive cases of female breast cancer were obtained from files of the Department of Pathology, Fukushima Medical University School of Medicine, Fukushima City, Japan, for 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 [25]. Clinicopathological variables and expression of major molecules that are related to aggressive behavior of cancers were examined: mean age, stage (tumor, node, metastasis [TNM], lymphatic invasion, venous invasion, lymph node metastasis (nodal status of regional lymph nodes), ploidy, estrogen receptor, progesterone receptor, p53, blood group type H [2], and Tn antigen. Expression of blood group type A was also examined because the data sheet for VVA from EY Laboratories (San Mateo, CA) describes VVA as reacting slightly with blood group A_2. However, VVA did not stain red cells from patients with blood group A_2, so we did not include results for this blood group. p53 protein was detected by means of immunohistochemistry with results classified into positive or negative according to the presence or absence of cells whose nuclei were stained, respectively [26].

Lymphatic invasion and venous invasions (ly and v factors) were judged positive or negative, respectively, on the basis of the presence or absence of cancer cell emboli in the vessels as seen on sections with elastica – Masson staining as well as hematoxylin and eosin staining. Estrogen receptor levels were determined by using standard biochemical methods (enzyme assay) for most patients, hormone receptor levels greater than 10 fmol/mg were considered positive. These materials had been resected from breasts and fixed in 20% formalin for about 1–2 weeks. Postoperative follow-up data were obtained by periodic outpatient examinations, or from mailed or telephone or questionnaires at the end of 2003. Patients whose death was from causes other than breast cancer or its complications were excluded from these analyses.

Frozen samples (39 cases) taken at operations, along with corresponding formalin-fixed, paraffin-embedded materials, were obtained from Chiba National Hospital. These materials were stored at −80°C until use. No postoperative follow-up data for these cases were available. The Ethics Committees of Chiba National Hospital approved the use of these materials.

Detection of carbohydrates in cancer cells via lectin histochemistry and immunohistochemistry

Immunohistochemistry was performed essentially according to a previously described method [27]. Sections 3 μm thick were incubated overnight at 4 °C with biotin-labeled VVA (about 0.4 μg/200 μl)(EY Laboratories) in 10 mM phosphate buffer (PB, Sigma, St. Louis, MO), pH 7.4, or with HB-Tn1 MAb (DAK0, Glostrup, Denmark) at a dilution of 1:500 in 50 mM Tris–HCl buffer (about 0.2 μg/200 μl). After sections were washed extensively with phosphate-buffered saline (PBS), those for lectin staining were treated with 200 μl of streptavidin-biotin-peroxidase complex (Nichirei, Tokyo, Japan) for 20 min at room temperature. Sections for HB-Tn1 staining were layered with about 200 μl of biotin-labeled anti-mouse immunoglobulin (Ig)(Nichirei) for 20 min at room temperature, after which they were treated with streptavidin-biotin-peroxidase complex (Nichirei) for 20 min at room temperature. Binding with lectins and MAb was detected with the use of hydrogen peroxidase plus diaminobenzidine. Sections were counterstained with Mayer's hematoxylin (Muto Pure Chemicals). As a negative control, use of lectins and MAb during the histochemical procedure was omitted: no staining was observed in the surface and cytoplasm of these cancer cells. Sections in which almost all cancer cells had stained strongly were stained simultaneously as a positive control.

We compared staining profiles and staining scores of VVA preparations from EY Laboratories, Vector, and Sigma ($VVA-B_4$). The staining profiles and staining scores of these preparations were quite similar (data not shown). We also compared molecular sizes of these lectins and reactivities of these lectins with bovine mucin, two cases of

Table 1. Characteristics of lectins and monoclonal antibodies used

Lectin or MAb	Abbreviation or usual name	Source	Clone	Ig subclass	Dilution	Carbohydrate specificity or epitope[a]
Vicia villosa agglutinin	VVA	EY Laboratories	-[b]	-	1:500	*N*-Acetylgalactosamine
Vicia villosa agglutinin	VVA	Vector	-	-	1:500	*N*-Acetylgalactosamine
Vicia villosa agglutinin-B_4	VVA-B_4	Sigma	-	-	1:500	*N*-acetylgalactosamine
Arachis hypogaea	PNA	EY Laboratories	-	-	1:300	Galactose β1-3*N*-Acetylgalactosamineα1-*O*-Ser/Thr
Anti-human serotransferrin MAb	-	Accurate Chemical	HT1/13.6.3	IgG	1:5000	-
Anti-human IgG MAb	-	DAKO	A57H	IgM, κ	1:1000	-
Anti-Tn antigen MAb	HB-Tn1	DAKO	HB-Tn1	IgM, κ	1:500	*N*-Acetylgalactosamine α1-*O*-Ser/Thr
VU-3C6 anti-MUC1 MAb	VU-3C6	Our own	VU-3C6	IgG	1:3000	GVTSPADTRPA[c]

[a]These data were taken from manufacture's data sheets.
[b]No relevant idem.
[c]The amino acid sequence in a MUC1 tandem repeat.

breast cancer materials, and Tn antigen epitopes, and we found no distinguishable differences among them (See Figure 5).

Characteristics of lectins and MAbs used here are summarized in Table 1. Biotin-labeled lyophilized VVA preparations from EY Laboratories and Sigma were dissolved according to manufactures' instructions and were stored at −80 °C.

Evaluation of immunohistochemical staining

Immunohistochemical methods were used to obtain the percentage of stained cells. At least 500 cells were examined and counted by means of a grid-equipped light microscope at high-power magnification (×400). The number of stained cells per total number of cancer cells in certain areas of cancer tissues was obtained. The result was 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 two categories: ≥5% and <5%, and cases with more than 5% stained cells regarded as positive.

Preparation and detection of glycoproteins carrying VVA-binding carbohydrates in cancer cells via sodium dodycyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting

Preparation of samples

Frozen materials were homogenized in an earthenware mortar with about 10 volumes of 0.5% ethylphenol-poly(ethyleneglycolether)$_n$, (Nonidet P40; Roche, Mannheim, Germany) in 0.25 M sucrose, 10 mM Tris–HCl, 50 mM $CaCl_2$, and 10 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.2, according to Matsushita et al. [28]. In some experiments, we used protease inhibitor cocktail tablets (complete; Roche) instead of PMSF. However we found no distinct difference in proteins from these samples (data not shown), so we have presented combined results.

