Role of GLRX3 as a novel secretory biomarker of pancreatic cancer based on pancreatic cancer stem cell characteristics

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Directed by Professor **Si Young Song**

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Moon Jae Chung

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ABSTRACT

Role of GLRX3 as a novel secretory biomarker of pancreatic cancer based on pancreatic cancer stem cell characteristics

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Pancreatic cancer is one of the most lethal diseases, which is difficult to diagnose and resistant to conventional treatment such as chemotherapy and radiotherapy. Cancer stem cells (CSCs) are implicated in carcinogenesis, cancer progression and recurrence. Several biomarkers for pancreatic CSCs were described but their function and mechanism were unclear. To find secretory biomarkers, pancreatic CSCs were enriched using sphere culture method. And secretome from culture medium of spheres and control adherent cells was analyzed by two-dimensional gel electrophoresis and MALDI-TOF. Total 200 spots were differentially expressed between spheres and adherent cells by at

least 2 fold, and up-regulated 55 spots were identified. Proteins known to be associated with cancer or CSCs such as HSP90AB1, ALDH, vimentin, and AKR were up-regulated in spheres and the expression was confirmed by western blot. Among up-regulated proteins, GLRX3 was selected for the new pancreatic CSCs marker. The overexpression of GLRX3 was confirmed in two individual CSCs populations, spheres and CD24+/CD44+/ESA+ cells. The overexpression of GLRX3 was demonstrated in pancreatic cancer patient tissues, blood and cell lines. The role of GLRX3 in pancreatic carcinogenesis and CSCs properties was assayed by shRNA transfection in pancreatic cancer cell lines. Compared to the control cells, knockdown of GLRX3 decreased in vitro proliferation, migration, clonogenicity, and sphere formation. In addition, knockdown of GLRX3 reduced tumor formation and growth in SCID mice. Furthermore GLRX3 regulated chemosensitivity to gemcitabine via Met/PI3K/AKT signaling. This study reveals that secretory protein GLRX3 may be a useful prognostic marker and inhibition of endogenous GLRX3 may be a new therapeutic strategy for pancreatic CSCs.

Key words: Pancreatic cancer, cancer stem cell, biomarker, Glutaredoxin3, chemosensitivity

GLRX3, a novel secretory biomarker of pancreatic cancer based on pancreatic cancer stem cell characteristics

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I. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis with a 5-year survival rate of less than 5% and invasive surgery is currently the only curative treatment[1]. However, only 10–20% of patients are candidates for surgery at the time of diagnosis. Over the past decade, several cancer-related genes have been identified in pancreatic cancer. However, pancreatic cancer remained worst progressive disease with poor prognosis and high frequency of recurrence or metastasis. In order to improve the detection rate of early cancer and the predictability of recurrence after treatment for patients with pancreatic cancer, accurate and sensitive biomarkers are needed. The only biomarker currently recommended for clinical use by the National Comprehensive Cancer Network guidelines for PDAC is carbohydrate antigen 19-9 (CA 19-9)[2]. But notable

limitations of CA 19-9 is that about 10% of the population do not generate the specific sialyl antigen and are thus termed nonsecretors[2,3] and the sensitivity is about only 75%. In addition, there have still been controversies about the correlation between CA19-9 and the prognosis of pancreatic cancer patients. The positive predictive value (PPV) was calculated at 0.9% in their asymptomatic population. A study in Japan screened 10,162 asymptomatic patients and found only 4 (0.4%) cases of PDAC[4]. Based on these data, screening asymptomatic individuals using CA19-9 is not possible for the early detection of PDAC. Development of targets for treatment of pancreatic cancer as well as biomarkers for early detection and prediction of prognosis after treatment are also needed. Recently cancer stem cells (CSCs) have been emerging as a new potential target.

CSCs are capable of self-renewal, tumor-initiating potential, and multi-lineage differentiation. Importantly, CSCs are resistant to radiation and chemotherapeutic drugs. From first identification of CSCs in myeloid leukemia[5], these have been described identification of CSCs in solid tumors including pancreatic cancer. For the isolation of pancreatic CSCs, several surface markers were reported; CD24, CD44, epithelial specific antigen (ESA), CD133, CXCR4, c-Met and combination of these markers[6-9]. And molecules involved in CSCs-related pathway were identified. However, there have been multiple populations with the ability of tumor formation and self-renewal in pancreatic cancer. And, CSCs population with defined each markers have been a partial

correlation with each other CSCs populations and many of the reported markers were not validated functionally. Therefore, understanding the function and relationship between markers, discovery of new marker candidates are required. For these purposes, differentially expressed proteins in CSCs were documented by proteomic methods. Kanojia et al. isolated breast CSCs derived from spheres of HER2/Neu transgenic mice, and identified the ferritin heavy chain 1(FTH1) as a potential therapeutic target using LC-MS/MS[10]. Emmink et al. and Van Houdt et al. performed proteomic analysis of colorectal CSCs from spheres of primary tumors using one-dimensional gel electrophoresis and nano LC-MS/MS and identified BIRC6 as a candidate target gene[11,12]. For pancreatic CSCs, Zhu et al. identified glycoprotein markers from CD24+/CD44+ cells of pancreatic cancer cell line as a prognostic marker. And they also suggest the co-expressed proteins with CD24 as a prognosis marker and therapeutic target from profiling of frozen pancreatic CD24+ adenoma tissues[13,14].

Glutaredoxin3 (GLRX3) was first identified as a protein-kinase C θ-interacting protein[15], and played a role in cell activation-dependent signal transduction pathways and cellular responses to stress mediated by ROS in T cells[16]. GLRX3 was negative regulator of cardiac hypertrophy and a positive inotropic regulator[17-19] and critical for cell cycle progression during embryogenesis[20]. Expression of GLRX3 in human cancer has been reported recently. Cha et al. reported that GLRX3 was overexpressed in colon and lung cancer than normal tissues, and expression level of GLRX3 mRNA had positive correlation with

survival of lung cancer patients[21-23]. Qu et al. reported that GLRX3 regulates cancer cell growth and metastasis via redox homeostasis[24]. However, the role of GLRX3 in pancreatic cancer remains unknown. Furthermore, the relationship between CSCs and GLRX3 in human cancer has yet to be reported.

In present study, we used sphere culture method for enrichment of pancreatic CSCs and analyzed secretome of pancreatic CSCs using two-dimensional gel electrophoresis and MALDI-TOF. Analysis of the data revealed several surface marker candidates of pancreatic CSCs or targets of drugs. Among them, we documented GLRX3 as a potential pancreatic CSCs marker and therapeutic target for pancreatic cancer.

II. MATERIALS AND METHODS

1. Clinical samples

Thirty two pancreatic cancer tissue samples were collected at Yonsei University Health System from January 2010 to December 2014. A pathological grading was made, and the tumor stage of the tissue samples was determined according to the American Joint Committee on Cancer (AJCC) staging system. After pathological evaluation, a tissue microarray (TMA) was generated using cores from tumors and adjacent normal tissue from each specimen. Healthy donors and pancreatic cancer serum samples were collected. The Ethics Committee for the Clinical Research of the Institutional Review Board of Yonsei Medical Center, Korea, approved this study protocol.

2. Cell culture, sphere culture and transfection

Eight pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, Cfpac-1, HPAC, MiaPaca-2, and Panc-1) were from ATCC. Human pancreatic duct epithelial cell line (HPDE) was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Ontario, Canada). All cells were grown in each conditioned medium and maintained in an atmosphere of 5% CO2/95% air at 37℃.

For formation of spheres, single cells were cultured in DMEM/F12 medium containing 0.5% FBS (Hyclone), 0.5% Bovine Albumin serum Fraction V (Gibco), Insulin-Transferrin-Selenium A (Gibco), 10ng/ml of hEGF (R&D systems, Wiesbaden-Norderstedt, Germany), 10ng/ml of hFGF (R&D) and 10ng/ml of hLIF (R&D) at density of $1x10^3$ cells/ml in ultralow attachment plate (Corning, Corning, NY, USA) for 7 days. Growth factors were added every 3 days. For secretory protein preparation, culture medium was changed to serum-free medium at post sphere culture 5 days and culture for fallowing 2 days.

