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Breast cancer detection in mice by *Helix pomatia* agglutinin lectin

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Abstract

All types of cancer cells possess aberrant glycosylation and different structures of oligosaccharides on surface membrane. To diagnose breast cancer, detection of glycan structure specific carbohydrate binding proteins, such as lectins, are one of the useful methods. Lectins are proteins that recognize and bind to specific carbohydrates. Lectins are found on the surfaces of cancer cell membranes. This research aimed to use the lectin *Helix pomatia* agglutinin (HPA) conjugated AlexaFlour647 (HPA-AlexaFlour647) to study its binding and follow up breast cancer treatment. *In vitro* model, the human normal breast Hs578Bst cell line and the human breast cancer MCF7 cell line were incubated with lectin HPA-AlexaFlour647 and then evaluated the specific binding by fluorescence imaging and flow cytometry. *In vivo* model, the mice were imaged at 1, 3, 6 and 24 h post injection with HPA-AlexaFlour647 by optical tomography. The light intensities were calculated with the MATLAB program. Furthermore, tumor tissues were removed for histological analysis. As the result of *in vitro* model, HPA-AlexaFlour647 on glycan structures were detected on Hs578Bst cells lower level than the MCF7 cells. HPA-AlexaFlour647 positive cells were 99% and 40% in MCF7 and Hs578Bst cells, respectively. In the mice model, the maximum intensity was highest at 1 h incubation time and then was decreased at 3 and 6 h. This finding revealed that HPA-AlexaFlour647 protein could be a candidate protein for monitor and imaging for breast cancer treatment in both *in vitro* and *in vivo* model.

Introduction

Breast cancer is the most common incident form of cancer in women around the world including Thailand [1, 2]. Screening and diagnosis of early stage breast cancer is very important. Patients who come to physicians in the early stages of the cancer and who have been treated with the standard protocol have an extremely good chance of being cured of breast cancer [3]. Glycosylation is one of the most commonpost-translational modification of proteins which plays an important role in the biological process [4]. All types of human cancer exhibit aberrant glycosylation and different structures of oligosaccharides on cell surface membrane [5]. Lectin is a glycoprotein that highly specific to carbohydrates on cell surfacemembrane. Lectin is found in many organisms. Lectin in plants such as *Ricinus communis* agglutinin-I (RCA-I) is found in castor bean and peanut agglutinin (PNA) is found in peanuts. Lectin in fungi such as *Aleuria aurantia* lectin (AAL) is found in the mushroom. Lectin in animals such

as *Helix pomatia* agglutinin (HPA) is found in snails [6]. Some lectins are used to examine the structure of carbohydrates and they are used as tools to identify malignant from benign tumor and to study cancer-related glycosylation changes [7, 8]. There is used lectins from plant for cancer researchsuch as Sakuma *et al*. used peanut agglutinin immobilized with fluorescent nanospheres to detection early colorectal cancer [9]. Soga *et al*. have studied lectin in legumes from *Wisteria japonica*in human lung cancer cells [10]. Zhou *et al*. have studied *Ricinus communis* agglutinin-I to assesse glycan on breast cancer cell surface [11]. In addition, there is used lectin from animal for cancer research such as *Helix pomatia* agglutinin (HPA) is a lectin which binds specific to *N*-acetylgalactosamine (GalNAc) and is found in the albumen gland of the roman snail [12]. HPA has been as interesting marker for studying the changed glycosylation in cancer. Binding of HPA to cancer cell membrane is associated with metastatic and poor prognosis in various human cancers [13]. HPA has been reported as the valuable prognostic indicator in breast cancer, gastric cancer, colorectal cancer, thyroid cancer, and other cancers [14-17]. HPA has been applied in various cancer studies. Based on the previously reported, this study focuses on using HPA to detect or bind to glycan structures on breast cancer cell membrane in both cell line and mice model. This may lead to the HPA application for breast cancer diagnosis and follow up the cancer treatment.

Methodology

Cell lines and culture conditions

In this study, two types of breast epithelial cell lines; human breast normal cell line Hs578Bst and human breast adenocarcinoma cell line MCF7were used as normal and cancer cell line models, respectively. Hs578Bst and MCF7cell lines were purchased from the American Type Culture Collection (ATCC, USA). The Hs578Bst cells were cultured in Hybri-Care medium (ATCC, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 30 ng/mL mouse epidermal growth factor (mouse EGF) (ThermoFisher, USA).The MCF7cells were cultured in RPMI 1640 medium (Gibco,USA) supplemented with 10% heat-inactivated FBS, 100 units/mLpenicillin and 100 µg/mLstreptomycin (Gibco,USA). The cells were seeded either in 25 or 75 cm^2 flasks and incubated at 37^oC, in a humidified incubator with 5 % CO_2.

