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N-acetylgalactosamine-6-sulfatase (GALNS), Similar to Glycodelin, Is a Potential General Biomarker for Multiple Malignancies

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Abstract. *Background/Aim: The aim of this study was to evaluate N-acetylgalactosamine-6-sulfatase (GALNS) as a new biomarker candidate for detecting lung cancer. Glycodelin or PAEP, the serum levels of which are known to be elevated in lung and other cancers, served as a benchmark for comparison. Patients and Methods: A total of 170 serum samples from healthy controls and patients with pneumonia, lung cancer, breast cancer, colon cancer, liver cancer, and head and neck cancer were analyzed for the levels of GALNS and PAEP by ELISA. Results: The median serum levels of GALNS and PAEP in all cancer types as well as pneumonia patients were significantly higher than those of the healthy controls. Conclusion: In addition to previously known cancers, the median serum levels of PAEP were also found to be higher in liver and head and neck cancer patients. GALNS and PAEP are promising general biomarkers for multiple cancers and deserve further evaluation.*

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Human lung cancer is one of the most prevalent and dreadful cancers and also a major health management problem worldwide (1, 2). According to the annual statistics of the Centers for Disease Control and Prevention, it has been the

leading cause of cancer death and the second most common cancer among both men and women in the United States (http://www.cdc.gov/) for many years. An estimation by the American Cancer Society estimated approximately 222,500 new cases of lung and bronchus cancer and 155,870 deaths from this disease in 2017 (3). These numbers correspond to 13.2% and 25.9% of new cases and cancer deaths, respectively, of all cancer cases. In a previous study, even with the advances in medicine in the past decade, the annual percent change of the incidence rate of lung and bronchus cancers was only reduced by 2.0% between 1992 and 2010 (4). Lung cancer is the leading cause of cancer death, and approximately 90% of all lung cancer deaths are due to metastases. In addition, 40% of patients present with brain metastases, and non-small cell lung cancer (NSCLC) is the most common primary tumor (5, 6).

Secreted proteins or proteins shed from the cell surface may be used as serological biomarkers for diagnosis, monitoring, or staging of cancers in relatively non-invasive blood tests. Representative examples include the FDA approved serum biomarkers PSA, CA125, α-fetoprotein, thyroglobulin, choriogonadotropin subunit β (CGB) (7) and WAP four-disulfide core domain protein 2 (WFDC2; Synonyms: HE4, WAP5) (8, 9). WFDC2 had been found to be overexpressed in ovarian cancer tissues (8).

Cancer cell secretomes (conditioned media) had been suggested as a good source for discovering biomarkers to develop serological tests (10-13). The most practical way to identify new biomarker candidates in secretomes is through proteomics-based analysis. Although there are significant advancements in mass spectrometry and bioinformatics technologies in the past two decades, the identification of biomarkers from secretomes remains a challenging task. Despite of this, a number of new cancer biomarker candidates have been identified *via* proteomics analysis of secretomes and examination of gene expression profiles in public databases in the post-genomic era. These included potential serological biomarkers annexin II for breast cancer (14), laminin β-1 (LAMB1) (15, 16), melanotransferrin (TRFM) (17), GDF15, S100A8/A9, and SERPINI1 (18) for colorectal cancer, and quiescin sulfhydryl oxidase (QSOX1) (19), importin subunit alpha-2 (KPNA2) (20), and stromal cell-derived factor 1 (SDF-1) (21) for lung cancer. ELISA tests results have shown that the above biomarkers had higher median serum levels in the analyzed cancer types than in healthy controls as well as a significant AUC (Area under the curve) value in ROC (Receiver operating characteristic) analysis.

In this study, we analyzed N-acetylgalactosamine-6-sulfatase (GALNS) as a new biomarker candidate. It was identified by proteomics analysis of the secretomes from the CL1-0 cell line with a low metastasis potential lung adenocarcinoma and its more aggressive derivative CL1-5. ELISA test was conducted to compare the serum levels of GALNS with the previously identified lung cancer biomarker glycodelin (GD; also known as progestagen associated endometrial protein, PAEP) in lung and four other types of cancer patients.