To inhibit endogenous GLRX3 mRNA expression, human pancreatic cancer cells were transfected with siRNAs using RNAiMAX reagent (Invitrogen) or shRNAs using Lipofectamine2000 reagent (Invitrogen) according to manufacturer's instructions and stable knockdown clones were selected by puromycin. Human GLRX3 specific siRNAs were purchased from Invitrogen.

Their sequences are follows: siGLRX3-1S, 5’-UGAGGGAGUUCUUUAGCUAACUCUG-3’and siGLRX3-1AS, 5’-CAGAGUUAGCUAAAGAACUCCCUCA-3’; siGLRX3-2S, 5’-AAGAAUUUCCACCAUCUGCUUGCUG-3’and siGLRX3-2AS, 5’-CAGCAAGCAGAUGGUGGAAAUUCUU-3’; siGLRX3-3S, 5’-AAACAUAGAGCUGAGGAUAGGUAGG-3’and siGLRX3-3AS, 5’-CCUACCUAUCCUCAGCUCUAUGUUU-3’. Stealth™ RNAi negative control duplex are used as a negative control. shRNA expressing plasmid targeting human GLRX3 and negative control plasmid were purchased from SABiosciences. The human GLRX3 shRNA sequence is 5’-GTGGAAATTCTTCACAAACAT-3’and control shRNA sequence is 5’-GGAATCTCATTCGATGCATAC-3’. For shRNA transfection, $5x10^4$ cells/well of HPAC were seeded onto 6 well plates the day before transfection. Transfection was performed using Lipofectamine2000 reagent according to manufacturer’s instructions and stable knockdown clones were selected by puromycin.

To inhibit endogenous Met mRNA expression, human pancreatic cancer cells were transfected with siRNAs using RNAiMAX reagent (Invitrogen). Human GLRX3 specific siRNAs were purchased from Santacruz.

3. Proteomic analysis

Equal amount of secretory proteins (1.0 mg) were isoelectrically focused on an

18-cm Immobiline Drystrip pH 3-10 NL (GE Healthcare) and separated on 9-17% SDS-PAGE gel. The gels were stained with CBB solution and scanned using a GS710 scanning densitometer (Bio-Rad, Hemel Hempstead, UK). The gel images were analyzed using Image Master Platinum 5 (GE Healthcare). Spot pairing of each gel image was performed as a control of adherent HPAC. Group analysis was performed with gel image of adherent of HPAC and CAPAN-1 as group A and gel image of spheres of HPAC and CAPAN-1 as group B. A spot was accepted with a cut-off ratio greater than 2.0-fold. Selected spots were excised manually from CBB-stained preparative gel, destained, and then digested with trypsin (Promega, Southampton, UK). Tryptic peptides were desalted and purified using a mixture of Poros R2 and Oligo R3, as described previously[25]. The MS spectra of peptides were generated via spectrometric analysis using a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA) in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV, with data summed from 500 laser pulses. The spectrum was calibrated against the tryptic auto-digested peaks (m/z 842.5090 and 2211.1046), and monoisotopic peptide masses were obtained with Data Explorer 3.5 (PerSeptive Biosystems). A mass range of m/z 800-4000 was used with 1000 shots per spectrum. For MALDI-TOF-MS, GPS 3.1 software (Applied Biosystems) was used for peak generation. MASCOT (Matrix Science, Boston, MA) was used to identify peptide sequences present in the protein sequence database (NCBInr)[25].

4. Semi-quantitative RT- PCR

The total RNA from cancer cells was extracted by RNAeasy extraction kit (Quagen) according to the manufacturer's instructions. To quantify the relative expression level of the gene, PCR was performed with β-actin primers as control. The PCR primers used were GLRX3 sense, 5'-GGGCGGCTGAGGCAGCT-3'; GLRX3 antisense, 5'-GCA GGGGGCAGCATGAGTC-3'; beta-actin sense, 5'-GGCATCCTCACCCTGAAGTA–3'; antisense, 5'-GGGGTGTTGAAGGTCTCAAA-3'.

5. Immunohistochemistry

All procedures were performed at RT unless otherwise specified. Paraformalin-fixed, paraffin-embedded tissue sections (3-5 ㎛ thickness) were deparaffinized in xylene and rehydrated in a graded ethanol series (100-90-80-70-50-30%) and the PBS washing. Endogenous peroxidase was blocked by immersing the slide in 0.3% (v/v) hydrogen peroxide in methanol for 15 min at RT. Microwave antigen retrieval was performed in citrate buffer (0.01M, pH 6.0). The sections were blocked by soaking in 10% (v/v) normal donkey serum for 1hr, and incubated in primary antibodies; anti-human GLRX3 (1:150, Sigma) for overnight at 4℃. The sections were incubated with EnVision/HPR, Rabbit/Mouse (DakoCytomation, CA, USA) and diaminobenzidine (DAB+) chromogen. The sections were counterstained with

hematoxylin (Sigma-Aldrich, Inc., St. Louis, MO, USA), dehydrated, and mounted.

6. ELISA

Serum GLRX3 and CA19-9 levels in healthy and pancreatic cancer patients were measured using ELISA. The ELISA kit for GLRX3 was purchased from USCNK (Wuhan, China). For comparison, serum CA19-9 level was measured with a commercial immunochemiluminescence kit (VITROS® ECiQ Immunodiagnostic System, Ortho Clinical Diagnostics). All assays were performed according to the manufactures' instructions.

7. Wound healing assay

Cells were seeded onto 12-well plates and allowed to grow to 100% confluence. A scratch was applied to the cell layer across each well and the cell layer was washed twice with PBS to remove loss cells from the scratch margins. The wells were refilled with fresh medium and culture for 17 hr. Cell morphology was captured by camera (Olympus DP12). The experiment was done in triplicate.

8. Soft agar assay

500 single cells suspension containing 0.3% agar medium was overlaid on 0.6% agar medium in 24 well plates (SPL). Each well was covered with complete

medium and the plates were incubated for 4 weeks. Colonies were stained with crystal violet and counted. The experiment was done in triplicate.

9. Flow cytometry and cell sorting

Cultured cells were detached with accutase solution (Sigma Aldrich) and washed in PBS with 0.5% FBS. Single cells were stained for 20 min on ice in the dark and washed twice in PBS with 0.5% FBS, and then fixed in 2% paraformaldehyde. Flow cytometric analysis was performed on FACSCalibur system (BS Biosciences, San Jose, CA) and cell sorting was performed using FACSAria II (BD Immunocytochemistry System, Franklin Lakes, NJ). Antibodies against CD44 (anti-CD44-FITC, BD pharmingen, Franklin Lakes, USA) and c-Met (anti-c-Met-FITC, eBioscience) were used. Antibodies for cell sorting against CD24 (anti-CD24-PE, BD), CD44 (anti-CD44-APC, BD) and ESA (anti-ESA-FITC, BD) were used. FITC-mouse IgG2b, κ isotype control (BD), rat IgG1 κ isotype control FITC (eBioscience), PE-mouse IgG2a, κ isotype control (BD) and APC-mouse IgG2b, κ isotype control (BD) were used as control.

10. Protein extraction and western blot

Cells were prepared in lysis buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 25 mM beta-glyserophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 0.1 mM PMSF and protease

inhibitor Protease Inhibitors cocktail (Leupeptin, Pepstatin, Aprotinin, and antipain each 5 μg/ml). For secretory protein preparation, culture medium was centrifuged and cellular components and debris were discarded. Culture medium was concentrated by 10k cut-off microcon (amicon) or by addition of ice-cold acetone, precipitated protein was resuspended with lysis buffer. Proteins were separated on SDS-PAGE and transferred to 0.45 ㎛ Immobilon P-transfer membrane (Millipore). Membrane was blocked in 5% (w/v) non-fat milk then probed with primary antibody; anti-human GLRX3 antibody, beta-catenin, E-cadherin, and GAPDH (Santacruz). Immunoreactive material was then visualized by west pico chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

11. Growth rate

2 x 10^3 cells were seeded per well into 24 well plates and the number of cells was counted at every 24 hr. The experiment was done in triplicate to determine the number of cells at each time point.

