

Differential expression of the α 2,3-sialic acid residues in breast cancer is associated with metastatic potential

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Abstract. Aberrant sialylation is closely associated with the malignant phenotype of cancer cells and metastatic potential. However, the precise nature of the molecules in breast cancers has not been unveiled. In this study, we investigated the expression levels of α 2,3-sialic acid residues of 50 primary tumor cases, 50 pair-matched lymph node metastasis tumor samples and in the MDA-MB-231, T-47D and MCF-7 breast cancer cell lines with different metastatic potential. The expression of α 2,3-sialic acid residues was analyzed by histochemistry, cytochemistry and flow cytometry with *Maackia amurensis* lectin (MAL). The invasion and migration abilities of cells were examined using cell adhesion and transwell *in vitro* assays. Pair-matched lymph node metastasis tumor samples exhibited higher levels of expression of α 2,3-sialic acid residues compared to that of primary tumors ($P=0.0432$). Furthermore, of 38 tumors cases in T1/T2 stages, 31 (81.58%) had weak staining for MAL, which specifically binds to α 2,3-sialic acid residues, whereas of 12 tumor cases in T3/T4 stages, only 1 (8.33%) had weak reactions for MAL. The highly metastatic breast cancer cell line MDA-MB-231 exhibited the strongest binding to MAL and the highest expression levels of α 2,3-sialic acid residues among the selected cell lines, depending on mRNA expression levels of α 2,3-sialyltransferase gene. The adhesion, invasion and migration activities confirmed that MDA-MB-231 exhibited the greater cell adhesion to, migration toward and invasion to Matrigel. Taken together, the high expression of α 2,3-sialic acid residues in breast cancer was associated with metastatic potential. This property may be important for developing new therapeutic approaches for breast cancer.

Introduction

Aberrant sialylation is most ubiquitous in malignant tumor cells. Sialic acids, as terminal monosaccharide were added to protein or lipid moieties, linked to Galb1,3(4)GlcNAc/Glc via α 2,3 or α 2,6 and mediated a variety of pathological process. The altered sialic acid residues are closely associated with the malignant phenotype of cancer cells and metastatic potential (1-4). The correlation between sialic acids content and tumor metastatic potential has been reported in many tumors and has received much attention recently. It has been proven that aberrant expression of terminal sialic acids, and in particular α 2,3-sialic acids are related to tumor adhesion and invasion (5,6). High levels of sialylation of cell surface glycoconjugates could significantly increase metastasis of colon carcinoma cells and human melanoma cells (7-9). Expressions of α 2,3-sialic acid residues of N-cadherin were altered in metastatic melanoma cell lines (10). This possibility is further supported in the experiment where soyasaponin I significantly impaired metastatic ability of breast cancer by decreasing expression of cell surface α 2,3-sialic acids (11). Inhibition expression of α 2,3-sialic acid residues may decrease the migratory ability of highly metastatic B16F10 melanoma cells (12).

To the best of our knowledge, the precise nature of the molecules has not been fully unveiled nor documented to be of clinical relevance, although the elevated expression of sialylation residues in breast cancer has been reported (13,14). In this study, we investigated the expression of α 2,3-sialic acid residues of 50 primary tumor cases, 50 pair-matched lymph node metastasis tumor samples and in three breast cancer cell lines with different metastatic potential (MDA-MB-231, T-47D, and MCF-7) using a specific lectin, and the association of invasion and metastasis was analyzed.

Materials and methods

Tumor samples. Tissue samples of 100 patients who underwent surgery were obtained from tissue array (Shanxi Chaoying Biotechnology Co., Ltd., China). There were 50 primary tumors and 50 pair-matched lymph node metastasis tumors. Age of the patients ranged from 28 to 80 years with a mean of 50.4 years. Depth of invasion, 10 tumors were staged T1, 28 as T2, 8 as T3, and 4 as T4, based on International Union Against Cancer (UICC) TNM staging system (15).