In vitro binding studies under fluorescence microscope

Cells were seeded into 6 well plates and maintained as above condition for 24 h. Cells were washed with phosphate buffer saline (PBS), fixed in 4% formaldehyde for 15 min, washed and incubated in 0.1% Triton X-100 for 15 min. After that the cells were washed with PBS and incubated in 1% bovine serum albumin (BSA) in PBS for 45 min at room temperature and incubated with 10µg/mL HPA-AlexaFlour647 (Invitrogen,USA) in dark, at 4^oC, overnight. In order to investigate the cell localization,the cells were washed with PBS and nuclei were labelled by briefly incubation with 0.1µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA). Confirmation of specificity of labelling were performed by taking the photographs under fluorescence microscope (ECLIPSE Ts2, Nikon).

Flow cytometry

The $5x10^5$ cells were washed in PBS and then incubated in 1% BSA in PBS for 15 min at room temperature and suspended in 3µg/mL HPA-AlexaFlour647 (Invitrogen, USA) in dark, at 4^oC, for 30 min and then washed with PBS. Unlabeled cells were included as a control group to assess levels of auto-fluorescence.Flow cytometry was performed using a CyAn ADP (Beckman Coulter, USA).

Animal model

Female BALB/cAJcl-nu/nu mice ages between 6 and 8 weeks at the beginning of the experiment were used (n=2). The mice were kept in filter-top cages and fed with sterile water and food *ad libitum*. All animal studies were approved byAnimal Care and Use Committee, Chiang Mai University (2560/MC-0001). All mice manipulations were performed under aseptic conditions in a laminar flow hood.

In vivo fluorescence imaging study in mice model

MCF7 cells suspension ($2x10^6$/200 µLPBS) was injected subcutaneously into the hips of nude mice for tumor development. When the tumors reached average size of 150-200 mm^3, the fluorescence imaging study was determined by optical tomography. HPA-AlexaFlour647 (final dose 500 µg/kg) was injected intravenously in tail vein. The fluorescence imaging in mice were observed at 1, 3, 6 and 24 h post injection and then sacrificed for histological analysis.

Pathological analysis

After the fluorescence imaging experiment, the tumors were removed and fixed in neutral-buffered formalin. Paraffin-embedded tumors were sectioned at 3-µm thicknessand stained with hematoxylin and eosin (H&E) to visualize tumor [18].

ResultsandDiscussion

Cellular localization of HPA biding in cell line model

Human breast cell lines were used as the *in vitro* model to study the specificity of HPA binding to glycan structures on normal breast cell (Hs578Bst) and breast cancer cell (MCF7) surface membrane. Fluorescence microscopy facilitated detail analysis of HPA binding. Nucleus was stained with DAPI (blue) and location of cell membrane was stained with HPA-AlexaFlour647 (red). As the result, HPA-AlexaFlour647 bound intensely in the MCF7 cell surface membrane while very low HPA binding was detected in the Hs578Bst cells (Figure 1). The results were similar to Brooks *et al*. which studied the expression of GalNac in breast cell lines. Seven breast epithelial cell lines such as HMT 3522, BT 474, MDA MB 435, MDA MB 468, ZR 75 1, MCF7 and DU 4475were labeled with HPA-FITC and were analyzed by confocal microscopy and flow cytometry. The labelling of HPA-FITCwas bound strongly in MCF7 and DU 4475 cell lines[19]. In addition, it was reported that the Cy5-labeled HPA in human colon cancer (HT-29), human non-small cell lung cancer (A549, H322, H358 and H1299), human ovarian cancer (IGROV1), murine breast cancer (B16F10 and TSA/pc), and human osteosarcoma (SAOS-2) cell linesand were detected by confocal microscopy. It was found that the strongest labelling was observed for colon and breast cancer cell lines [20].