Homogenized materials were centrifuged at 3000 rpm for 10 min and supernatants were shaken gently at 4°C overnight. These solutions were centrifuged at 10,000 rpm for 15 min and supernatants were dialyzed against 10 mM PB at 4 °C overnight. After buffer was exchanged twice in 1 day, the solutions were centrifuged at 10,000 rpm and supernatants were concentrated about 100 times via filtration membranes with a molecular size cut-off of 10 kDa (Ultracent-10; Tosoh, Tokyo, Japan). The protein concentration for each case was measured by using a BCA Protein Assay kit (Pierce, Rockford, IL) and was adjusted to 1 μg/μl. These materials were kept at −80 °C.

SDS-PAGE and Western blotting

SDS-PAGE was performed according to the method described in our previous study [29]. A 5-μl sample (1 μg/1 μl) was diluted twice by use of 2× sample buffer and was warmed at 100 °C for 3 min. Materials were then fractionated, usually with 5–15%, and sometimes with 10% gels or 10–20% acrylamide gradient gels with 3.0% or 3.5% stacking gels. Electrophoresis was

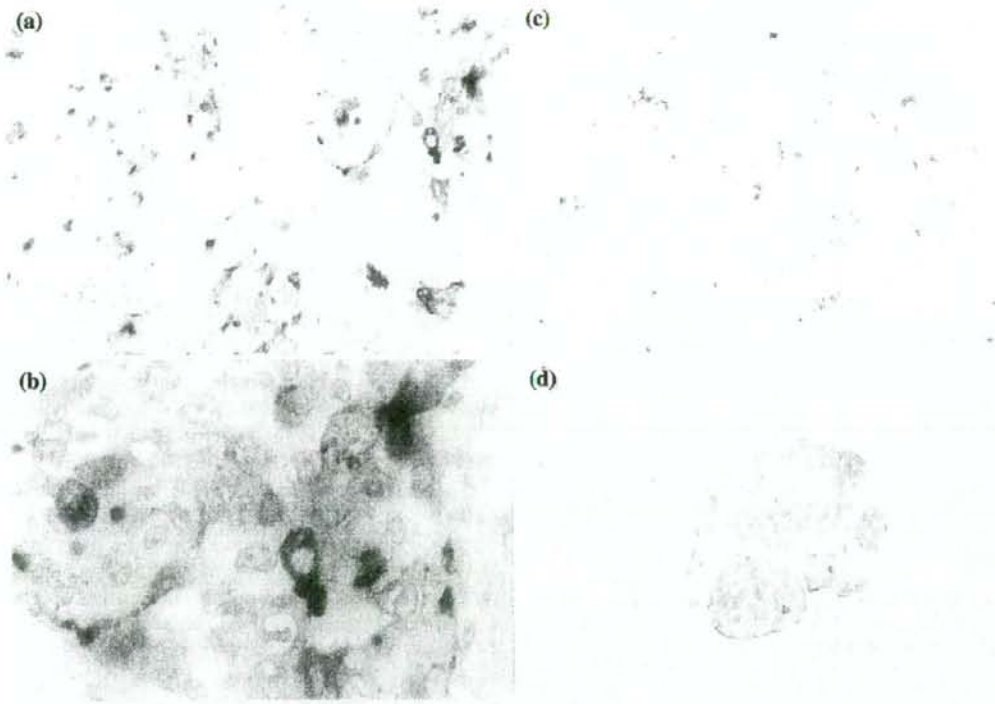

Figure 1. VVA staining in primary breast cancer. (a) Strong positive staining was found mainly on cancer cell surfaces. Original magnification × 200. (b) In some cases, the cytoplasm of the cancer cells stained intensely. Original magnification × 400. (c) Some positive VVA staining seen in the cytoplasm and/or around the cancer cells had a secretary, 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.

performed with Tris-glycin-SDS running buffer including 10% methanol at a constant current of 30 mA. Separated proteins were transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Bedford, MA) with Tris-glycin-SDS running buffer at a constant current of 0.28 mA/cm^2 of membrane and were kept in a tank for 15 h at 4 °C. These membranes were washed briefly with 50 mM Tris buffer containing 0.1% Tween-20 (TBS-T), followed by incubation at room temperature for 1–2 h with 1–2% bovine serum albumin (Sigma) supplemented with 0.125 M NaCl.

These membranes were allowed to react with biotin-labeled VVA (EY Laboratories) or HB-Tn1 at 4 °C overnight. In some experiments, the transferred membranes were also allowed to react with biotinylated VVA from Vector (1:500), biotinylated-VVA-B_4 (Sigma, 1:500), digoxigenin (DIG)-labeled VVA, biotinylated peanut agglutinin (PNA) (EY Laboratories, 1:300), or VU-3C6 anti-MUC1 MAb (Table 1). Membranes for HB-Tn1 or VU-3C6 assays were washed with TBS-T and were reacted with biotinylated anti-mouse Ig (1:2000; Amersham, Buckinghamshire, England) for 15 min at room temperature. All membranes were incubated in streptavidin-alkaline phosphatase conjugate (1:3000; Amersham). Reactive bands were visualized by using H_2O_2 as substrate in NADH-NBT solution, consisting of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (Roche).

Partial characterization of VVA-binding carbohydrates and their possible carrier proteins

Effect of formalin fixation on VVA staining of glycoproteins blotted on PVDF membranes

Erythrocytes, serum, and endothelial cells in formalin-fixed and paraffin-embedded thin sections from surgical specimens rarely showed VVA staining, whereas serotransferrin and IgG heavy chain (~50 kDa) and IgG light chain (~25 kDa) manifested strong VVA staining. Therefore, we examined the effect of formalin fixation of these proteins blotted on PVDF membranes. SDS-PAGE and Western blotting were performed as described above. PVDF membranes were immersed in 50 ml of 20% formalin 47 h, and then they were washed extensively with distilled water and stained with VVA. PVDF membranes on which these proteins were blotted and were immersed in Tris buffer were used as controls.

Hapten inhibition test

We conducted the hapten inhibition test according to our previous publication [29]. Briefly, a solution of VVA

(1 μg/1 μl; EY Laboratories) or HB-Tn1 (0.1 μg/0.1 μl) was incubated for 1 h at room temperature with 1 ml of a solution of 0.01, 0.1, 1, 10, or 100 mM of GalNAc (Research Organics, Cleveland, OH), 1 mM Tn antigen (epitope)(GalNAc α 1-serine; Toronto Research Chemicals, North York, Ontario, Canada) or 10 or 100 mM methyl-α-D-mannopyranoside (Sigma) in 10 mM PBS including 0.15 M NaCl, followed by incubation at 4 °C overnight. The solutions were then centrifuged at 10,000 rpm for 15 min at 4 °C, and supernatants were used for staining. Solutions without hapten sugar were used as controls. Bovine mucin (Calbiochem, Darmstadt, Germany) and lysate from metastatic breast cancer foci, which we prepared [7], were used as controls for GalNAc residues. Purified human transferrin (Serva, Heidelberg, Germany) served as control mannose residues.