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MAL histochemistry. Paraffin-embedded specimens were dewaxed and repaired for 10 min at 121°C. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide/methanol at room temperature. The sections were incubated for 20 min in goat serum to block non-specific binding and then incubated with 20 μ g/ml biotinylated MAL (Vector Laboratories, Inc., USA) at 4°C overnight. The bound biotinylated MAL was detected by biotin-streptavidin-peroxidase (Beijing Zhongshan Biotechnology Co., Ltd.). 3,3'-diaminobenzidine (DAB) was used as chromogen and haematoxylin was used for nucleus counterstain. One thousand cells of each section were randomly selected and counted blindly. The positive percentage of counted cells was semi-quantitated according to a 2-tier scoring system: weakly positive, 1-40%; and strongly positive, 41-100% (16). The slides were photographed under a Nikon Eclipse TE2000-S Inverted microscope (magnification, \times 200).

Cell lines and cell culture. Human breast cancer cells lines, MCF-7, T-47D and MDA-MB-231, were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). MCF-7 and T-47D were cultured in DMEM medium with high glucose. MDA-MB-231 cells were cultured in L15 medium (Gibco™ Invitrogen Corp., USA). Medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin (100 IU/ml-100 μ g/ml). Cells were fed every 3 days at 37°C in a humid atmosphere 5% CO₂) and harvested by 0.25% trypsin.

Cytochemical staining. Cells were grown on glass coverslips, after achieving confluence, they were treated with or without neuraminidase (Sigma) for 1 h at 37°C, coverslips were washed three times with PBS and fixed with 95% alcohol at 4°C for 20 min. The cells labeling with biotinylated MAL (10 μ g/ml) were performed as detailed above. One hundred cells were randomly selected and photographed from representative fields of each coverslip, cells were evaluated as previously described.

Flow cytometric analysis. To detect sialylation structures of cell surface glycoproteins and glycolipids, we used FITC-conjugated MAL (Vector Laboratories, Inc.), which is specific for binding to α 2,3-sialic acid residues. Cells were detached with 0.25% trypsin-EDTA solution, then washed three times with PBS, adjusted cells density 3×10^6 /ml, stained with 10 μ g/ml FITC-MAL in PBS (contain 0.5% BSA and 0.05% sodium azide) at 4°C for 1 h, then washed three times with PBS. The fluorescence intensity of the stained cells was measured with a FACScan flow cytometer and analyzed with CellQuest (BD, Bioscience, USA).

RT-PCR. Sialic acids, as terminal sugars were added by sialyltransferase. Reverse transcriptase polymerase chain reaction for studying mRNA level expression of α 2,3-sialyltransferase (α 2,3-ST) gene in breast cancer cell lines. Cellular total RNA was extracted using TRIzol (Invitrogen). Subsequently, RNAs were reverse-transcribed using RNA Miniprep Kit (Takara Biotechnology Co., Ltd.). PCR amplification reaction was conducted with the following primers: α 2,3-ST forward, 5'-CGC AAA CTT TTC AGA GGG AG-3'; reverse, 5'-AAG

AGA GAA TCG CGC TCG TA-3'. The β -actin forward, 5'-GTG GAC ATC CGC AAA GAC-3'; reverse, 5'-GAA AGG GTG TAA CGC AAC T-3'. PCR products were run on 1.0% agarose gel and the intensity of each band was quantified using Scion Image software (Scion, Frederick, MD). Results for each detected band intensity were normalized to β -actin band intensity values.

Cell-Matrigel adhesion assay. The 96-well plates were precoated with 25 μ g/well of Matrigel basement membrane extract (BD Bioscience, USA). The 1×10^5 cells (control or neuraminidase-treated) were labeled by Chloromethyl-benzamidodialkylcarbo-cyanine (CM-Dil) 3 μ g/ml for 2 h at 37°C. They were seeded into the precoated wells and incubated for 30 min at 37°C in 5% CO₂ incubator. The cells were washed twice with PBS gently to remove unattached cells, the numbers of cell attached to the Matrigel were counted in five randomly selected microscopic fields per well and photographed under a fluorescence microscope. Independent experiments were performed at least three times.