Patients and Methods

Cell lines and cell culture conditions. Lung adenocarcinoma cell lines CL1-0 and its more aggressive derivative CL1-5 (A kind gift from Prof. Pan-Chyr Yang, National Taiwan University) were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 1% HEPES, 1% sodium pyruvate (HyClone, Logan, UT, USA), and 1% penicillin-streptomycin (Gibco, Waltham, MA, USA) at 37˚C in a 5% CO_2 incubator.

Isolation of secretomes. When the cells reached 70 to 80% confluency (~1×10^7) in ten 10-cm dishes, the medium was discarded and the cells were gently washed with serum-free medium four times. Conditioned media were harvested following further incubation of cells in serum-free medium for 24 h. The proteins were concentrated and the medium was exchanged twice with 10-ml PBS (HyClone) and then two times with 0.5 ml 0.1% RapiGest SF solution (Waters, Milford, MA, USA) in an Amicon Ultra-15 tubes (10K device) (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Western blot analysis. SDS-PAGE, silver staining of protein gels, and western blot analysis were carried out using standard procedures (22). Polyclonal anti-GALNS (GeneTex, Irvine, CA, USA) and monoclonal anti-PAEP, anti-β-actin, and anti-calreticulin (CALR) (Sigma-Aldrich, St. Louis, MO, USA) antibodies were used in western blot analysis according to procedures suggested by the manufacturer.

Serum samples. Healthy controls and patient blood samples were collected and the sera were obtained with a standard procedure similar to that described by Tuck *et al*. (23). This study was approved by the institutional review board (IRB no.: 103-3009B) of Chang Gung Medical Center and all patients signed a written consent form.

ELISA test. The serum protein levels were analyzed with human GALNS/Chondroitinase and PAEP/Glycodelin/Gdf Sandwich ELISA kits (LifeSpan BioScience, Seattle, WA, USA) according to procedures recommended by the manufacturer.

Bioinformatics analyses. The subcellular locations of proteins were analyzed using the COMPARTMENTS tool (24). The expression profiles of the candidate genes in normal and lung cancer tissues were obtained from the Oncomine database (25).

Results

Candidate biomarker GALNS. GALNS was found to be upregulated in CL1-5 cells by LC-MS/MS analysis of secretome samples from CL1-0 and CL1-5 cells. Among the differentially expressed candidates, GALNS peptides were identified only in the CL1-5 secretome. In accordance with