12. In vivo tumorigenesis

Cells were suspended with 50% Matrigel (BD biosciences) in HBSS (invitrogen Inc.) to a final count of 3 x 10^7/ ml. 200 μL of cell suspension was injected subcutaneously into 6-week-old NOD/SCID male mice. Tumor formation was

monitored twice a week. Tumor volumes were calculated by the formula V (mm3) = A x B^2, where A is the largest dimension and B is the perpendicular diameter. After 14 weeks, tumor xenografts were recovered from mice, fixed in 4% paraformaldehyde, and embedded in paraffin.

13. MTT assay

After incubation at 37˚C overnight, cells were treated with various concentrations of gemcitabine in complete growth media and incubated for 72 h at 37˚C. A 3-(4,5-dimenthelthiazol-2-ly)-2,5-diphenyltetrasolium bromide-based assay (absorbance 570 nm) was used to measure the number of metabolically active cells.

14. Statistical analysis

Serum GLRX3 and CA19-9 levels were compared between normal and pancreatic cancer patients using Kruskal-Wallis test, which is non-parametric statistics. Cox regression, Cut-off value, Receiver operating characteristic (ROC) curve, area under the ROC curve (AUC), and 95% confidence intervals (CI) were performed using SAS, version 9.2 (SAS Institute Inc., Cary, NC, USA) and R package, version 3.1.3 (http://www.R-project.org). All data were expressed as median ±SD.

III. RESULTS

1. Differential expression of GLRX3 in spheres and adherent cells of pancreatic cancer cell lines

For enrichment cancer stem cells, we cultured two pancreatic cancer cell lines, HPAC and CAPAN-1 in sphere conditioned media on ultralow attached plate for 7 days; spheres of HPAC (HS) and spheres of CAPAN-1 (CS). For control, HPAC and CAPAN-1 cells were cultured in sphere conditioned media on normal cell culture plate for 7 days; adherent cells of HPAC (HA) and adherent cells of CAPAN-1 (CA).

To investigate novel marker for the pancreatic cancer stem cells, secretory protein profiles of spheres and adherent cells from cultured media were analyzed. Figure 1A showed 2D gel images for secretory proteins extracted from spheres and adherent cells of HPAC and CAPAN-1. A total of 626, 576, 642 and 515 spots were noted in the culture media from HS, HA, CS and CA cells and 587 spots in the four gels were matched, respectively. To compare two spheres and adherent cells, gel image of HA and CA were classified as group A and gel image of HS and CS were classified as group B. As a result, totally 200 spots of group B including 55 increases and 145 decreases were differentially expressed at least two fold compared with the control group A. For identification of differently expressed proteins, 55 up-regulated spots in spheres were further subjected to MALDI-TOF analysis. Total 53 spots were identified

to 46 proteins and these up-regulated proteins in spheres then adherent cells. All proteins were analyzed with secretomeP 2.0 and SignalP 4.1 to predict their secretory potential. About 52% of 46 proteins were potentially secreted by means of the classical or non-classical secretion pathways. Actually, heat shock protein27 (HSP27) (spot no. 16193) was reported as a potential serum marker and an inducer of gemcitabine resistance of pancreatic cancer[26-30], and overexpression of neutrophil gelatinase associated lipocalin (spot no. 16663 and 16246) was reported in transgenic mice model and human pancreatic cancer patient serum as a potential biomarker[31,32]. Furthermore, 35 proteins were previously as up-regulated in cancer including pancreatic cancer, and 19 proteins were reported to be correlated with CSCs. HSP90 (spot no. 15391 and 15602), Grp78 (spot no. 15413), Grp94 (spot no.15538) and HSP27 (spot no. 16193), belong to heat shock protein family were reported as therapeutic target for pancreatic cancer and targeted drugs were developed[33-35]. Overexpression of aldo-keto reductase proteins, AKR1B1 (spot no. 15977, 15978, and 16000) and AKR1C2 (spot no.15965) was observed in various cancer tissues including pancreatic cancer[36-38]. Also, proteins involved in tumor metastasis and invasion were overexpressed in spheres than adherent cells including cathepsin D (spot no. 16095), vimentin (spot no. 15687) and keratin 9 (spot no.16276)[39,40]. KRAS mutation is hallmark of pancreatic cancer. PIK3CA (spot no. 16432) was downstream effector of RAS and mutant of PIK3CA was observed in breast, ovarian, colorectal cancer and coexisted with RAS (KRAS and NRAS) and

BRAF mutation[41-44]. And aldehyde dehydrogenase (ALDH) (spot no. 16610) activity was increased in serum of pancreatic cancer patient, ALDH activity was enhanced in tumor initiating population related to CD133 or CD44 and contribute to chemotherapy and radiation resistance in pancreatic cancer, breast cancer and lung cancer[45-48]. Transferrin (spot no. 16676) was used for supplement of sphere culture as iron supply, and its receptor, transferrin receptor was reported as potential diagnostic and therapeutic target for pancreatic cancer[49,50]. Prominin-1/CD133 was reported as one of pancreatic CSCs markers, and recent report demonstrated that CD133 has important role in transferring uptake through CD133-Tf-iron network[51]. These data indicated that our proteomic results have reliability for searching of new secreted protein candidates of the pancreatic cancer stem cells.

Next, to further validate these proteomic data, 5 representative proteins, aldo-keto reductase 1, B1 (AKR1B1), heat-shock protein90 (HSP90), ALDH, vimentin, and GLRX3 were selected and confirmed by western blot analysis. As shown in figure 1B, expression of all the selected proteins was increased in culture media from spheres of HPAC or CAPAN-1 than these adherent cells. Among them, ALDH was identified as a potential CSCs target in solid tumor including pancreatic cancer[45-47,52]. The overexpression of ALDH in spheres than adherent cells demonstrated the validity of our proteomics approach to identify up-regulated proteins in cancer stem cells. Among these five proteins, we selected GLRX3 for further investigation.

2. Expression of GLRX3 is in pancreatic CSCs population

We hypothesized that GLRX3 may play a functional role in maintaining self-renewal or pancreatic cancer stem-like properties given its involvement in other stem cell systems. So we identified the GLRX3 as a potential pancreatic CSCs marker. Overexpression of GLRX3 mRNA and protein in spheres than adherent cells were confirmed by semi-quantitative PCR and western blot analysis (Fig. 1B and 1C). However, the reported markers of CSCs were varied and were partially overlapped to another populations[53]. Therefore, we verified whether GLRX3 was overexpressed in other pancreatic CSCs population. CD24+/CD44+/ESA+ population is one of the pancreatic CSCs[6]. So, we isolated the CSCs through the combination of positive CD24, CD44 and ESA cell surface markers from HPAC cells by fluorescence-activating cell sorting (FACS) and performed semi-quantitative PCR to measure the GLRX3 levels in the CSCs (CD24+/CD44+/ESA+) and surface marker negative cancer cells (CD24-/CD44-/ESA-). As a result, GLRX3 mRNA was overexpressed in CD24+/CD44+/ESA+ cells compared to CD24-/CD44-/ESA- cells (Figure 1D). This data suggest possibility that GLRX3 may be overexpressed in other pancreatic CSCs populations.