In vitro migration and invasion assay. Tumor cell migration and invasion were measured using 24-well transwell chamber (Costar Corp., USA), which consists of upper and lower chambers that were separated by Millipore membranes with 8- μ m pore sizes. Cells able to move from the top surface (or precoated with Matrigel) to the under surface of the membrane were considered migration (or invasion). In brief, MCF-7, T-47D, and MDA-MB-231 cells (2×10^5 or 2.5×10^4 for invasion) were added to the upper chamber in serum-free medium, the lower compartment was filled with media supplemented with 10% FBS. After incubation at 37°C for 24 h, the non-migrating cells on the upper surface of the filter were removed with cotton swabs, and the migrating cells on the lower surface were fixed with methanol for 30 min. After drying, the fixed cells were stained with 0.1% crystal violet for 30 min, then washed three times with PBS, photographed under a fluorescence microscope and incorporated dye was dissolved in 10% acetic acid. The optical densities of each well were measured by ELISA reader at 570 nm. Experiments were performed in triplicate.

Statistical analysis. The data were expressed as the mean \pm SD and analyzed by SPSS 13.0 statistical software to evaluate the statistical difference. P-value <0.05 was considered statistically significant. The relationship between the expression of α 2,3-sialic acid residues and patient's clinical characteristics was analyzed by the χ^2 -test. One-way ANOVA analysis was used to evaluate the results of cell adhesion assays and transwell chamber assay.

Results

Expression of α 2,3-sialic acid residues in breast cancer samples correlated with clinicopathological characteristics. The expressions of α 2,3-sialic acid residues were observed by using MAL staining. The result showed positive MAL binding reaction in breast cancer regions, whereas there was negative staining in the stromal parts (Fig. 1A). Pair-matched lymph node metastasis tumors exhibited more frequent and

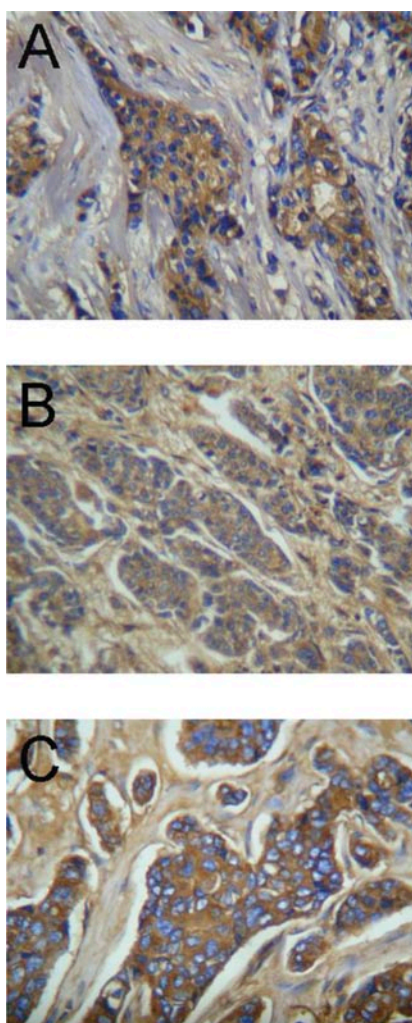


Figure 1. Histochemical staining with MAL in breast cancer tissues. (A) MAL staining is the positive reaction in breast cancer regions, whereas negative MAL staining was observed in the stromal part. (B and C) MAL staining in primary cancer (B) and pair-matched lymph node metastasis (C), the latter exhibited more intense positive reaction (magnification, $\times 200$).

stronger expression of $\alpha 2,3$ -sialic acid residues than those in primary tumors (Fig. 1B). Furthermore, of 38 tumor cases in T1/T2 stages, 31 (81.58%) had weak staining for MAL, whereas of 12 tumors cases in T3/T4 stages, only 1 (8.33%) had weak reactions for MAL. High expression of $\alpha 2,3$ -sialic acid residues was closely associated with tumor invasive depth ($P < 0.05$) and lymphatic metastasis. However, the relationship between intensity of $\alpha 2,3$ -sialic acid residues and age was not observed.