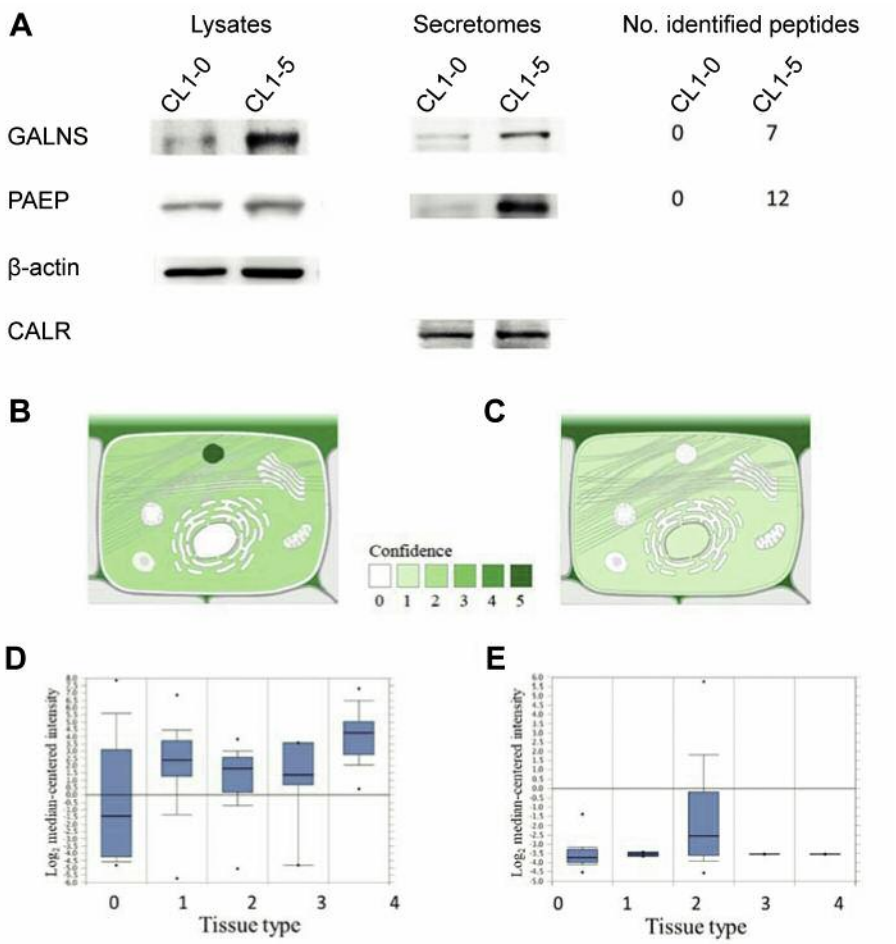

Figure 1. *GALNS and PAEP had higher expression in lung cancer tissues. Western blot results of GALNS and PAEP in CL1-0 and CL1-5 whole cell lysate and secretome samples are shown with the number of peptides identified by LC-MS/MS on the right (A). β-actin and CALR are loading controls for lysates and secretomes, respectively. Compartments analysis results showed that GALNS (B) and PAEP (C) had a high confidence of extracellular distribution. Oncomine data showing the distribution of the log_2 median-centered intensities of GALNS expression in tissue type 0: normal lung tissues (n=17), 1: lung adenocarcinoma (n=139), 2: lung carcinoid tumor (n=20), 3: small cell lung carcinoma (n=6), and 4: squamous cell lung carcinoma (n=21) (p=0.013; Dataset: Bhattacharjee lung) (26) (D) and PAEP in tissue type 0: normal lung tissues (0; n=31), 1: large cell carcinoma (n=2), 2: lung adenocarcinoma (n=31), 3: lung cancer (n=1), and 4: non-small cell lung carcinoma (n=1) (p=0.001; Dataset: Su lung) (52) (E).*

the proteomics data, western blot results also showed that CL1-5 cells had relatively higher expression levels of GALNS than CL1-0 (Figure 1). Analysis of subcellular location using the COMPARTMENTS database indicated that GALNS had a high confidence level of extracellular distribution. Further cancer expression analysis found that GALNS had higher median mRNA levels in lung cancer than normal lung tissues in a Oncomine dataset (26). Thus, GALNS was chosen for further examination together with a previously identified lung cancer biomarker PAEP (27).

GALNS and PAEP ELISA tests. A total of 170 serum samples from 21 healthy controls, 18 patients with pneumonia, 62 lung cancer (49 adenocarcinomas, 10 squamous cell carcinomas, and 3 pleomorphic carcinomas), 14 breast cancer, 29 colon cancer, 21 liver cancer, and 5 head and neck cancer patients were analyzed using commercial ELISA kits (Figure 2). The median values of GALNS and PAEP levels were significantly higher in lung, colon, breast, and hepatic cancer patients than healthy controls. Although the sample size is relatively small, the median levels of both proteins were also found to be higher in head and neck cancer patients. In addition to cancer, the median levels of these proteins were also higher in pneumonia patients.