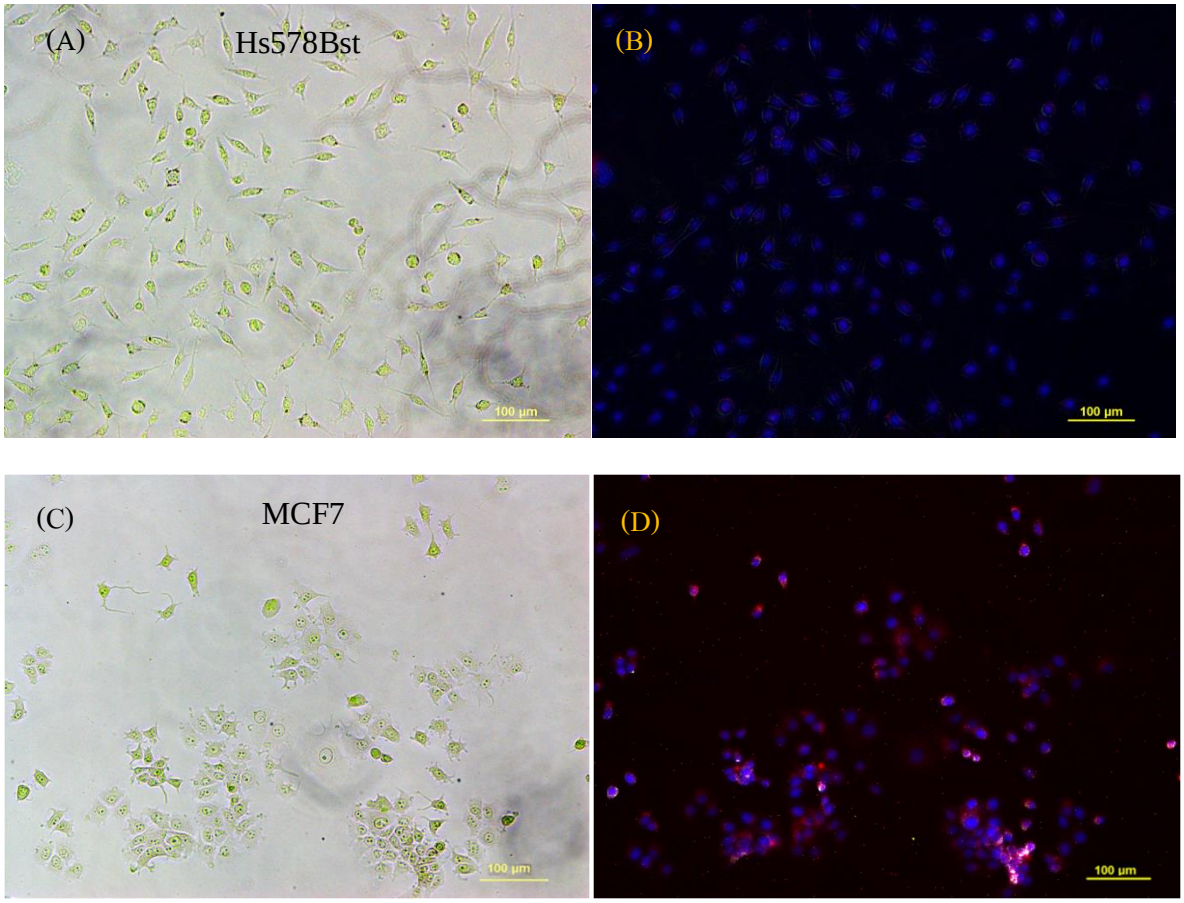

Figure1. Fluorescence images of the binding of HPA-AlexaFlour647 in normal breast cell line (Hs578Bst) and breast cancer cell line (MCF7). (A) Bright field image of Hs578Bst cells. (B) Merged image of DAPI and HPA-AlexaFlour647 in Hs578Bst cells. (C) Bright field image of MCF7 cells. (D) Merged image of DAPI and HPA-AlexaFlour647 in MCF7 cells.

Analysis of cell surface HPA-AlexaFlour647 labelling by flow cytometry

Flow cytometry was used to investigate the HPA-AlexaFlour647 binding to glycan in both normal breast cell lines and breast cancer cell line (Figure 2). The results showed the higher percentage binding (99.43 ± 0.21%)in breast cancer cell line, when compared tonormal breast cell line(40.16± 4.8%).Breast cancer cells were labelledgreater than normal cells about 2-fold. Flow cytometry also confirmed the level of cell surface labelling with HPA consistent with fluorescence microscope.