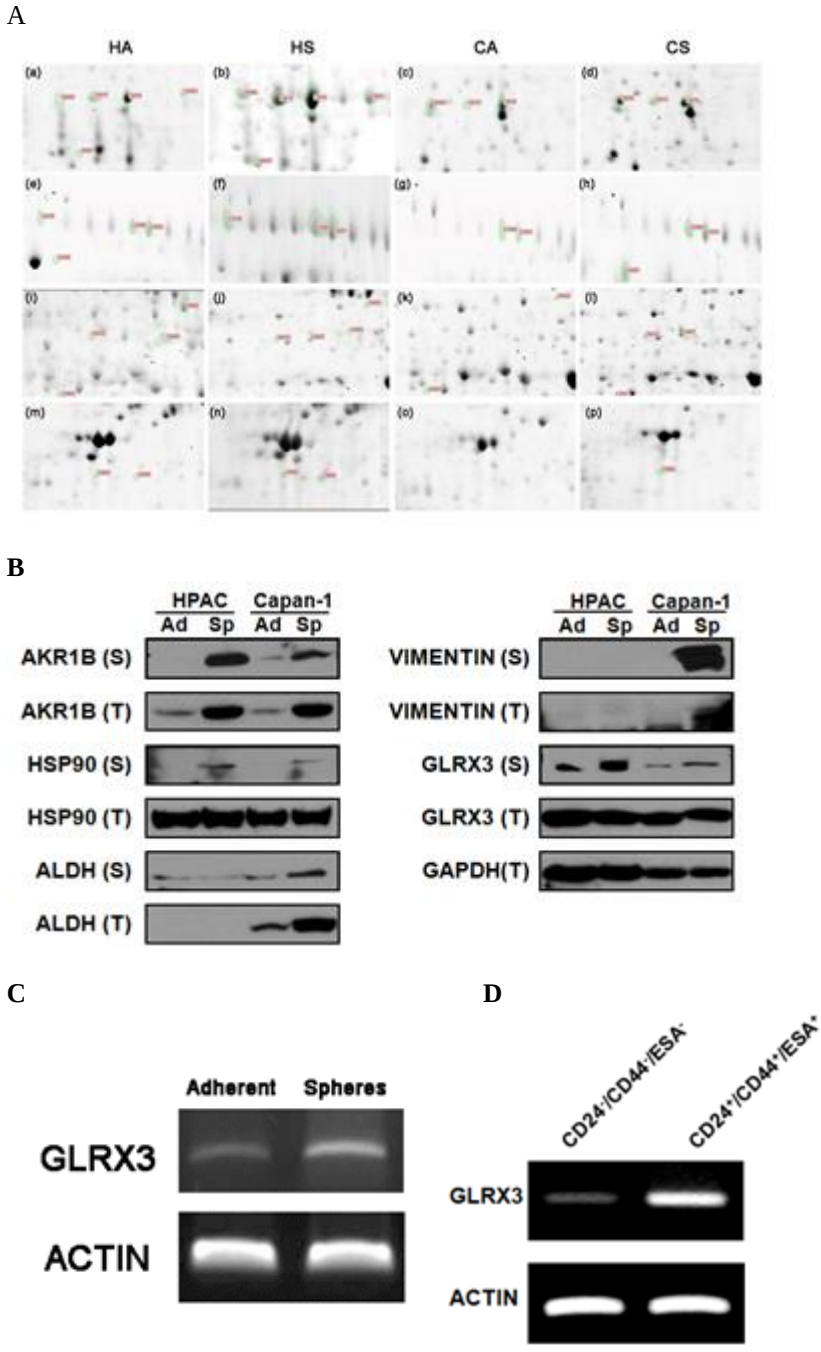


Figure 1. Differentially expressed spots in adherent cells and spheres. (A) 2-DE gel

image of secretome from adherent cells and spheres; adherent of HPAC (HA), spheres of HPAC (HS), adherent of CAPAN-1 (CA), and spheres of CAPAN-1 (CS). Representative five differentially expressed spots were highlighted in the adherent cells and spheres of HPAC 2DE gel image. (B) Confirmation of five differentially expressed spots in western blot analysis. (C) Semi-quantitative RT-PCR of GLRX3 levels in adherent cells and spheres. GLRX3 mRNA was overexpressed in spheres than adherent HPAC cells. (D) Semi-quantitative RT-PCR of GLRX3 levels in CD24+/CD44+/ESA+ cells and CD24-/CD44-/ESA- cells. HPAC cells were fluorescent stained with CD24-PE, CD44-APC, and ESA-FITC and isolated by FACS. GLRX3 mRNA was overexpressed in CD24+/CD44+/ESA+ cells than CD24-/CD44-/ESA- cells.

3. Expression of GLRX3 in pancreatic cancer patients and cell lines

To determine the expression of GLRX3 in human pancreatic cancer tissues, we performed immunohistochemical staining in pancreatic TMA for GLRX3. Immunohistochemical staining revealed strong cytoplasmic expression of GLRX3 in cancer cells (Fig. 2A(b)-(d)). Of 32 cases, 20 cases (62.5%) showed positive GLRX3 expression in cancer tissues. The islet cells also showed positive immunoreactivity. However, normal pancreatic ducts, acinar cells in normal tissues did not react with the antibody (Fig. 2A (a)).

In this study, GLRX3 was identified as a potential secretory biomarker for pancreatic CSCs, so we experimented to detect the GLRX3 in blood samples.

We detected GLRX3 in patient plasma samples by western blot analysis. To discard the 6 high-abundant proteins (albumin, transferrin, IgG, IgA, haptoglobin and anti-trypsin) within plasma, we used the multiple affinity removal column system[54]. The plasma samples from five healthy person, five patients with chronic pancreatitis, and twenty patients with pancreatic cancer were used for western blot analysis (Table 1). As shown in Figure 2B, expression of GLRX3 was increased in plasma of pancreatic cancer patients compared to plasma of normal or chronic pancreatitis patients. Expression levels of GLRX3 in pancreatic cancer were larger 8.8 fold than control plasma and 2.8 fold than chronic pancreatitis. Moreover, expression levels of GLRX3 in chronic pancreatitis were larger 3.1 fold than control plasma. These data confirmed that GLRX3 was secretory biomarker protein detected in human blood samples and up-regulated in pancreatic cancer patient's plasma than healthy person or chronic pancreatitis patients.

Next, to investigate level of GLRX3 in human pancreatic cancer cell lines, we performed western blot analysis. In human pancreatic cancer cell lines, GLRX3 was abundantly expressed (Fig. 2C). mRNA and protein expression profiles of GLRX3 in various pancreatic cancer cells were analyzed. Also, GLRX3 protein was expressed in cell lysates and culture medium from pancreatic cancer cell lines, but GLRX3 was not detected in normal pancreatic epithelial cell line; HPDE, which means GLRX3 expression and secretion from pancreatic cancer cell. All these results suggest that overexpressed and secreted GLRX3 in

pancreatic cancer has a potential as a biomarker for pancreatic cancer.