In additional to lung cancer, receiver operating characteristic area under curves (ROC AUC) analysis of the protein levels in sera of cancer patients and healthy controls indicated that GALNS and PAEP had significant AUC values suggesting they

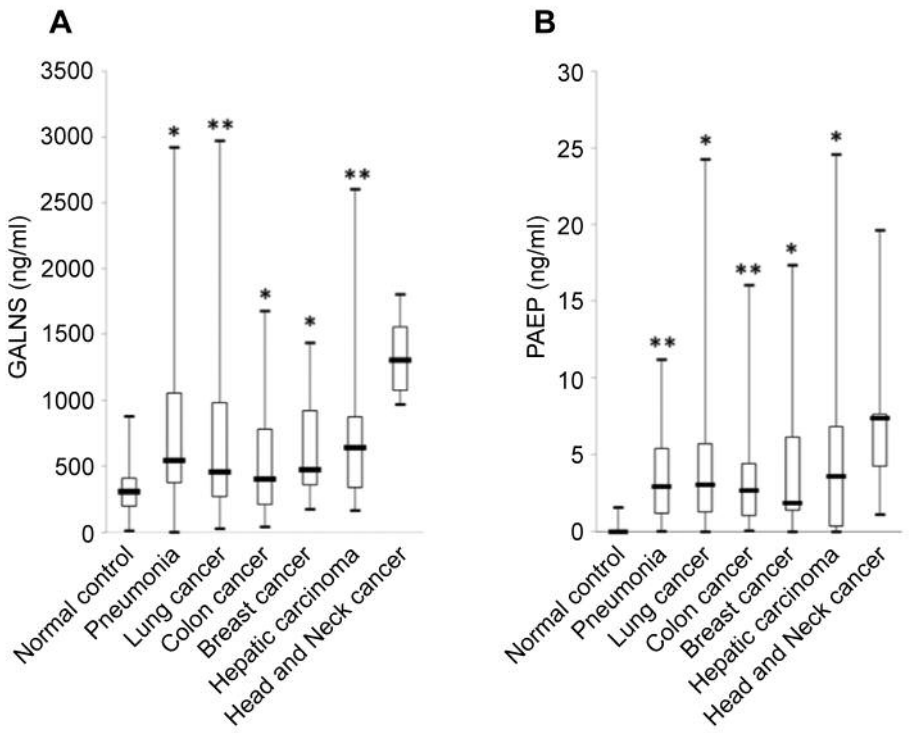

Figure 2. *Serum levels of GALNS and PAEP in healthy and disease samples. The serum levels of the GALNS and PAEP in samples from healthy controls, pneumonia patients, lung cancer, breast cancer, colon cancer, liver cancer, and head and neck cancer patients were determined by sandwich ELISA. Thick horizontal lines represent median values of the control and cancer groups. (p-value of pneumonia/cancer versus healthy control: *p<0.05 and **p<0.005).*

may be helpful for the discrimination of patients with several other types of cancers (Figure 3). The AUC of GALNS and PAEP in lung cancer patients were 0.710 and 0.895, respectively. For breast, colon, and liver cancer, the AUC of GALNS were 0.765, 0.647, and 0.780, and PAEP were 0.905, 0.977, and 0.905, respectively. The AUC values above 0.990 in head and neck cancer may not be conclusive because a limited number of samples was available for this cancer type.

Discussion

GALNS is a lysosomal enzyme involved in the hydrolysis of the 6-sulfate groups of the N-acetyl-D-galactosamine 6-sulfate units of chondroitin sulfate and of the D-galactose 6-sulfate units of keratan sulfate (28-30). As suggested by the expression data from Human Protein Atlas (HPA), GTEx (The Genotype-Tissue Expression project), and FANTOM5 (The functional annotation of the mammalian genome 5 project) in the Tissue Atlas of HPA, *GALNS* is an ubiquitously expressed gene which has no tissue specificity (31). Mutations of *GALNS* had been reported to be the cause of the inherited lysosomal storage autosomal-recessive disorder mucopolysaccharidosis IV A (MPS IVA; Morquio syndrome) (32). Deficiency of the enzyme led to the accumulation of keratan sulfate within lysosomes. Because keratan sulfate was predominantly found in cartilage and cornea, the abnormal storage causes skeletal abnormalities and cloudy corneas (33). In addition to lysosome, Reactome (a knowledgebase of biological pathways and processes), text mining, and PSORT analysis of protein localization sites in cells in COMPARTMENTS resource suggested that GALNS might also be present in the extracellular space (24). A previous study by Parkinson-Lawrence *et al*. has shown that GALNS protein could be detected in the serum from normal control but not from MPS IVA patients (34).