Different expression of $\alpha 2,3$ -sialic acid residues in breast cancer cell lines. The expression of $\alpha 2,3$ -sialic acid residues was examined in three human breast cancer cell lines with different metastatic potential, MDA-MB-231, T-47D, and MCF-7. As shown in Fig. 2, MAL staining was on cell membrane or in cell cytoplasm. The highly metastatic breast cancer cell line MDA-MB-231 shows the most intensive staining compared with T-47D and MCF-7 (Fig. 2A-C). All the binding reactions were inhibited by neuraminidase treatment, indicating that the staining of cells was due to

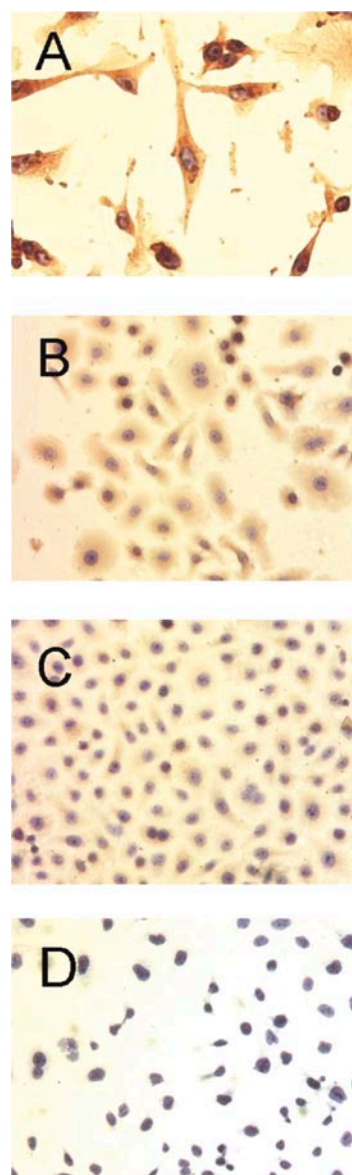


Figure 2. Cytochemical analyses of MAL staining in breast cancer cells. MDA-MB-231 (A) shows the most intense staining compared with T-47D (B) and MCF-7 (C). (D) MAL reaction was inhibited by pre-treatment with neuraminidase (magnification, $\times 200$).

the interaction between $\alpha 2,3$ -sialic acid residues and MAL (Fig. 2D).

Furthermore, flow cytometric analysis was performed to study $\alpha 2,3$ -sialic acid contents on cell surface by using FITC-MAL. A typical image is shown in Fig. 3. Results from three independent experiments demonstrated that the mean fluorescence intensities of MAL-labeling cells of MDA-MB-231, T-47D, and MCF-7 were 1091.38 ± 10.78 , 733.80 ± 9.47 and 434.58 ± 10.19 , respectively. Significant differences were seen across cell lines (MDA-MB-231 to T-47D, $P < 0.01$; MDA-MB-231 to MCF-7, $P < 0.01$). Those results suggest that high level of $\alpha 2,3$ -sialic acid residues was expressed in the highly metastatic breast cell lines.

In breast cancer, the $\alpha 2,3$ -sialyltransferase ($\alpha 2,3$ -ST), is mainly responsible for catalyzing sialic acids to form sialylated residues. We analyzed the mRNA levels of $\alpha 2,3$ -ST by