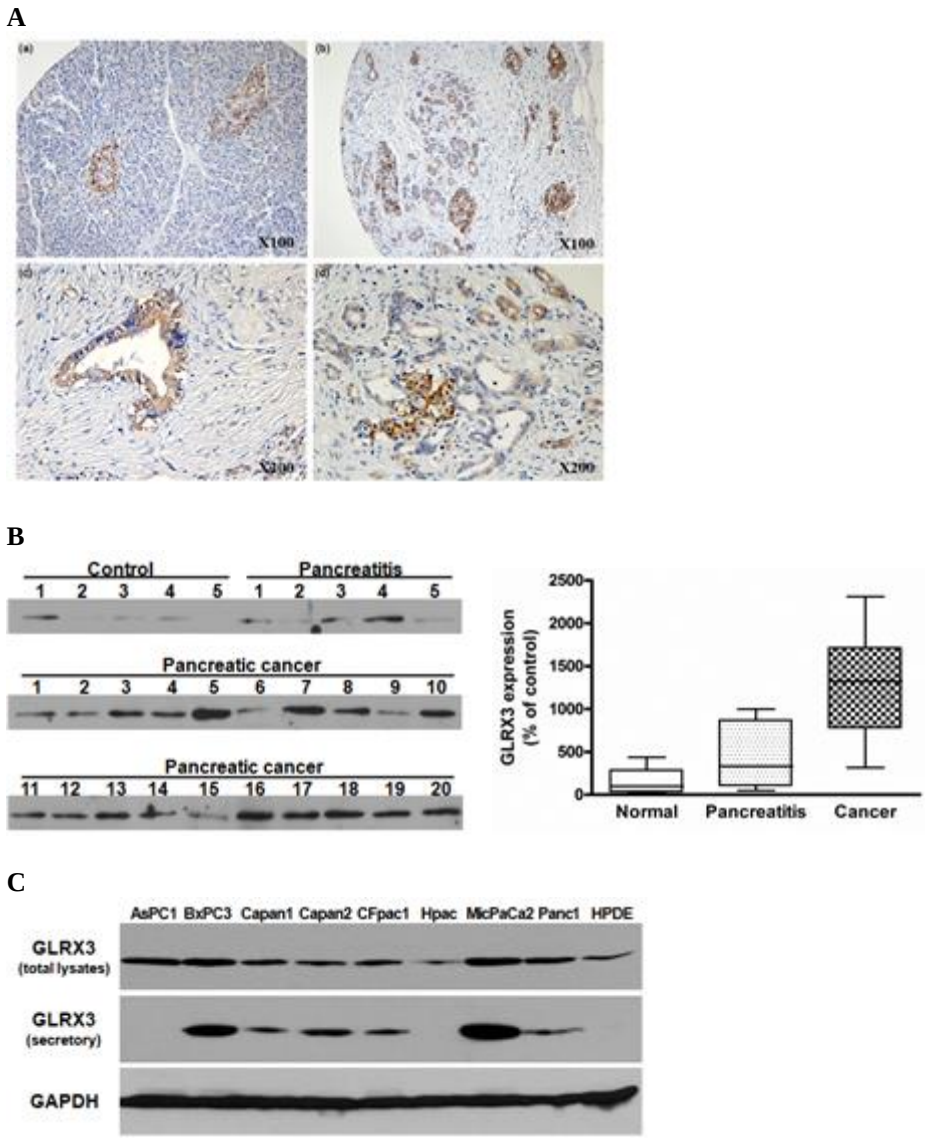


Figure 2. GLRX3 is overexpressed in pancreatic cancer patients and cell lines. (A) GLRX3 is overexpressed in pancreatic cancer tissues. Immunohistochemical staining

was performed on pancreatic TMA. Representative images show islet cells expressing GLRX3 in normal pancreas tissue (a), and overexpression in adenocarcinoma (b-d). (B) Western blot analysis was performed in depleted plasma of healthy person (n=5), chronic pancreatitis patients (n=5) and pancreatic cancer patients (n=20). GLRX3 is overexpressed in plasma of pancreatic cancer patients compared to plasma of healthy person and chronic pancreatitis patients. Values of GLRX3 expression were estimated by image analysis system (BAS2500, Fujifilm, Tokyo, Japan) and normalized to mean value of control group (regarded as 100%). (C) GLRX3 expression in pancreatic cancer cell lines. GLRX3 protein was expressed in cell lysates and culture medium from pancreatic cancer cell lines, but not detected in normal pancreatic epithelial cell line; HPDE.

Table 1. Plasma samples that were used for validation

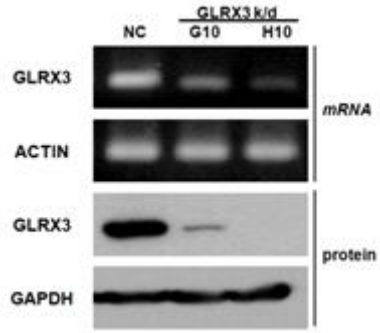
	Cases (n)	Gender	Age range (median)
NL	5	Male = 3	29-33 (33)
		Female = 2	
CP	5	Male = 4	30-63 (47)
		Female = 1	
PC	20	Male = 9	39-78 (60)
		Female = 11	

4. Knockdown effect of GLRX3 in pancreatic cancer cells by shRNA

To determine the role of GLRX3 in pancreatic cancer cells, shRNA targeting human GLRX3 (shGLRX3) or control vector (shControl) were stably transfected into HPAC cells and selected by puromycin. Selected clones of shGLRX3 transformed cells (G10 and H10) showed the similar manners of GLRX3 expression. mRNA and protein level of GLRX3 was reduced in GLRX3 knockdown cells, compared to control shRNA transfected cells (NC) (Fig. 3A).

The biological function of GLRX3 was evaluated by comparing cell growth and wound healing ability between control and GLRX3 knockdown cells. Cell proliferation was reduced in shGLRX3 cells than control cells (Fig. 3B). As shown in figure 3C, wound healing ability was reduced in shGLRX3 cells than control cells. To verify the function of GLRX3 in pancreatic cancer tumorigenicity in vivo, we injected GLRX3 knockdown or control cells subcutaneously into SCID mice and measured the resulting tumor growth after 14 weeks (Fig. 3D). GLRX3 knockdown cells (H10 clone) showed tumor formation in only 60% (3/5) of mice, while control cells showed tumor formation in 100% (5/5). Moreover, tumors derived from GLRX3 knockdown cells were 0.22 fold smaller than tumor derived from control cells (mean ±SEM; 83.61蘟.89 ㎣ vs. 379.9되.2 ㎣).

A



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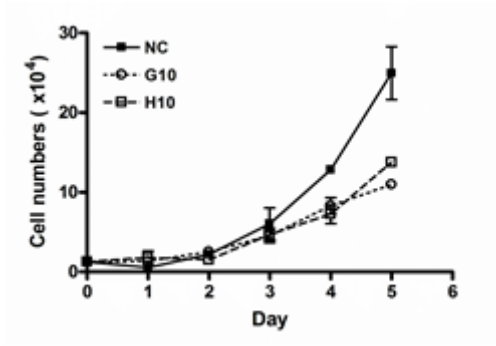

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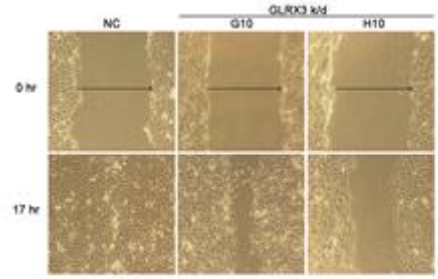

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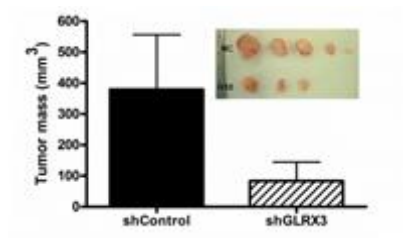

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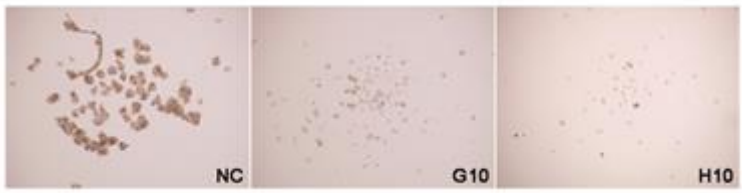

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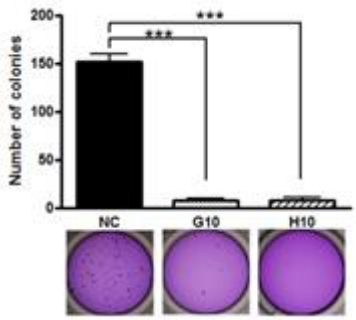