Binding of VVA or HB-Tn1 to GalNAc-α-1-O-p-aminophenyl (PAP)-human serum albumin (HSA) or GalNAc-β-1-O-PAP-HSA

We examined binding abilities of VVA from EY Laboratories, Vector and Sigma ($VVA-B_4$) and those of HB-Tn1 to GalNAc-α-1-O-PAP-HSA (IsoSep, Tullinge, Sweden) and GalNAc-β-1-O-PAP-HSA (IsoSep). These proteins were transferred to PVDF membranes by using of Western blotting. Mucin from bovine submaxillary gland (Calbiochem) was used as a positive control.

Affinity chromatography for isolation of VVA-binding molecules

Affinity chromatography with the agglutinin from *Arachis hypogaea* (PNA; Sigma) was used for a sample from one case, essentially according to our previous paper [29]. A solution of 10 mg of proteins/ml PB was loaded on a 5.0-ml bed volume column packed with NHS-activated beads (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's data sheet. The column was first washed with 20 ml of PB containing 0.125 M NaCl for equilibration, and binding proteins were eluted with a solution of 0.5 M D-lactose (β-D-Gal-[1 → 4]-D-Glc) (Sigma) supplemented with 0.125 M NaCl in 10 mM PBS, with a flow rate of 1 ml/min. The lactose fraction (about 10 ml) was concentrated as described above and used for analysis.

Statistical analysis

Statistical comparisons on data between groups were performed by using Fisher's exact probability test. Overall disease-free survival rates were calculated by use of the Kaplan-Meier method. The difference between the survival curves was analyzed statistically via the log-rank test. Factors related to survival were analyzed by means of the Cox's proportional hazards regression model. Differences were statistically significant when $p < 0.05$.

Result

Localization of VVA-staining carbohydrate in primary breast cancer

VVA staining appeared almost exclusively in cancer cells. Intensive staining was usually found in the cell periphery or on cell membranes at luminal and/or lateral surfaces, but often the cytoplasm was also stained (Figure 1a, b). In some cases, VVA -positive substances in the cytoplasm or around the cancer cells appeared to be secretory (Figure 1c). These substances showed faint staining and sometimes made accurate estimation of positive staining rates difficult. With regard to invasion and metastasis, dilated lymphatic vessels contained floating cancer cells, which demonstrated VVA-positive staining that was usually seen on outer cell surfaces (Figure 1d).

Normal mammary ducts also stained for VVA but this staining was weak and usually restricted to apical duct surfaces (data not shown). Few lymphocytes showed VVA staining; macrophages sometimes contained many VVA-positive droplets. VVA-positive staining was rarely found in blood vessels, lymphatic vessels, fibroblasts and other normal counterparts.

Features with anti-Tn antibody (HB-Tn1) staining resembled those with VVA staining. On serial thin sections, VVA-stained features demonstrated staining that correlated exactly with HB-Tn1 staining, but HB-Tn1 stained only parts of the VVA-stained features (data not shown).

Correlation of VVA- or HB-Tn1-positive staining of cancer cells and clinocpathological parameters

Table 2 (Panel a) summarizes correlations between VVA-positive staining and several clinicopathological parameters related to aggressive growth of breast cancer. VVA-positive staining was significantly correlated with tumor stage, lymphatic invasion, and lymph node metastasis ($p = 0.085$, $p = 0.0010$ and $p = 0.0430$, respectively). VVA-positive staining and disease-free survival or 5-year survival rate showed no significant correlation ($p = 0.66$ and $p = 0.53$, respectively) (data not shown). Similarly, no significant correlations were found between VVA-positive staining and estrogen receptor status, progesterone receptor status, p53 expression, blood group type A status or type H2 expression, or ploidy pattern of cancer cells. Strong correlations were also found for VVA-positive staining and staining for HB-Tn1-staining ($p < 0.0001$).

Relationship between HB-Tn1-positive staining and the clinicopathological variables mentioned above are presented in Table 2 (Panel b). These results demonstrated marked differences from results for VVA-positive staining. For example, no relationships were found for HB-Tn1-positive staining and tumor stage, lymphatic invasion, and lymph node metastasis. However, there

Table 2. Relationships between VVA-positive staining and clinicopathological variables (a); Relationships between HB-Tn1-positive staining and clinicopathological variables (b); or major parameters related to aggressive breast cancer

Variable	No. of case	No. of VVA-positive case	%[a]	χ^2	*p* value
a. *Relationships between VVA-positive staining and clinicopathological variables*					
All cases	322	140	43.5		
Age (years)	322			3.5345	0.0601
50 ≤	172	88	51.2		
51≥	150	52	34.7		
Tumor stage	317			8.3962	0.0385
(TNM)					
I	120	58	48.3		
II	146	57	39		
III	38	15	39.5		
IV	13	10	76.9		
Lymphatic invasion	318			10.8212	<0.0010
Negative	231	88	38		
Positive	87	51	58.6		
Venous invasion	317			3.1917	0.074
Negative	239	98	41		
Positive	78	41	52.6		
Lymph node metastasis	322			4.09	0.043
Negative	161	61	37.9		
Positive	161	79	49		
Estrogen receptor	295			1.2307	0.2672
Negative	126	50	39.7		
Positive	169	78	46.2		
Progesterone receptor	284			0.8217	0.3464
Negative	154	67	43.5		
Positive	130	56	43		
p53	213			0.846	0.3567
Negative	127	51	40.2		
Positive	86	39	45.3		
Blood group type A	270			0.917	0.3383
Negative	248	109	44		
Positive	22	12	54.5		
Blood group type	176			0.0675	0.8037
H2					
Negative	81	36	44.4		
Positive	95	44	46.3		
Ploidy	117			0.066	0.7972
Diploid	57	27	47.4		
Aneuploid	60	27	45		
Tn	291			50.81	<0.0001
Negative	140	37	26.4		
Positive	151	91	65.6		
MUC1	338			1.269	0.2609
Negative	71	27	38		
Positive	267	101	37.8		
b. *Relationships between HB-Tn1-positive staining and clinicopathological variables*					
Variable	No. of cases	No. of Tn-positive cases	%[b]	χ^2	*p* value
All cases	322	151	46.9		
Age (years)	322			0.0902	0.7639
50 ≤	172	82	47.7		
51≥	150	69	46		
Tumor stage	317			6.1442	0.1048
(TNM)	120	48	40		
I	146	75	51.3		