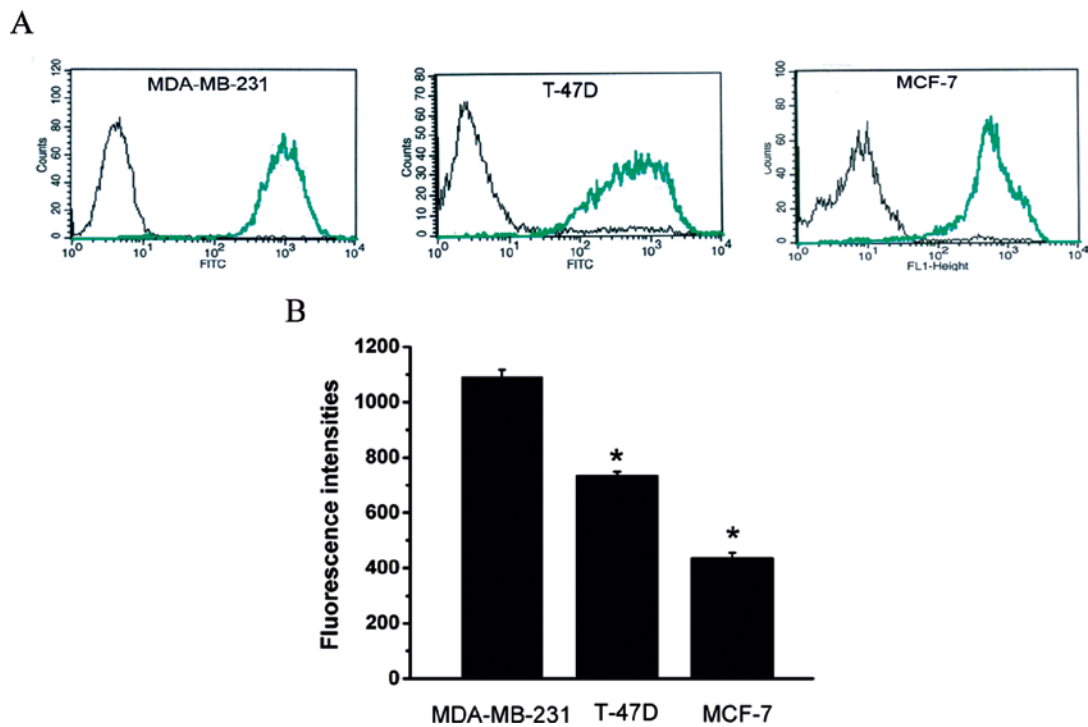


Figure 3. Flow cytometric analysis α 2,3-sialic acid residues with FITC-MAL. Cells were incubated with FITC-MAL at 4°C for 1 h. The fluorescence intensities of FITC-MAL labeled cells were determined. (A) the expression of α 2,3-sialic acid residues on the surface of MDA-MB-231 was markedly increased compared with that in T-47D and MCF-7 (green line). (B) Values of fluorescence intensity are shown as the mean \pm SD.