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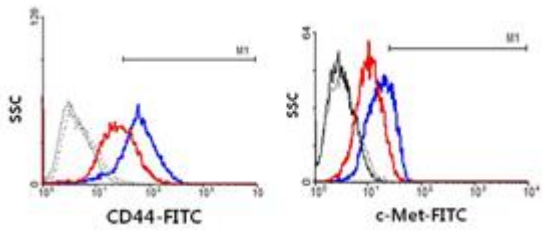
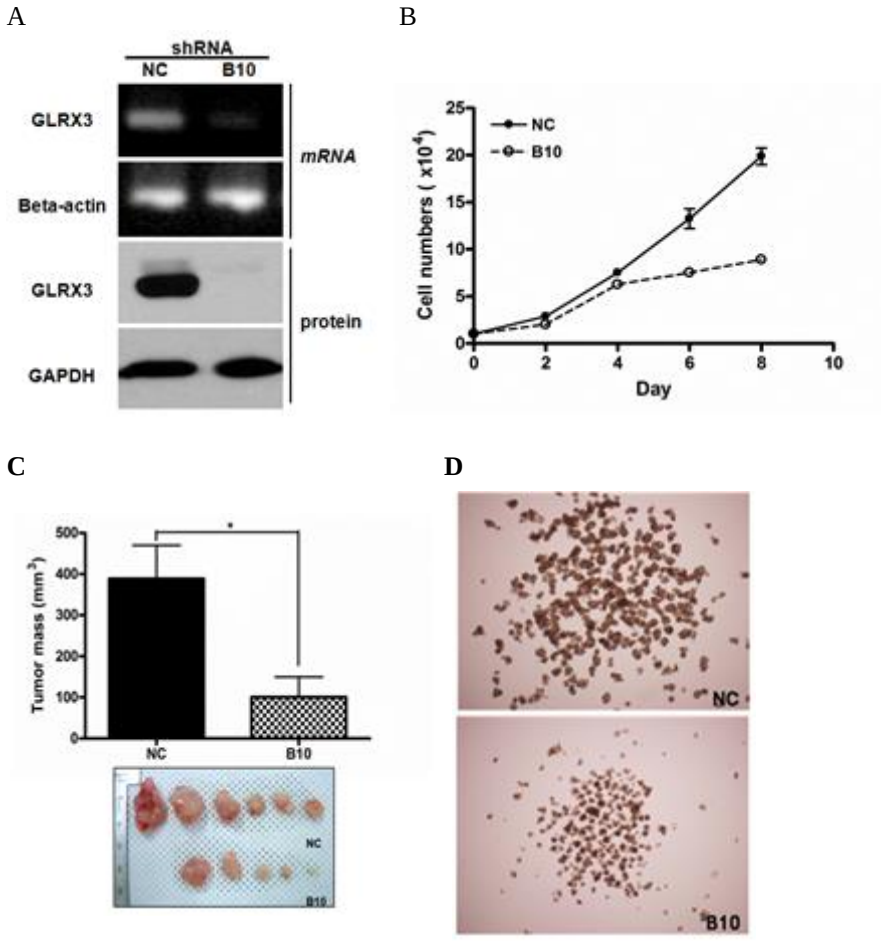


Figure 3. Effect of inhibiting GLRX3 in HPAC pancreatic cancer cells (A) Establishment of GLRX3 knockdown cells in pancreatic cancer cell line, HPAC. mRNA and protein level of GLRX3 was down-regulated by shRNA transfection. B-actin and GAPDH served as a loading control. (B) Cell proliferation was reduced in shGLRX3 transfected cells. The transfected cells (2×10^3 cells per well) were counted every 24 h using a hemocytometer. The experiment was done in triplicate and data are shown as the mean ±SEM. (C) Wound-healing ability was reduced by GLRX3 knockdown. (D) shGLRX3 cells formed no or smaller tumor than shControl cells in vivo. The shGLRX3 and shControl cells were injected into the flank of 6 week-old male SCID mice (n=5/each group) and monitored for 14 weeks. Representative grafts and image shows the results of tumor xenografts at the end of experiments. Data are shown as the mean ±SEM. (E) Formation of spheres was reduced by GLRX3 knockdown. $1x10^3$ cells/ml of shControl and shGLRX3 cells were cultured in sphere conditioned media on ultralow attachment plate for 7 days. Representative 4x photomicroscope images showed spheres 7 days after culture. (F) Colony formation was reduced by GLRX3 knockdown. shControl and shGLRX3 cells were cultured on agar media for 4 weeks. The experiment was done in triplicate and data are shown as the mean ±SD ($p<0.001$). Representative image (0.8x) and graph were achieved at the end of experiment. (G) Flow cytometry analysis of CD44 and c-Met expression. Histograms are for CD44-FITC or c-Met-FITC, respectively. CD44 positive cells were reduced in GLRX3 knockdown cells than control cells. c-Met positive cells were reduced in GLRX3 knockdown cells than control cells.

Knockdown effects of GLRX3 were subsequently confirmed in other pancreatic cancer cell line, CFPAC-1, In CFPAC-1, knockdown of GLRX3 lead to reduced cell proliferation and in vivo tumorigenic ability (Fig. 4). These data proposed that GLRX3 was involved in tumor initiation and growth of pancreatic cancer cells.



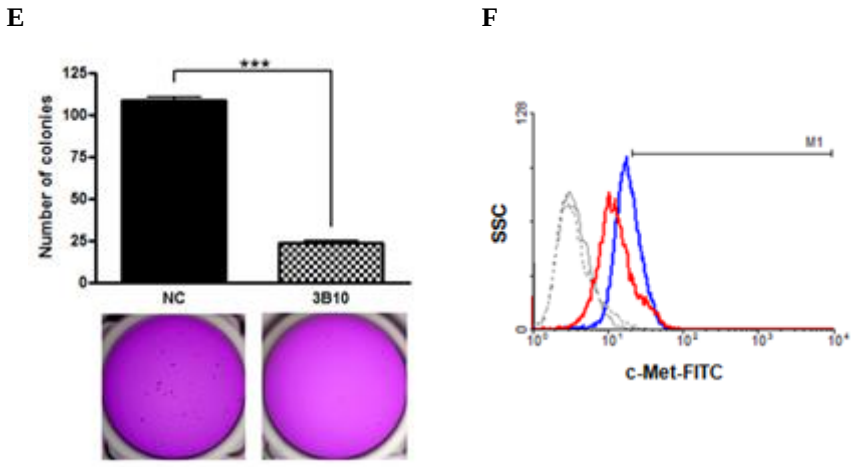


Figure 4. Effect of inhibiting GLRX3 in CFPAC-1 pancreatic cancer cells (A) Establishment of GLRX3 knockdown cells in pancreatic cancer cell line, CFPAC-1. mRNA and protein level of GLRX3 was down-regulated by shRNA transfection. B-actin and GAPDH served as a loading control. (B) Cell proliferation was reduced in shGLRX3 transfected cells. The transfected cells (2×10^3 cells per well) were counted every 24 h using a hemocytometer. The experiment was done in triplicate and data are shown as the mean ±SEM. (C) shGLRX3 cells formed no or smaller tumor than shControl cells in vivo. The shGLRX3 and shControl cells were injected into the flank of 7 week-old male SCID mice (n=6/each group) and monitored for 9 weeks. Representative grafts and image shows the results of tumor xenografts at the end of experiments. Data are shown as the mean ±SEM. (D) Formation of spheres was reduced by GLRX3 knockdown. 1×10^3 cells/ml of shControl and shGLRX3 cells were cultured in sphere conditioned media on ultralow attachment plate for 7 days. Representative 4x photomicroscope images showed spheres 7 days after culture. (E) Colony formation was reduced by GLRX3 knockdown. shControl and shGLRX3 cells were cultured on agar

media for 4 weeks. The experiment was done in triplicate and data are shown as the mean ±SD ($p<0.001$). Representative image (0.8x) and graph were achieved at the end of experiment. (F) Flow cytometry analysis of c-Met expression. Histograms are for c-Met-FITC, respectively. c-Met positive cells were reduced in GLRX3 knockdown cells than control cells.

5. Effect of GLRX3 on pancreatic CSCs phenotype

To evaluate the role of GLRX3 in pancreatic CSCs self-renewal and long-term growth potential, we performed in vitro tumorspheres assay and colony forming assay using GLRX3 knockdown and control HPAC cells. As a result, GLRX3 knockdown cells did not form tumorspheres, while control cells formed tumorspheres (Fig. 3E). Moreover, colony formation was inhibited significantly in GLRX3 knockdown cells compared to control cells in soft agar (mean ±SD; H10; 8.000̓.944 vs. G10; 25.50̦.873 vs. NC;152ꞌ.874, Fig. 3F). In GLRX3 knockdown CFPAC-1 cells, sphere and colony formation were also reduced than control cells (Fig. 4D and E). These results suggest that GLRX3 has a role in self-renewal and long-term survival in pancreatic CSCs.

Next, the expression of known pancreatic CSCs markers was documented by flow cytometry. As shown in Figure 3G, expression of CD44+ in GLRX3 knockdown HPAC cells was significantly reduced to 24.87ꞌ.410% (mean ±SD) compared with 88.95ɜ.850% positive expression in the control cells. The expression of c-Met was reduced to 5.7ः.301% (mean ±SD) in GLRX3

knockdown HPAC cells, compared with a 23.81±.348% in the control cells. CD44 and c-Met were well known CSCs markers and CD44+/c-Met high cells were the most tumorigenic in vivo[55]. Furthermore CD44 was reported to promote c-Met activity[56]. These results suggested GLRX3 may be involved in CD44 and c-Met signaling pathway. In GLRX3 knockdown CFPAC-1 cells, the expression of c-Met was also reduced to 17.317±.887%, compared with a 33.873±.602% in the control cells (Fig. 4F).