Table 2. (Continued)

Variable	No. of case	No. of VVA-positive case	%[a]	χ^2	*p* value
II	38	23	60.5		
III	13	5	61.5		
IV					
Lymphatic invasion	318			0.8258	0.3635
Negative	231	105	45.5		
Positive	87	45	51.7		
Venous invasion	317			0.2983	0.5849
Negative	239	111	46.4		
Positive	78	39	50		
Lymph node metastasis	322			2.1079	0.1466
Negative	161	69	42.9		
Positive	161	82	49.1		
Estrogen receptor	295			0.6763	0.4108
Negative	126	62	49.2		
Positive	169	75	44.3		
Progesterone receptor	284			1.9107	0.1669
Negative	154	79	51.3		
Positive	130	56	43		
p53	213			4.6167	0.0318
Negative	127	53	41.7		
Positive	86	54	62.8		
Blood group	270			0.3853	0.5347
type A					
Negative	248	120	48.4		
Positive	22	20	54.1		
Blood group	176			1.3845	0.2393
type H 2	81	41	50.6		
Negative	95	51	53.7		
Positive					
Ploidy	117			1.0983	0.2946
Diploid	57	23	59.6		
Aneuploid	60	30	54.1		
VVA	291				< 0.0001
Negative	140	52	28.6		
Positive	151	99	70.7		
MUC1	290			11.7	0.0006
Negative	144	94	65.3		
Positive	146	121	82.9		

[a]No. of VVA-positive cases/no. cases × 100.
[b]No. of Tn-positive cases/no. of cases × 100.

was a marginal relationship between HB-Tn1-positive staining and 5-year survival rate ($p = 0.061$). HB-Tn1-positive staining was strongly related strongly to staining for p53 ($p = 0.0318$), VVA (< 0.0001), and MUC1 ($p < 0.0006$).

Correlation of VVA-binding protein expression and breast cancer aggressiveness

We analyzed expression of VVA- and HB-Tn1-binding proteins in a stock of frozen breast cancer materials (total 39 cases) by SDS-PAGE followed by Western blotting. VVA bound mainly relatively small molecules, less than 100 kDa, and produced distinct positive bands at ~26, 30, 33, 50 and 75 kDa under reducing and denaturing conditions (Figure 2a). Cases showed no distinct differences in VVA reactivity for the ~26, 50, and 75 kDa molecules but did demonstrate marked differences for the ~30 kDa and 33 kDa bands (Figure 2a). We did not examine in detail the VVA reactivities for molecules of > 200 kDa, because of a lack of material but we were able to perform this analysis for eight cases.

Of the 39 cases examined, 11 (28.2%) and 23 (60.0%) showed expression of ~30 and ~33 kDa molecules, respectively. No statistically significant differences were found for cases with expression of the ~30 kDa VVA-positive band, compared with cases

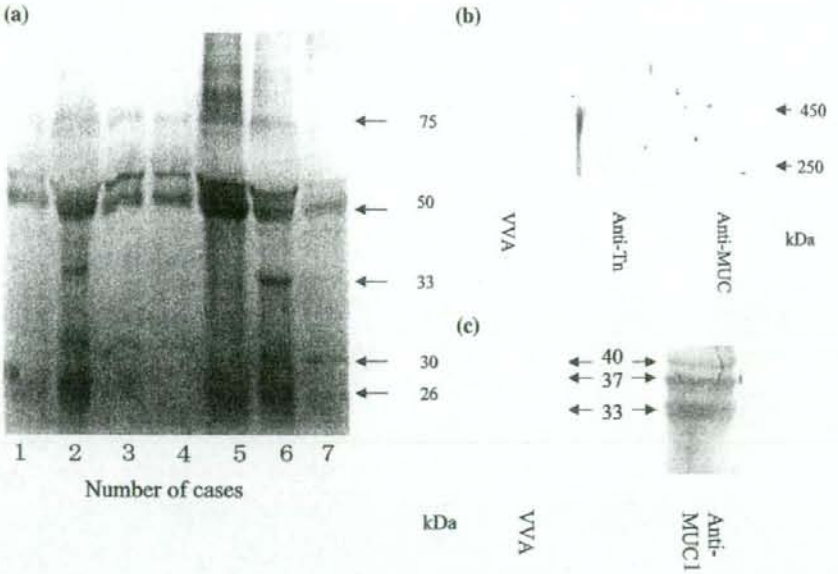

Figure 2. (a) Expression of VVA-binding proteins of human breast cancers demonstrated by Western blotting. Results for proteins of relatively small low molecular size from seven cases of primary cancer are shown. Among the proteins, the one of ~33 kDa was most relevant to lymph node metastasis. A sample of 5 μg/5 μl in each lane (one case per lane) was analyzed via SDS-PAGE, which was consisted of a 5–15% gradient gel and was conducted at 30 mA. Separated proteins were blotted on PVDF membranes at the rate of 0.28 mA/cm^2 for 15 h at 4 °C. Staining was performed as described in Materials and methods. (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. These sizes were estimated via comparison with electrophoresis of the standard proteins (Precision Plus Protein standards, Bio-Rad, Hercules, CA) and rat laminin (Chemicon) (data not shown). (c) VU-3C6-positive molecules of ~33, 37, and 40 kDa, but not ~30 kDa, were found in this breast cancer case. VVA staining from the same case was shown in Figure 3c. The VU-3C6-positive bands resembled the VVA-positive bands.

without expression, with regard to ly factor, v factor, skin invasion and lymphatic metastasis although about half the cases (6 of 11) with the ~30 kDa band had aggressive tumors. In contrast, expression of ~33 kDa VVA-positive band was related to cancer aggressiveness, especially in lymphatic invasion: cases with ~33 kDa molecule more often had lymphatic invasion (ly factor) than cases without the ~33 kDa molecule ($p = 0.0076$) (Table 3).

Of the eight cases that were evaluated for expression of VVA-binding molecules of > 200 kDa, almost all cases (seven) had a VVA-positive band (See Figure 2b). No distinct tendency for cancer aggressiveness was found, however. Of these seven cases, one demonstrated strong expression (about four times greater compared with other cases) of the VVA-positive molecule.