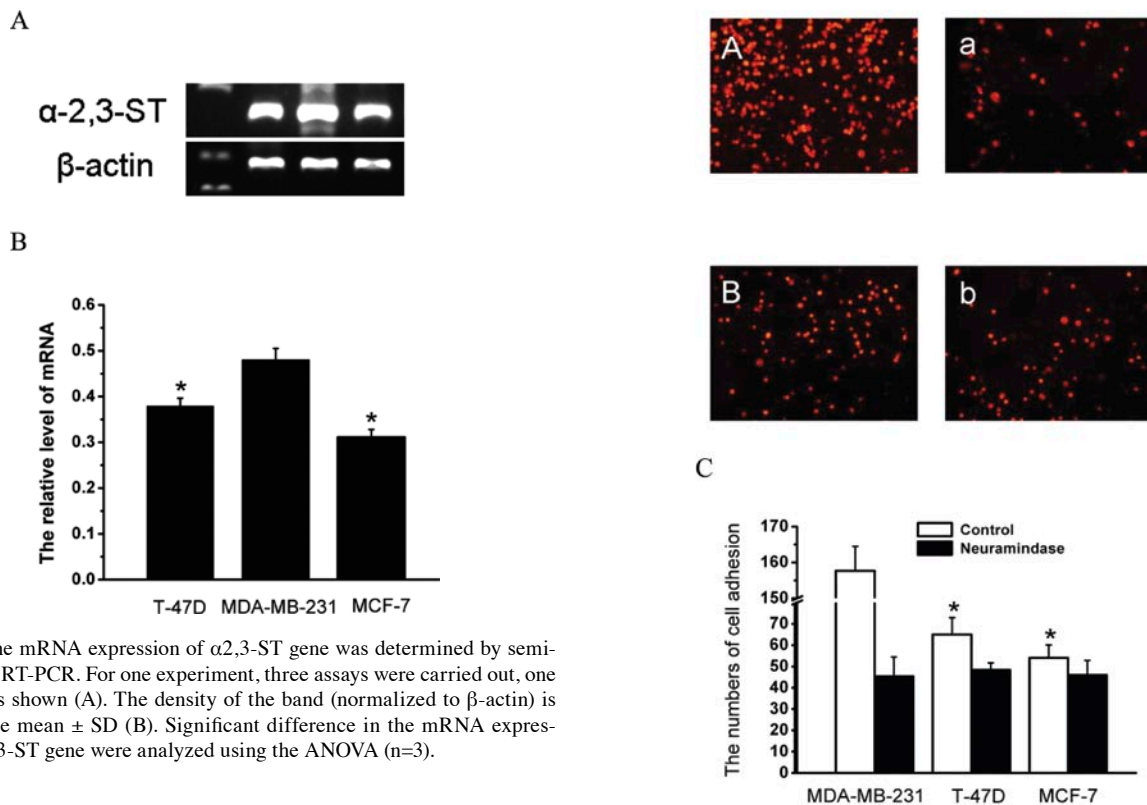


Figure 4. The mRNA expression of α 2,3-ST gene was determined by semi-quantitative RT-PCR. For one experiment, three assays were carried out, one set of gels is shown (A). The density of the band (normalized to β -actin) is shown as the mean \pm SD (B). Significant difference in the mRNA expressions of α 2,3-ST gene were analyzed using the ANOVA (n=3).

semi-quantitative RT-PCR in breast cancer cell lines. The relative mRNA level of MDA-MB-231, T-47D, and MCF-7 was 0.48 ± 0.027 , 0.3791 ± 0.017 , 0.311 ± 0.018 , respectively (Fig. 4). Significant differences were seen across cell lines (MDA-MB-231 to T-47D, $P < 0.01$; MDA-MB-231 to MCF-7, $P < 0.01$).

Figure 5. Cell adhesion assay. Cells were stained with Dil at 37°C for 2 h before adding to the 96-well culture plate pre-coated with Matrigel. The adhesive cells were counted and photographed in five random fields. MDA-MB-231 (A) had increased adhesion ability compared with T-47D (B). After neuraminidase treatment, a significant adhesion reduction was observed from MDA-MB-231 cells (a). However, the number of adhered T-47D cells did not show a significant change (b). (C) The cell adhesion is shown as the mean \pm SD.