6. Regulation of GLRX3 on chemosensitivity via c-Met signaling

To evaluate if there were any changes in c-met level and downstream signal by GLRX3 knockdown, we assayed c-met downstream signal molecules by western blot analysis. As a result, c-Met, PI3K and phosphorylation of AKT were reduced in GLRX3 knockdown HPAC and CFPAC-1 cells. These results indicated that GLRX3 was involved in Met/PI3K/AKT pathway. Furthermore another chemosensitivity related molecule, ABCG2 was reduced in selected clones of shGLRX3 transformed cells (G10 and H10). On the other hand, siRNA targeting Met was reduced the expression level of c-Met, PI3K, phosphorylation of AKT but not GLRX3 (Fig. 5B and Fig. 6A). These results demonstrated that GLRX3 regulates MET/PI3K/AKT pathway.

Since gemcitabine is the standard treatment for pancreatic cancer and high level of c-Met has been reported to be correlated with poor survival and gemcitabine resistance in pancreatic cancer patients[57], we examined the

chemosensitivity to gemcitabine in GLRX3 knockdown cells. As a result, GLRX3 knockdown induced the treatment response to gemcitabine in HPAC and CFPAC-1 cells (Fig. 5C and Fig. 6B). Taken together, these results indicated that GLRX3 regulate chemosensitivity to gemcitabine via MET/PI3K/AKT/ABCG2 pathway.

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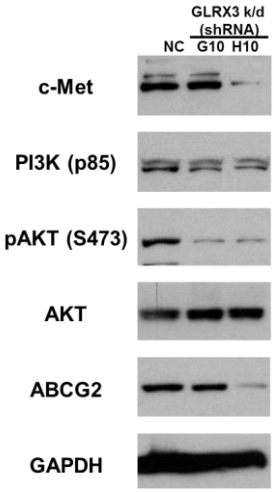

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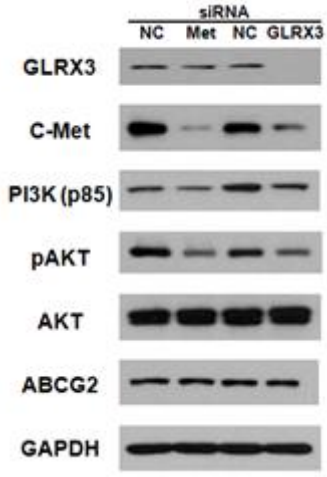

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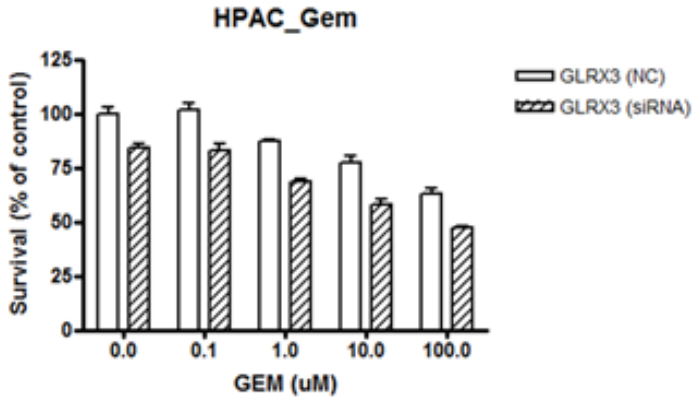

Figure 5. GLRX3 regulates chemosensitivity via c-Met signaling in HPAC cells

(A) ABCG2 expression was down-regulated by shRNA transfection. (B) Effect of GLRX3 or Met silencing on Met/PI3k/AKT singling. The expression of c-Met, PI3K, phosphorylation of AKT was reduced by siRNA transfection. (C) Effect of GLRX3 knockdown on the cytotoxicity of 72 hours inoculation with gemcitabine in GLRX3 or Met silenced HPAC cells compared to negative control cells (NC). GLRX3 knockdown induced the treatment response to gemcitabine. Data are shown as the mean ±SD.

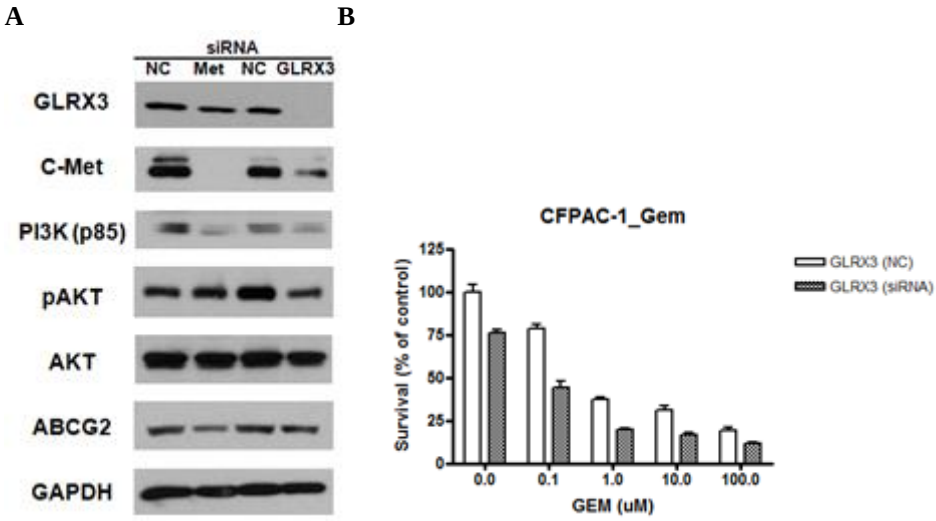

Figure 6. GLRX3 regulates chemosensitivity via c-Met signaling in CFPAC-1 cells

(A) Effect of GLRX3 or Met silencing on Met/PI3k/AKT/ABCG2 singling. The expression of c-Met, PI3K, phosphorylation of AKT was reduced by siRNA transfection. (B) Effect of GLRX3 knockdown on the cytotoxicity of 72 hours inoculation with gemcitabine in GLRX3 or Met silenced CFPAC-1 cells compared to negative control cells (NC). GLRX3 knockdown induced the treatment response to gemcitabine. Data are shown as the mean ±SD.

7. GLRX3 is a potential diagnostic marker for pancreatic cancer

To compare the expression level of GLRX3 and CA19-9, we assayed GLRX3 in patient serum samples by ELISA using 70 individual serum samples, healthy person (n=10) and pancreatic cancer patients (n=60) (Table 2). Since used commercial ELISA kit was more sensitive to serum than plasma, we used serum sample for ELISA. Median serum levels of CA19-9 in healthy person and pancreatic cancer patients were 7.0 (0.9-21.5 U/ml) and 491.5 (4-20000 U/ml), and the difference was statistically significant ($p<0.0001$) (Fig. 7A). Median serum levels of GLRX3 in healthy person and pancreatic cancer patients were 13.27 ng/ml (range; 1.94-27.18 ng/ml) and 70.84 ng/ml (range; 7.5-357.64 ng/ml), respectively. Post-hoc analysis (bonferroni correction) revealed that the difference between serum level of GLRX3 in healthy person and pancreatic cancer patients was significant ($p<0.0001$) (Fig. 7B). To evaluate the diagnostic performance of serum GLRX3 and CA19-9 levels for pancreatic cancer, we calculated the AUC using a ROC curve. For GLRX3, the AUC was 0.869 (95% CI: 0.8490, 0.9510), and that of CA19-9 was 0.962 (95% CI: 0.8532, 0.9499) with no difference ($p=0.3462$) (Fig. 7C). With the cut-off value of 28.067 ng/ml, the sensitivity and specificity of GLRX3 for diagnosis of pancreatic cancer were 80.0% and 100%. For the CA19-9, the sensitivity and specificity were 80.3% and 100% at a cut off value of 37 U/ml. When GLRX3 and CA19-9 were combined, the AUC (0.978: 95% CI: 0.9753, 0.9999) was increased compared with GLRX3 or CA19-9 alone ($p<0.0001$) (Fig. 7C). These results indicated

that GLRX3 alone or combined with CA19-9 is a potential biomarker for pancreatic cancer.