We also examined 29 cases for expression of HB-Tn1-binding proteins and found 2 and 7 cases

Table 3. Relationship between ~33 kDa VVA-binding carbohydrate expression and aggressive cancer cell growth

Variable	No. of VVA-positive cases	No. of VVA-negative cases	%	χ^2	*p*-value
Lymphatic invasion				7.1185	0.0076
Negative	12	14	46.2		
Positive	11	1	91.7		
Venous invasion				0.7759	0.3786
Negative	15	11	57.7		
Positive	6	2	75		
Skin invasion				0.3412	0.5591
Negative	21	14	60		
Positive	3	1	75		
Lymph node metastasis				0.8274	0.3629
Negative	13	10	56.5		
Positive	8	3	72.7		

with HB-Tn1-positive bands at ~30 and 33 kDa, respectively (data not shown). Seven of the eight cases with VVA-binding molecules of >200 kDa had HB-Tn1-positive bands (Figure 2b). Expression of HB-Tn1-binding proteins was not correlated with cancer aggressiveness.

Comparison of staining, molecular size, and Tn epitope-binding property of different VVA samples

We compared staining characteristics for VVA samples from EY Laboratories, Vector and VVA-B_4 from Sigma but we found no clear differences among these lectins (data not shown). We also observed no distinct differences for molecular sizes (data not shown) and reactivities to GalNAc-α-PAP-HSA or GalNAc-β-PAP-HSA among these VVA samples (Figure 5).

Partial characterization of VVA-binding carbohydrates from breast cancers in comparison with HB-Tn1-recognizing antigen

Effect of formalin fixation on VVA staining

On the basis of results from another experiment with affinity chromatography and amino acid sequencing of proteins separated by SDS-PAGE [30], we assumed the VVA-binding ~26 and 50 kDa proteins to be IgG light chain and heavy chain, respectively, and the VVA-binding ~75 kDa protein to be serotransferrin. Western blotting detected these proteins in every case, although differences in staining strength were noted. However, we did not find VVA-positive erythrocytes and blood vessels in surgical materials fixed with formalin and embedded in paraffin.

We thought that the discrepancy in staining might be caused by the formalin fixation. In order to examine this possibility, the PVDF membranes, on which samples from two breast cancer cases, human serotransferrin, human IgG, and bovine mucin were blotted, were fixed with formalin for 48 h and then they were stained with VVA. We found that human serotransferrin and human IgG rarely stained with VVA, whereas samples from breast cancers and bovine mucin were stained with VVA to the same degree as did untreated materials (data not shown).

Hapten inhibition test

The hapten inhibition test revealed that VVA staining and HB-Tn1 staining in thin sections were completely abolished by preincubation of VVA with 1, 10, and 100 mM GalNAc (data not shown). This test was also applied to molecules, obtained from breast cancer tissues and blotted on PVDF membranes. VVA binding to ~30 and 33 kDa molecules was completely absorbed by preincubation with either 10 or 100 mM GalNAc (Figure 3a,b) or 1 mM Tn antigen (Figure 3c,d). VVA binding to molecules of >250 kDa was not examined. VVA-binding to IgG (heavy chain and light chain) was absorbed with GalNAc but not Tn antigen. VVA-binding to serotransferrin was not absorbed after preincubation of VVA with GalNAc or Tn antigen, but binding was absorbed completely by preincubation with 100 mM mannose (data not shown).

Binding ability of VVA to GalNAc residue – carrying molecules

VVA samples from EY Laboratories, Vector, and Sigma bound strongly to either GalNAc-α-1-O-PAP-HSA or GalNAc-β-1-O-PAP-HSA 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 (Figure 4).

Characterization of proteins of ~30, 33, and >200 kDa carrying VVA-binding carbohydrates

The ~30 and 33 kDa molecules were positive for VU-3C6 staining (Figure 2c). We tried to isolate these molecules by using VVA affinity chromatography with samples from one 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 our previous study revealed that the ~30 kDa molecule was an atypical MUC1 [31] and MUC1 is known to have many PNA-binding carbohydrates [32]. The results in Figure 6 show a band of ~30 kDa found by PNA affinity chromatography. The ~30 kDa protein isolated by PNA was stained with VVA, HB-Tn1, PNA, and VU-3C6.

VVA-binding molecules of >200 kDa were detected in samples from several cases. These samples also stained for HB-Tn1 and VU-3C6 anti-MUC MAb (Figure 2b).

Discussion

Breast cancer is one of the most widespread cancers in the world. An important characteristic of this cancer is its aggressive growth, especially metastasis, which leads to death of 20-30% of patients. Lymph node metastasis is the most important predictor associated with the prognosis of breast cancer, so molecules involved in lymphatic metastasis have been extensively investigated. We have been studying cancer metastasis to elucidate its mechanisms and to develop effective therapy [7,33]. We believe, on the basis of findings from a previous study of invasive ductal carcinoma of the breast examined via combination analysis [7], that VVA-binding carbohydrates have an important role in lymphatic invasion by primary breast cancer cells, and the present results supported this.

One purpose of the present study was to determine whether expression of VVA-binding carbohydrates

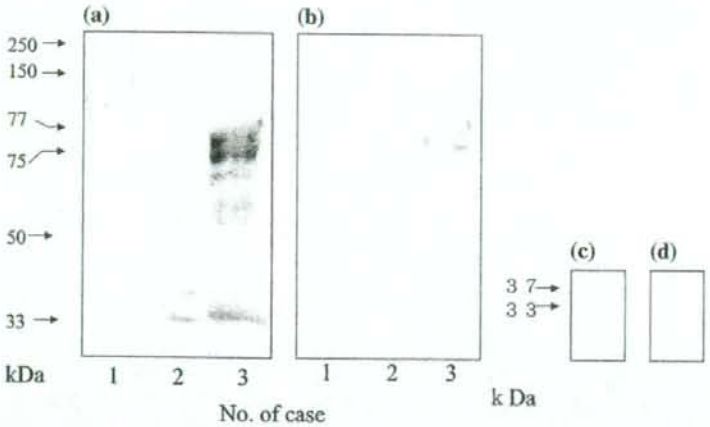

Figure 3. Hapten inhibition test for VVA-binding molecules. (a) Original VVA staining profile. Lanes 1, 2, and 3 are bovine mucin as a positive control for VVA; a breast cancer; and a positive control for the VVA-positive ~33 kDa molecule, respectively. (b) Preincubation of VVA with 100 mM GalNAc caused VVA staining in lane 1 and the ~33 kDa molecule in lane 2 and lane 3 to disappear completely. The ~33 kDa VVA-positive band (c) disappeared completely with after preincubation of VVA with 1 mM Tn antigen (d). Molecular sizes were estimated from a combination of molecular size markers RPN800 (Amersham, Buckinghamshire, UK) and Precision Plus Protein standards (Bio-Rad).