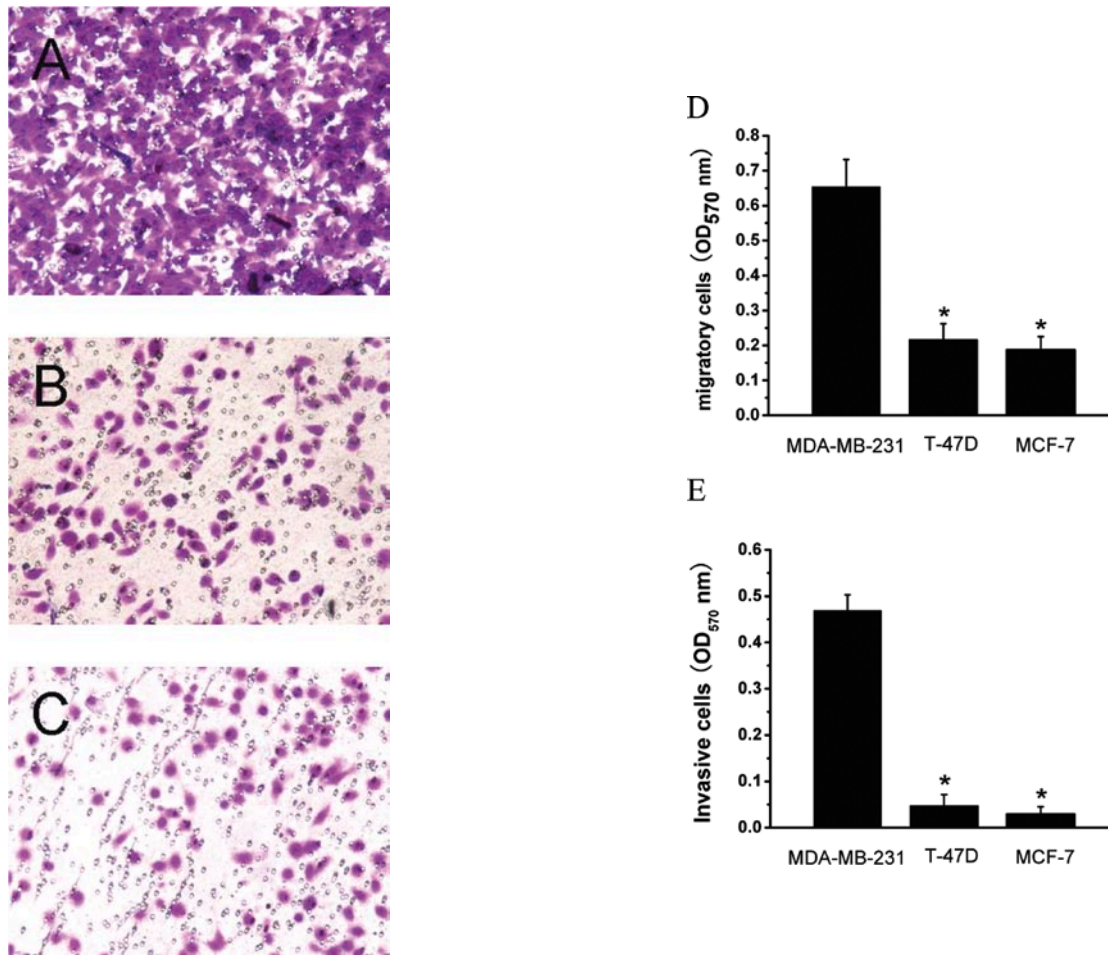


Figure 6. Cell migration and invasion were examined using a transwell chamber. The images show the crystal violet-stained breast cancer cells migrating through transwell chamber, MDA-MB-231 (A) displayed increased migratory potential compared with T-47D (B) and MCF-7 (C). (D and E) The optical densities of migration and invasion were read at 570 nm and are shown as the mean \pm SD. Statistical analysis was done using the ANOVA.

Cell adhesion to ECM relation to α 2,3-sialic acid residues.

The ability of adhesion to ECM was examined in different cell lines. As shown in Fig. 5, obvious differences in the cell number adhered to Matrigel were observed among cell lines. The mean number of adhesive cells of MDA-MB-231, T-47D, and MCF-7 was 157.66 ± 6.79 , 65 ± 7.87 , 54 ± 6.16 , respectively. Significant differences were seen among cell lines (MDA-MB-231 to T-47D, $P < 0.001$; MDA-MB-231 to MCF-7, $P < 0.001$; T-47D to MCF-7, $P > 0.05$).

After treatment with neuraminidase, the number of adherent MCF-7 and T-47D cells did not show a significant change. However, a significant adhesion reduction was observed in MDA-MB-231 cells ($73.55 \pm 9.13\%$ inhibition, $P < 0.001$).

High levels of α 2,3-sialic acid residue association with promotion of cell invasion and migration.

The abilities of cancer cell migration and invasion play important roles in evaluating tumor metastasis. The invasion and migration of breast cancer cell lines were measured using the transwell chamber. As shown in Fig. 6, the optical density of migration and invasion of MDA-MB-231 was 0.654 ± 0.078 , 0.469 ± 0.034 , while T-47D and MCF-7 were 0.216 ± 0.046 , 0.047 ± 0.024 and 0.187 ± 0.038 , 0.029 ± 0.016 , respectively. Significant differences

were seen in the different metastatic potential breast cell lines (MDA-MB-231 to T47D, $P < 0.01$; MDA-MB-231 to MCF-7, $P < 0.01$; T-47D to MCF-7, $P > 0.05$).

Discussion

Aberrant sialylation is closely associated with the malignant phenotype of cancer cells and metastatic potential. The content of sialic acid have been elevated on some malignant tumor cells, a positive correlation between cell metastatic potential and total sialic acid content has been reported in many types of tumors. Most of these studies suggested that high level of sialylation may contribute to invasion and metastasis (17-19). The tumor dissemination is a multistep process involving aberrant biological behavior of tumor cells, such as adhesion, migration and invasion. So far, few studies on the relationship between the expression of α 2,3-sialic acid residues in breast cancer and invasion and metastasis have been reported. Hence, we examined the expression of α 2,3-sialic acid residues in breast cancer using MAL staining and the significance of metastatic potential were analyzed. The results suggest that high levels of α 2,3-sialic acid residues was closely associated with lymph node metastasis and invasive depth in breast cancer patients. Then we measured the

expression of α 2,3-sialic acid residues in three human breast cancer cell lines with different metastatic potential and the abilities of invasion and migration were analyzed. A positive correlation between the levels of α 2,3-sialic acid and ability of invasion and migration was observed (Figs. 2-6).