The roles of glycodelin (GD or PAEP; Other names: PP14, PAEG, PEG, ZIF-1, *etc*.; Gene name: *PAEP*) in cancer development and progression have recently been reviewed by Cui *et al*. (35). Glycodelin is a glycoprotein with four different glycosylated forms (*i.e*., Glycodelin A, C, F, and S) that has various biological activities in human reproduction and also immunomodulatory effects (36-40). It is a secreted protein mainly expressed in secretory endometrium, placenta, and ductus deferens (41, 42). Endometrial expression has been found in normal premenopausal but not in postmenopausal endothelium (43). Overexpression of *PAEP* has been reported in tissues of several malignancies including female-specific endometrial, ovarian, and breast cancers, and non-gender specific lung adenocarcinoma and squamous cell carcinoma,

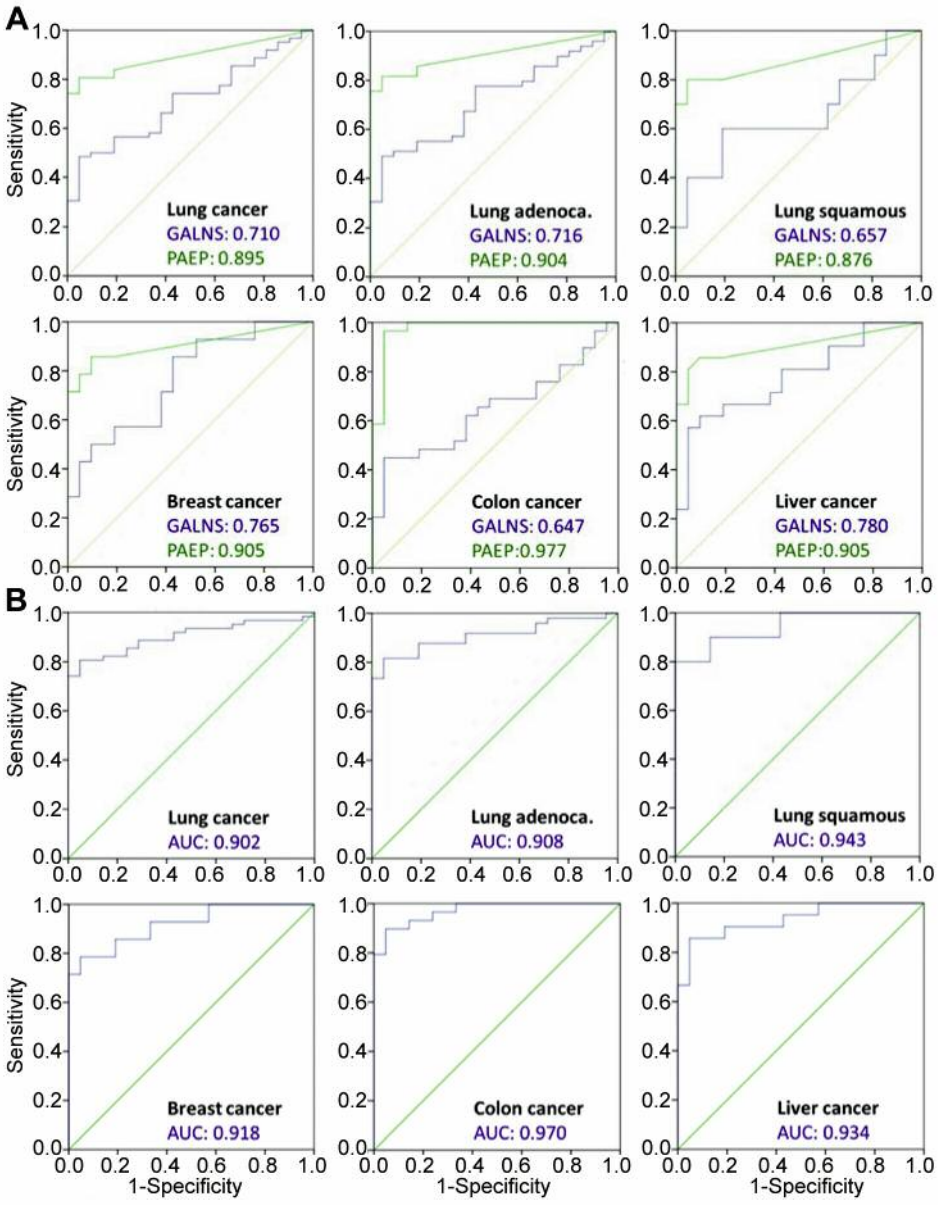

Figure 3. *ROC curves analysis indicated GALNS and PAEP alone (A) or combined (B) may be helpful for discriminating healthy from the indicated cancer types. Lung cancer group included 49 adenocarcinoma, 10 squamous cell carcinoma, and 3 pleomorphic carcinoma patients. The lung adenocarcinoma and squamous cell carcinoma data were also analyzed separately.*

and lung metastases of colon cancers (43-47). Several independent studies have reported that PAEP may serve as a serum biomarker of endometrial, colorectal, lung, and ovarian cancers (27, 48, 49). In addition, higher expression of the immunosuppressive isoform Glycodelin A (GdA) was a prognostic marker for poor outcome in endometrial and in advanced stage ovarian cancer patients. The overall survival was significantly reduced in female but not male NSCLC patients with high glycodelin mRNA levels (27, 50, 51).

Using ELISA, we found that patients with different types of cancers had higher serum levels of GALNS. The serum levels of GALNS in normal controls and patients with lung, colon, breast, hepatic, and head and neck cancers as well as pneumonia were examined side by side with those of PAEP. Box plot showed that both proteins had higher median values in all cancer types (Figure 2). Similar to PAEP, whose serum levels are known to be higher in lung and colon cancers as mentioned above, the median levels of GALNS in patients