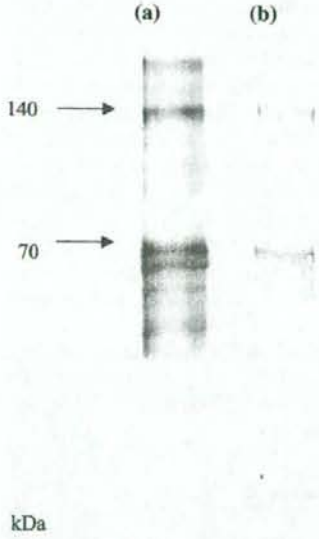

Figure 4. Comparison of VVA- and HB-Tn1-binding molecules. Bovine mucin purified from submandibular gland (20 μg/lane) was separated by SDS-PAGE, followed by Western blotting. HB-Tn1 bound to two molecules with ~70 and 140 kDa (b), whereas VVA bound several other molecules in addition to these (a).

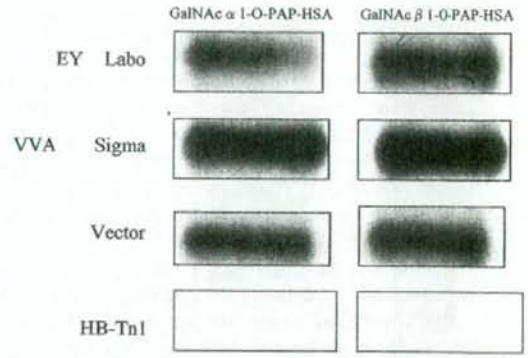

Figure 5. Binding capabilities of VVA and HB-Tn1to GalNAc-residues. We tested the binding of VVA and HB-Tn1 to GalNAc-α-1-*O*-PAP-HAS and GalNAc-β-1-*O*-PAP-HAS, which were transferred to PVDF membranes. GalNAc-α-1-*O*-PAP-HSA and GalNAc-β-1-*O*-PAP-HSA (10 μg/lane) were separated by 10% SDS-PAGE and transferred to PVDF membranes, which were stained with VVA labeled with DIG. VVA clearly bound the ~65 kDa band independent of the α or β anomers, but HB-Tn1 showed no binding. We examined VVA samples from different sources - E.Y Laboratories, Vector and Sigma - but found no difference in staining.

differs from that of HB-Tn1-binding carbohydrates. Terasawa et al. [14] and Avichezer and Arnon [34] initially posed this question in 1996. The former group found that expression of VVA-binding carbohydrate in uterine cervical cancer was different from that of HB-Tn1-binding carbohydrate with respect to the relationship to clinicopathological parameters. The latter discovered a discrepancy in reactivities of breast cancer cells to VVA and anti-Tn antibody for *in vitro* and *in vivo* studies [34]. We have also thought that too few clinicopathological reports support the concept proposed by Springer et al. [35], that Tn antigen expression is crucial for aggressive growth of cancer cells [36,37].

In the present study, we demonstrated that expression of VVA-binding molecules in primary breast cancer cells was different from that of HB-Tn1-binding molecules as related to clinicopathological parameters. Furthermore, we defined VVA-binding molecules at the Western blotting level: we discovered a ~33 kDa VVA-binding molecule whose expression directly corresponded to lymphatic vessel invasion. The possibility remains that expression of VVA-binding molecules of ~30 kDa and >200 kDa was related to breast cancer aggressiveness, but we could not demonstrate this

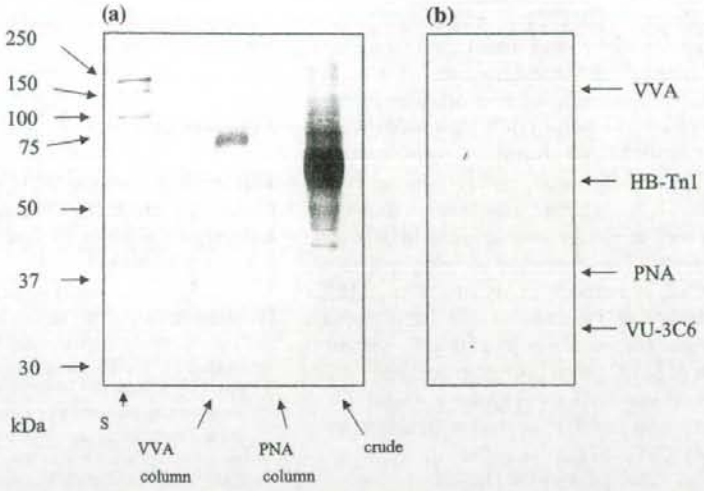

Figure 6. Separation and characterization of the ~30 kDa VVA-binding molecule by using PNA affinity chromatography. (a) Profiles of affinity chromatography-purified proteins via SDS-PAGE. VVA column chromatography did not produce significant amounts of the of ~30 or 33 kDa proteins. In contrast, PNA column chromatography produced a single ~30 kDa band stained with Coomassie brilliant blue. The "crude" lane is an original lysate from a case of primary beast cancer. Lane S shows standard proteins. (b) The ~30 kDa molecule stained with VVA (1:500), HB-Tnl (1:3000), PNA (1:1000) and VU-3C6 (1:3000).

because of an insufficient number of cases examined. Avichezer and Arnon also found ~35 and 38 kDa VVA-binding molecules in the human ovarian cancer cell line IGROV-1, but they did not discuss a relationship to cancer aggressiveness [34].

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 Tn antigen, because VVA binding completely disappeared after preincubation of VVA with Tn antigen. This possibility is supported by the fact that VVA specifically recognizes Tn antigen (18–23). A remaining question concerned the reason why the binding carbohydrate of VVA was not consistent with that of HB-Tnl, although both VVA and HB-Tnl MAb bind specifically to Tn antigen. This question would be resolved if VVA could bind both single (or free) and clustered Tn antigens and HB-Tnl could bind to only Tn antigen clusters. Actually, this possibility is likely: Nakada et al. [38,39] and Oppezzo et al. [40] demonstrated that several anti-Tn MAbs could not bind to single (free) Tn antigen but could bind to clusters of Tn antigen. Therefore, we believe that VVA may bind to molecules of ~30, 33 and more than >200 kDa, which expose both single Tn antigens and their clusters, whereas HB-Tnl may bind to molecules that expose only Tn antigen clusters. No evidence exists, however, that HB-Tnl MAb could not bind to single Tn antigen, so the final answer to this must await further studies.