Many studies suggested that an increased cell surface sialic acid content in cancer cells is caused by up-regulating of sialyltransferase (20-24) and depended on the mRNA levels of sialyltransferase gene (25). In breast cancer, the sialyltransferase (α 2,3-ST), is mainly responsible for catalyzing sialic acid to form α 2,3-sialic acid residues (26). In this study, we analyzed the mRNA level of α 2,3-ST gene in breast cancer cell lines. The results indicated that the mRNA level of α 2,3-ST is higher in MDA-MB-231 than that in T-47D and MCF-7. Similarly, the α 2,3-sialic acid residues on cell surface, the products of α 2,3-ST is highest in MDA-MB-231 by flow cytometric analysis among the selected cell lines. The highly metastatic breast cancer cell line MDA-MB-231 had higher expression of α 2,3-sialic acid residues compared to T-47D and MCF-7 depending on the mRNA levels of α 2,3-ST genes. The MAL (lectin from *Maackia amurensis*) specifically binding the sialyl α 2,3Gal β 1,4GlcNAc/Glc trisaccharide sequence (27). Furthermore, it can bind to certain sialylation residues that carry out various functions such as cell adhesion, invasion and migration. MAL, however, did not react with neuraminidase treatment. Neuraminidase cleaves terminal sialic acid residues on cell surface and changes glycan chain structures. In our study, positive MAL staining was detected in breast cancer tissue, while staining was negative in the stromal part (Fig. 1A). MAL staining was on cell membrane or in cell cytoplasm (Fig. 2A-C). All the binding reactions were inhibited by neuraminidase treatment (Fig. 2D). These proved that the staining of cells was due to the interaction between α 2,3-sialic acid residues and MAL.

Cell migration is an essential process in metastasis. The extracellular matrix (ECM) serves as the molecular scaffold for cell adhesion and migration. In the first phase of migration, adhesion of cells to the ECM is critical. Matrigel is reconstituted basement membrane has been proved useful in the study of the molecular mechanisms of basement membrane invasion. An increasing amount of evidence proved that cell-surface carbohydrates promote the emergence of migration (28). However, the nature of the molecular have not been fully elucidated. In the present study, MDA-MB-231 with high levels of α 2,3-sialic acid residues, greater adhesion to Matrigel and the most number of cells migrated and invasion were observed. Effects of adhesion to Matrigel was significantly reduced by neuraminidase treatment, the high level of α 2,3-sialic acid residues in MDA-MB-231 were largely inhibited (73.55 \pm 9.13% inhibition) relative to that of MCF-7 (14.81 \pm 6.76%) and T-47D (25.64 \pm 3.29%). Similar results were observed in migration and invasion assay *in vitro*. Thus implying that α 2,3-sialic acid residues in human breast cancer play a pivotal role in cell adhesion, migration and invasion. We infer that the α 2,3-sialic acid residues in human breast cancer are involved in the metastasis process.

In addition to directly modulating cell motility, α 2,3-sialic acid residues are involved in the synthesis of sialyl Lewis X determinants, which are the major ligands for endothelial

E-selectin (24). The sialyl Lewis X structure on malignant cells is suggested to facilitate tumor cell dissemination by mediating the tumor-endothelial cell interactions (29,30).

On the other hand, integrins represent a particularly important adhesion receptors that mediate attachment to ECM protein ligands (31,32). Hypersialylation of β 1 integrins can up-regulate cell motility in colon cancer. This increased negative charged properties of sialic acids was correlated with reduced adhesiveness of tumor cells and may be suitable for conformational change of integrin and enhances its function in cell-ECM interactions (33). Similar studies have also indicated that increased sialylation could activate β 1 integrin, mediate its adhesion to ECM proteins (34) and stimulating cell migration through host ECM (35). Most of these studies and our finding supported the hypothesis that increase of sialylation of cancer cells play an important role in tumor metastasis.

In conclusion, our studies suggested that different expression of α 2,3-sialic acid residues was associated with promoting adhesion, migration, and invasion *in vitro*, possibly contributing to breast cancer metastasis. This property may be meaningful to breast cancer patients in seeking novel target for therapeutic approaches.

Acknowledgements

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