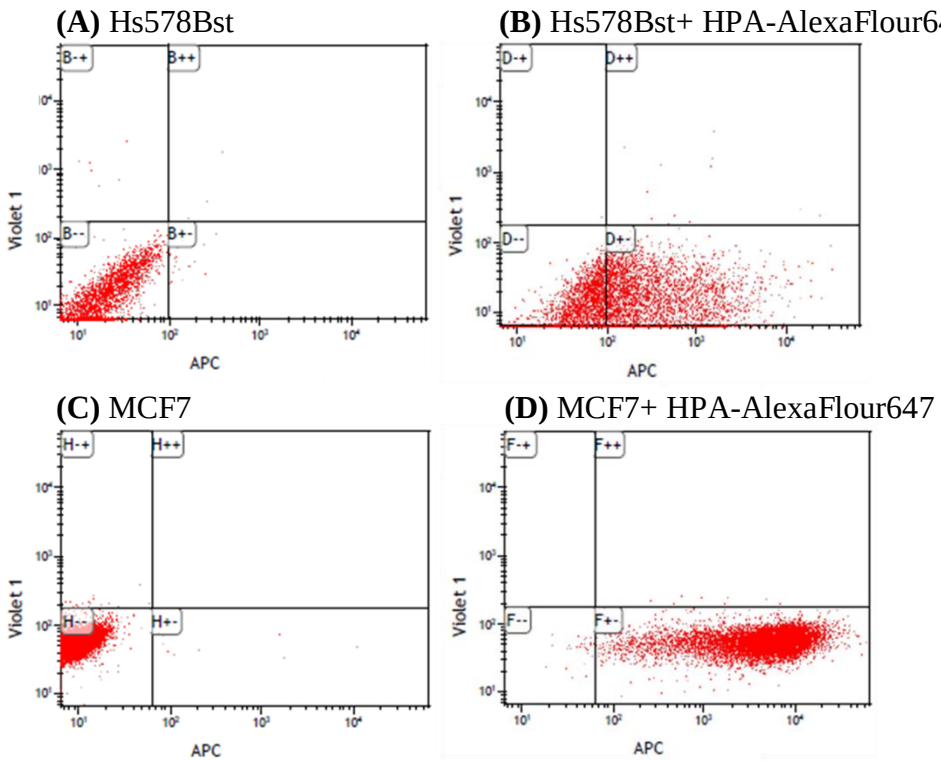

Figure 2. Flow cytometric analysis of HPA positive in human breast cell lines. (A) Unstained Hs578Bst cells. (B) HPA-AlexaFlour647 stained Hs578Bst cells. The population in quadrant 4 (D+ –). (C) Unstained MCF7 cells. (D) HPA-AlexaFlour647 stained MCF7 cells. The population in quadrant 4 (F+ –).

Table 1. The summary of HPA-binding of cell lines using flow cytometry.

Cell line	Flow cytometry (cell surface labelling)
	% cells
human breast normal cell line (Hs578Bst)	99.43 ± 0.21%
human breast adenocarcinoma cell line (MCF 7)	40.16 ± 4.80%

In vivo fluorescence imaging

A sedated mouse was placed with the animal holder for fluorescence imaging (Figure 3).The mice were imaged by optical tomography beforeand after injection with HPA-AlexaFlour647. The area of each scanning was determined by the size of the cancer lesion in each mouse. The cross-sectional image was the back-projection image from scanning where the bright spot was tumor area of HPA-AlexaFlour647 binding. *In vivo*HPA-AlexaFlour647 was detected in the tumor area as evidenced by changing of fluorescence signals at four different time points (Figure 4). The regions of interest (ROI) was drawn in each slice of each time point image. The light intensity in each ROI was calculated with MATLAB program. The fluorescence signal could be detected at 1 h with highest light intensity and then the signals were decreased at 3, 6 and 24 h incubation time, respectively (Figure 5). Currently, molecular functional imaging was clinically used in nuclear medicine by SPECT or PET. However, the disadvantage was the use of radiopharmaceuticals. Therefore, there were radioactive waste, radiation exposure to patients and medical personnel. Radiation safety was the primary concern. Molecular functional imaging, based on the differences feature between normal and tumor but observed by optical system were alternative technique which was available. This technique was useful for patients and medical personnel to screening and diagnosis cancer in future.