It may also be important that Tn antigen is one of the epitopes with which VVA reacts. As demonstrated by previous workers and the present study, VVA bound not only to terminal α-linked GalNAc but also to terminal β-linked GalNAc [19]. In addition, VVA can bind to various kinds of GalNAc residues such as Cad antigen, Forssman antigen, GalNac α1 → 3Gal [18,19,22]. More important, the present study demonstrated that VVA did bind to mannose residues of serotransferrin and that this binding was modulated by formalin fixation. The diverse binding properties of VVA may explain the data of Avichezer and Arnon [34], who determined that VVA was distributed in various tissues in an *in vivo* exnograft model, although Tn antigen was rarely found in most organs.

Another important finding from the present study concerned the molecules that carry the VVA-binding carbohydrates. VVA-binding molecules of ~30, 33, and >200 kDa appeared to react with VU-3C6 anti-MUC1 MAb, which suggests that these molecules were MUC1 or MUC1 fragments containing MUC1 tandem repeats [41]. The >200 kDa molecule is likely to be native MUC1, because the molecular size is consistent with that of MUC1, and because breast cancer cells expose MUC1 with much Tn antigen [33,42]. We now believe that the VVA-binding molecule of ~30 kDa is MUC1, because the molecule was isolated via PNA affinity chromatography followed by elusion with lactose and it reacted with VVA and VU-3C6 anti-MUC1 MAb. We do not know the reason why VVA affinity chromatography failed to isolate VVA-binding protein, but it may be likely that the ~30 kDa VVA-binding protein was too little to be stained with Coomassie brilliant blue. We believe that the ~33 kDa VVA-binding molecule is also MUC1 because Western blotting with VU-3C6 detected the molecule with same size (Figure 2c). More studies are needed to confirm that the ~33 kDa VVA-binding molecule is the same as the ~33 kDa VU-3C6-binding molecule.

The molecular size of MUC1 varies between 200 and 450 kDa under reducing and denaturing conditions according to number of tandem repeats, as it is well known [30,43,44]. However, we and other researchers have discovered a very small MUC1 [30,45–48]. We are interested in small MUC1 forms in relation to the metastatic potential of breast cancer cells in lymph nodes and liver [7,30]. We currently have no direct evidence for the way in which these atypical MUC1 molecules are generated. However, the following options are possibilities. One is reduced or dysfunctional MUC1 glycation, although this possibility is unlikely because the size of mucin core is about 68 kDa [45]. Second is fragmentation of MUC1 by a proteolytic enzyme, which may yield several small MUC1 fragments [43,44]. Third is splice variants of MUC1: several workers recently discovered MUC/Y, which included small sizes of MUC1s such as ~30 and 33 kDa [46–48].

These atypical MUC1 molecules with VVA-binding carbohydrate, which is presumably Tn antigen, may be utilized for adhesion of cancer cells to lymphatic vessels, because 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 for them to pass from a primary lesion into lymph nodes [7,30]. In fact, cancer cells that strongly expressed MUC1 embolized frequently in lymphatic vessels [49,50]. MUC1 with Tn antigen may interact with intercellular adhesion molecule-1 of endothelial cells as Rhan et al. suggested [51]. In the present study, expression of the VVA-binding carbohydrate in cancer cells was independent of several important aggressiveness- related factors, such as estrogen receptor, progesterone, p53, blood group H2, and ploidy pattern, as well as 5-year survival rates. 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 and clinicopathological parameters, including prognosis, related to cancer cell aggressiveness.

In conclusion, we examined the relationship between expression of VVA-binding carbohydrates in cells in primary foci of breast cancer and breast cancer aggressiveness-related clinicopathological parameters including survival. We demonstrated that expression of VVA-binding carbohydrate in cancer cells correlated with lymphatic metastasis of breast cancer, particularly invasion of with lymphatic vessels. We believe that one of the VVA-binding carbohydrates is nonclustered Tn antigen, which is carried by MUC1 that has an atypical molecular size of ~30 or 33 kDa, as well as *in situ* MUC1. We previously reported that 250 or 450 kDa MUC1 with HB-Tn1-binding carbohydrate, which is probably Tn antigen clusters, was related to lymph nodes metastasis of breast cancer. On the basis of all these findings, we believe that MUC1 and its splice variants with Tn antigen or Tn antigen clusters is implicated in the molecular mechanism of lymphatic metastasis of breast cancer.

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Prognostic impact of p53 protein overexpression in patients with node-negative lung adenocarcinoma

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Abstract

Prognostic value of p53 protein expression in node-negative lung adenocarcinoma is still controversy. The expression of p53 protein was examined immunohistochemically in lung adenocarcinoma using monoclonal antibody BP53-12. A total 131 cases of primary lung adenocarcinoma were examined. Relationship between expression of p53 protein and clinicopathologic factors were studied. Overexpression of p53 protein was found in 19 patients (14.5%). Univariate and multivariate analysis showed that overexpression of p53 protein was an independent prognostic factor in node-negative lung adenocarcinoma. p53 alteration could be a valuable predictor for prognosis in node-negative lung adenocarcinoma.

Keywords: Lung adenocarcinoma; p53 protein; prognostic value; node-negative

1. Introduction

Lung cancer is the leading cause of cancer death in the majority of developed country [1,2]. In spite of advances of diagnostic and theraptic modalities, the prognosis is still poor. The overall 5-year survival rate is still only 15% [2]. Even though node-negative patients only have about 60% of 5-year survival rate [3–5]. Definite prognostic factor is needed to improve the prognosis or to help in the management of patients with lung cancer. To date, several kinds of prognostic factors for node-negative lung cancer, such as integrins, hepatocyte growth factor, endothelial cell growth factor, some kinds of oncogenes and inactivation of tumor suppressor gene have been reported and are still controversy [6–9].

p53 tumor suppressor gene is located on chromosome 17p13.1.The product of this gene is nuclear protein, which regulates cell cycle, apoptosis cellular differentiation and DNA repair [2,3,10,11]. Mutation of p53 is commonly found in many kinds of human cancer including lung cancer [12,13]. Missense

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ıtation of the p53 gene usually but not always olongs the half-life of the protein from minutes to ıurs and results in nuclear accumulation of the p53 otein, which can be detected by immunohistochemry as the overexpression [14]. In non-small cell lung ncer, the incidence of p53 overexpression using ımunohistochemistry is 17.5–77% [2,13]. Accordg to recent several studies including meta-analysis, 'erexpression of p53 is thought to be significant ognostic factor at least in lung adenocarcinoma 3]. However, in node-negative patients, the prog-ıstic value of p53 overexpression is still controversy 5,16]. In this report, we suggest that p53 over-:pression is significant prognostic factor in patients ith node-negative lung adenocarcinoma using ımunohistochemistry.