with these cancers were also higher than those in normal controls. Interestingly, the serum levels of both proteins were also higher in breast, hepatic, and head and neck cancer patients, which had not been reported before. However, we also found that GALNS and PAEP were elevated in serum specimens from pneumonia patients due to tuberculosis or to other bacterial infections. Despite of this, non-invasive blood test of these proteins independently or combined with a panel of other biomarkers may still be useful in routine health examination for cancers. Patients with high serum protein levels may refer to ultrasound (US), X ray computed tomography (CT), and nuclear magnetic resonance imaging (NMR) examination for more precise diagnosis.

In summary, GALNS and PAEP may serve as general biomarkers for diagnosis or monitoring of the therapy of more than one type of cancers including the relatively common breast, colon, lung, and ovarian cancers. Since only a limited number of specimens from each cancer type was examined in this work, the serum levels of PAEP in hepatic and head and neck cancers and GALNS in the analyzed cancer types deserves further validation with a larger sample size.

Conflicts of Interest

The Authors declare that they have no competing interests regarding this study.

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Authors' Contributions

CC Hua and WV Ng: Conceived the study, participated in its design and supervised the study; WK Kuo, CW Fan, CL Yuan, HY Chen and SW Yang: Access to patients and sample collection; LC Chang: Evaluated all pathological data; ML Ho, LJ Chu, IH Yeh, HI Chang and TY Chou: Performed experiments and interpretation of data; M Luo, TH Wu and YI Chang: Bioinformatics analysis; CJ Yu: Sample collection; WV Ng: Prepared the manuscript. All Authors read and approved the final article.

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