Figure3. The animal holder

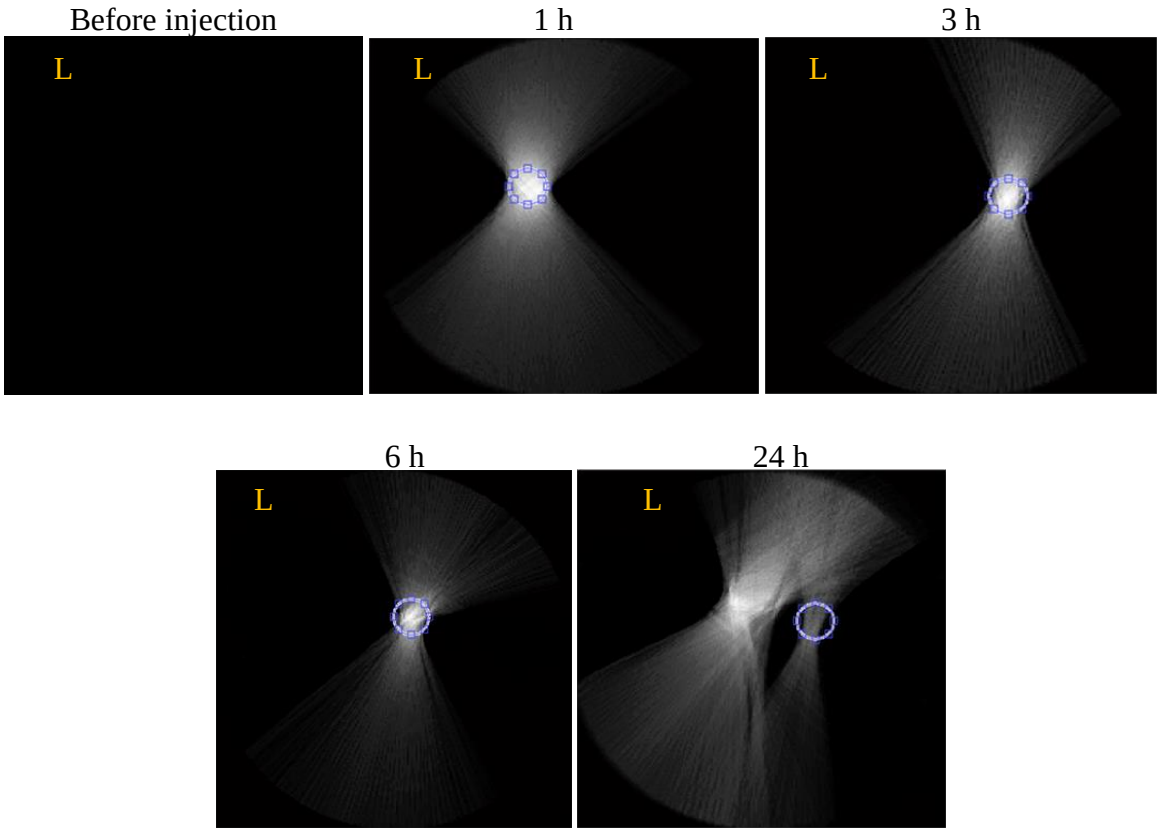

Figure 4. Optical tomographic images before HPA-AlexaFlour647 injection and at incubation time1, 3, 6, 24 h after the HPA-AlexaFlour647 injection.