Materials and methods

1. Patients

A total 131 cases of primary lung adenocarcinoma ere examined in the present study. These samples :presented the material obtained from all patients ho had undergone complete resection and regional 'mph nodes dissection at the Department of Surgery Fukushima Medical University, School of Medi-ıne, Japan, between 1979 and 1993. The resected rimary tumors were macroscopically and micro-:opically examined to determine the location of ımor, tumor size, and extent of lymphatic and venous ıvasion, metastasis to lymph nodes, and histological ıbtype, according to the International Staging ystem for Lung Tumors. The major clinicopathoio-ical characteristics of the patients enrolled in this :udy are summarized in Table 1. Lymphatic (ly ıctor) and venous (v factor) invasion were deter-ıined with Elastica-Masson staining as well as ematoxylin and eosin staining. Invasion was con-idered positive or negative based on the presence or bsence of cancer cell emboli in the respective vessels fter examining all fields of cancerous tissues.

.2. Immunohistochemistry

Immunohistochemistry was performed according ɔ the methods described previously using monoclonal antibody BP53-12. Briefly, 3 μm-thick sections were cut from blocks and were mounted on glass slides precoated with 0.05% poly-L-lysin solution (Muto Pure Chemicals, Tokyo, Japan). Sections were dewaxed in xylene and dehydrated through graded alcohol solutions. Endogenous peroxidase activity was quenched by 20-min incubation with 0.3% (v/v) solution of hydrogen peroxidase (Wako, Osaka, Japan) in 100% methanol. After incubation with 5% dry skim milk in phosphate-buffered saline for 30 min at room temperature, the sections were incubated overnight at 4 °C with primary antibody (BP53–12, anti-human p53 protein monoclonal antibody, kindly provided by Dr. Shunsuke Imai, Nara Medical University) [17] at a dilution of 1:30,000. Then the primary antibody was detected using biotinylated secondary anti-mouse IgG antibody, by the avidin-biotin complex method, using 3.3′-diaminobenzidine as a chromogen. Sections were

Table 1
p53 expression according to patient clinical characteristics

Clinical characteristics	p53-negative	p53-positive	*P*value*
Age	61.6±9.7	62.6±9.0	N.S.
Gender			
Male	52 (85.2)	9 (14.8)	
Female	60 (85.7)	10 (14.3)	N.S.
T factor			
T1	52 (82.5)	11 (17.5)	
T2	46 (88.5)	6 (11.5)	
T3	10 (83.3)	2 (16.7)	
T4	4 (100)	0 (0)	N.S.
N factor			
Negative	75 (86.2)	12 (13.8)	
Positive	37 (84.1)	7 (15.9)	N.S.
p-Stage			
stageI-II	87 (87.9)	12 (12.1)	
stage III	25 (78.1)	7 (21.9)	N.S.
Ly factor			
Negative	70 (90.9)	7 (9.1)	
Positive	42 (77.8)	12 (22.2)	0.05
v factor			
Negative	78 (88.6)	10 (11.4)	
Positive	34 (79.1)	9 (20.9)	N.S.
Differentiation			
Well	43 (82.5)	5 (17.5)	
Moderate	52 (85)	11 (15.0)	
Poor	17 (89.6)	3 (10.4)	N.S.

Data between parentheses are percentage numbers. *Data are the P values of χ^2 square test. NS: not significant.

Table 2
Univariate analysis for prognostic factor

Factor	Total patients N=131	P value	Node-negative patients N=80	P value
T factor				
T1	66.4		78.5	
T2	49.3		57.7	
T3	50		83.3	
T4	0	0.0024	0	0.1538
N factor				
Negative	68.8		–	
Positive	25.8	0.0001	–	–
ly factor				
Negative	69.4		76.8	
Positive	34.6	0.0001	48.8	0.0559
v factor				
Negative	62.9		78	
Positive	38	0.0164	50.2	0.0153
Differentiation				
Well	70.2		79.9	
Moderate	57		55.7	
Poor	42	0.0217	72.7	0.1164
P53 protein				
Negative	59.7		74.1	
Positive	23.9	0.0163	37.5	0.022

Data are percentage of 5-year survival rate using Kaplan–Meier analysis. P values are estimated using logrank test.

washed several times in phosphate buffered saline after each step. Sections were counterstained with Mayer's hematoxylin (Muto Pure Chemicals) before dehydration through graded alcohol solutions and mounting.

2.3. Evaluation of immunohistochemical staining

The entire field of the cancer tissues was examined, and the percentages of positively stained cancer cells were estimated. The degree of staining was divided into positive or negative according to the percentage of cells showing staining of nuclei, among the total number of cells; more or less than 50%, respectively [18].

2.4. Statistical Analysis

Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using the χ^2 test with Fisher's exact correction. Survival curves were drawn according to Kaplan-Meier method. The log-rank test was used to perform univariate survival analysis. Multivariate analysis for survival was performed by the Cox's proportional hazard model. All analysis was performed using Stat View 5.0 software (Abacus Concepts, Berkeley, CA). A significant difference was established when P value of <0.05 was obtained.

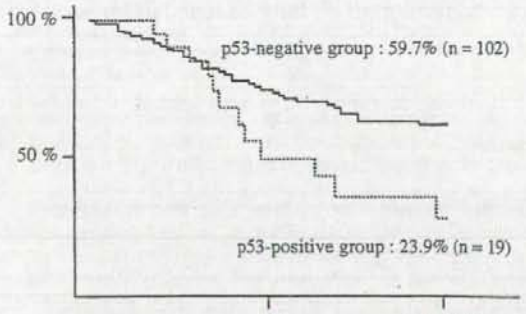
3. Results

3.1. p53 immunohistochemistry

p53 immunohistochemical staining was always localized in nuclei. Patient characteristics according to p53 immunohistochemical staining are shown in Table 1. Nineteen (14.5%) of the 131 patients showed positive immunohistochemical staining in cancer tissues. The expression of p53 protein expression correlates with lymphatic vessel invasion.

3.2. Survival analysis

A five-year survival rate of all over the patients and node-negative patients are 54.8% and 70.9% respectively. Overexpression of p53 protein is one of the prognostic factors in adenocarcinoma besides T factor, nodal involvement, lymphatic vessel invasion, blood vessel invasion and differentiation using



No. of patients at risk

	0M	12M	24M	36M	48M	60M
p53-negative (N=102)	102	29	80	72	66	65
p53-positive (N=19)	19	18	13	10	8	7

Fig. 1. Survival curves for patients with and without p53 protein expression. P value=0.0163