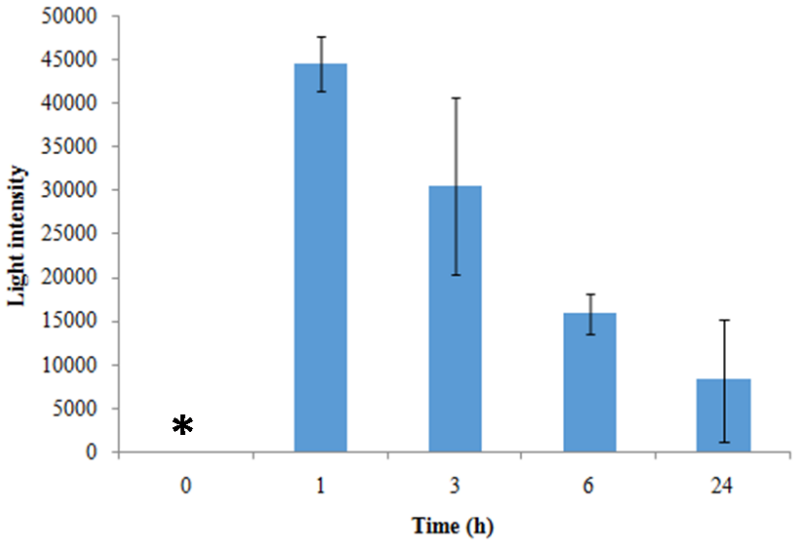

Figure 5. The light intensity of the tumors at the different time points after the HPA-AlexaFlour647 were injection.Before the HPA-AlexaFlour647 injection (star mark) showed no light intensity.

Assessment of pathological analysis

After the fluorescence imaging experiment, the mice were sacrificed and the tumors were removed, fixed in neutral-buffered formalin for histological analysis. Paraffin-embedded tumors were sectioned at 3 µm thickness. After the tumor samples were stained with hematoxylin and eosin, the tumor samples were assessed under microscope. Figure 6 showed the tumor sample slice from mice stained with hematoxylin and eosin. Hematoxylin and eosin stains were very important dyesfor recognize tissue types and the morphologic changes that formed the basis of cancer diagnosis [21]. Hematoxylin has a deep blue-purple color and stains nucleus. Eosin is pink and stains cytoplasm, contrasting colors to easily differentiate cellular components. Normal tissues have large regions of cytoplasm (pink regions) and small nucleus.Malignant tissues have a large nucleus and irregular shapes (purple regions) [22]. The pathological examination confirmed the cancerous of biopsy tissue which corresponded to the recorded image from optical tomography.

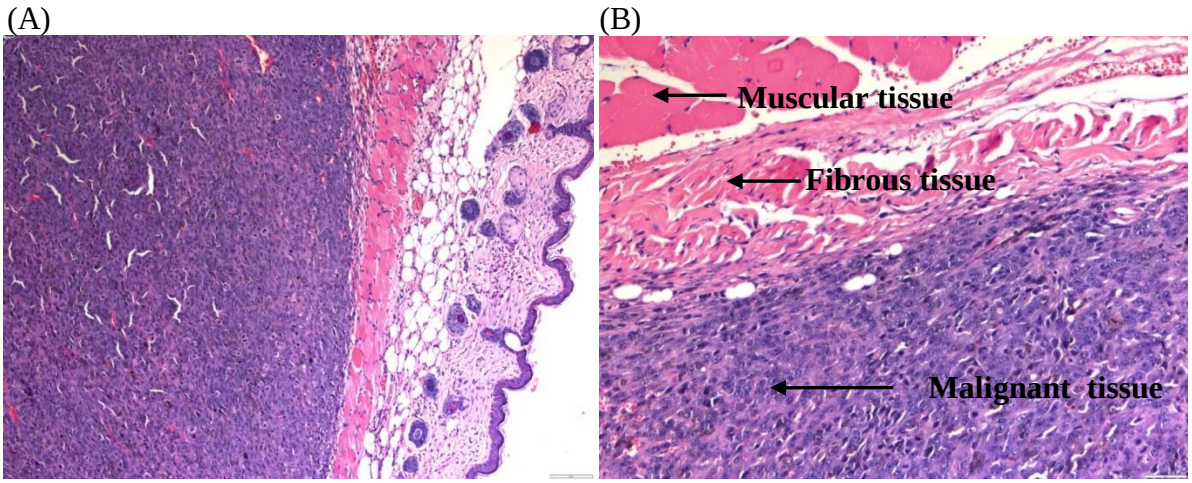

Figure 6. Images oftissuefrom micestained withhematoxylin and eosin. With 10X (A) and 20X (B) magnification.

According to results of this research, HPA-AlexaFlour647 could be used as an indicator for screening patients and diagnosisearly breast cancer. In addition, this could be applied to screen and diagnosis other cancers in the future.

Conclusion

In vitro study, HPA could bind to oligosaccharide structureson cancer cell surface membrane greater than normal breast cells. For the study in mice model, the appropriate time for recording image was 1 h after HPA-AlexaFlour647 injection and the localization of the tumorcould be indicated. The results of biopsy confirmed that the cancer actually corresponded to the fluorescence images. In this finding, HPA-AlexaFlour647 may be another alternative technique for screening and imaging for breast cancer in diagnosis and treatment.

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