Table 2. Demographics and clinical characteristics of the healthy person and patients' cohort

GLRX3	Cases (n)	Gender	Age range (median)	Stage (AJCC)
NL	10	Male = 6	40-63 (47.5)	
		Female = 4		
PC	60	Male = 40	38-89 (64.5)	Ⅰ = 0
		Female = 20		Ⅱ = 25
				Ⅲ = 11
				Ⅳ = 24

CA19-9	Cases (n)	Gender	Age range (median)	Stage (AJCC)
NL	40	Male = 29	26-63 (45.5)	
		Female = 11		
PC	66	Male = 44	38-89 (65)	Ⅰ = 0
		Female = 22		Ⅱ = 27
				Ⅲ = 13
				Ⅳ = 25

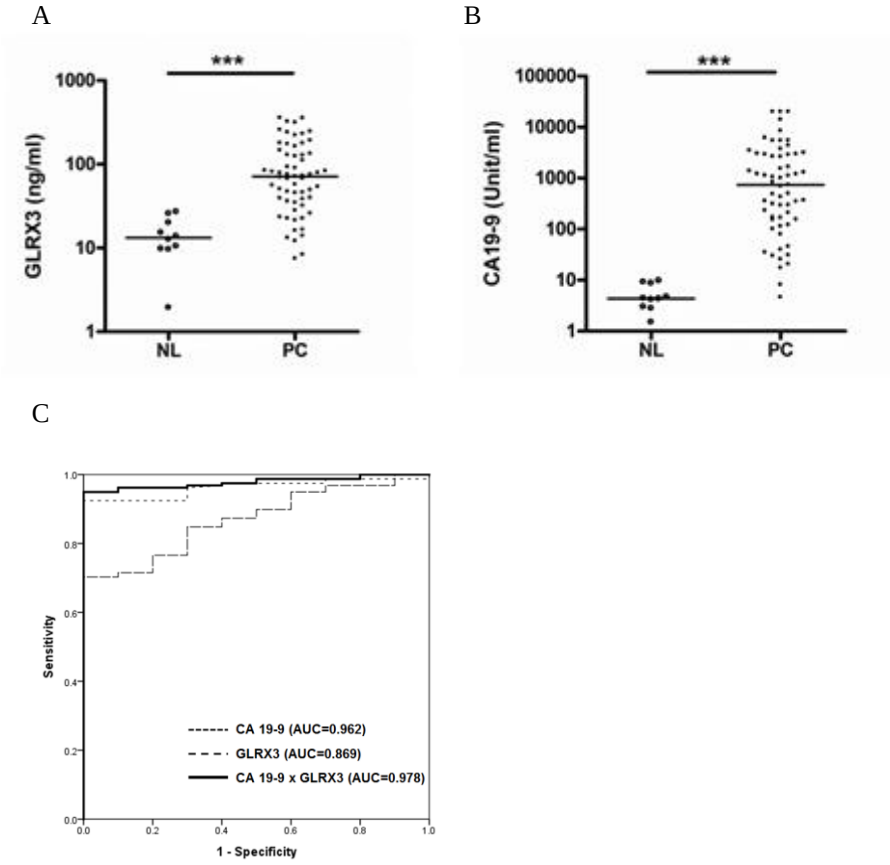

Figure 7. GLRX3 is overexpressed in serum of pancreatic cancer patients compared to healthy person. (A, B) Dot plot for the serum level of GLRX3 and CA19-9. The horizontal line represents the median. The serum level of GLRX3 and CA19-9 were significantly different between pancreatic cancer patients and healthy person ($p<0.001$). (C) ROC curves of pancreatic cancer patients vs. healthy person for GLRX3, CA19-9, as well as their mathematical combination. When GLRX3 and CA19-9 were combined, the AUC was increased compared with GLRX3 or CA19-9 alone ($p<0.0001$).

IV. DISCUSSION

Sphere culture method is one of useful methods for CSCs enrichment including side population and sorting method by specific markers. In previous study, sphere cells were characterized by increased Hedgehog, Notch, and WNT signaling molecules as well as increased tumorigenic and metastatic potential than adherent cells. In this study, we identified novel secreted markers for the pancreatic CSCs in spheres compared with adherent cells. GLRX3 was first identified as a PKC θ-interacting protein in early 2000s, and studied about stress response in immune cells and hypertrophy in heart[15,16,18]. Recently, correlation of GLRX3 expression and human cancer has been reported. GLRX3 was overexpressed in colon, lung, breast and nasopharyngeal cancer, and GLRX3 expression was reported to have positive correlation of patient survival[21,22,24,58]. Although GLRX3 was potential secretory protein, there was no report about detection of GLRX3 in patient blood or cell culture medium. In our study, overexpression of GLRX3 was consistently detected in blood of pancreatic cancer patients and media of cultured cells as well as in tissues of pancreatic cancer patients. Furthermore, expression level of GLRX3 was increased in plasma of pancreatic cancer patients than in plasma of healthy person or chronic pancreatitis patients. Furthermore using commercial ELISA kit, GLRX3 was highly secreted into serum of pancreatic cancer patients than healthy control serum. As a diagnostic marker, GLRX3 was similar sensitive as CA19-9 and when combined with CA19-9 for pancreatic cancer. These data indicated that

GLRX3 is a potential diagnostic biomarker for pancreatic cancer.

Previous reports did not fully demonstrate the function and role of GLRX3 in cancer progression and cancer cell stemness. In our study, GLRX3 was identified as a marker for pancreatic CSCs as well as a regulator for maintenance of CSCs phenotype. In GLRX3 knockdown study, GLRX3 had a role in cell proliferation, metastasis, in vivo tumor formation and tumor growth as well as spheres formation and colony formation. There were several populations for pancreatic CSCs, but these CSCs populations were reported to be partially overlapped. In our study, GLRX3 was increased in spheres than adherent cells, and also increased in CD24+/CD44+/ESA+ cells than CD24-/CD44-/ESA- cells among pancreatic cancer cells. Knockdown of GLRX3 reduced the proportion of CD44 and c-Met positive cells and also decreased tumor formation in SCID/NOD mice. CD44, CD24 and ESA was first described as markers for pancreatic CSCs, and CD24+/CD44+/ESA+ cells were reported to be more tumorigenic than other populations. CD44 is a membrane receptor for hyaluronan, and is involved in cell motility and metastasis[59]. CD44 is frequently used for the isolation of CSC in various type of cancer, and expression level of CD44 is correlated with poor prognosis of pancreatic cancer with gemcitabine resistance[60]. c-Met is the receptor tyrosin kinase for hepatocyte growth factor/scatter factor and was its activity was promoted by CD44[55,56]. CD44+/c-Met high cells were the most tumorigenic than low c-Met expressed cells without CD44 in vivo[9]. Recent study has shown

that GLRX3 regulates cell growth and metastasis via EGFR/AKT pathway in nasopharyngeal carcinoma[58]. c-Met is an upstream molecule of PI3K/AKT signaling pathway. Previous studies reported that targeting of Met pathway overcomes chemo-resistance and stem cell signaling in pancreatic cancer[61,62] and Met inhibitor resulted in chemosensitivity of cancer stem cells in gastric cancer[63]. In our study, knockdown of GLRX3 down-regulated Met/PI3K/AKT pathway and enhanced chemosensitivity to gemcitabine in pancreatic cancer cells.

In summary, our study describes the secretory proteomic profile for the pancreatic CSCs including already known markers and novel marker, GLRX3. Level of GLRX3 expression was elevated in pancreatic cancer patient tissues, and blood and cancer cell lines. GLRX3 was involved in cancer cell proliferation, migration, invasion, tumorigenesis, and maintenance of CSC properties. GLRX3 regulated CSCs phenotype through CD44 and c-Met signaling pathway. These suggest that GLRX3 is a new potential biomarker for pancreatic cancer as well as a target for therapeutics of pancreatic CSCs properties.

V. CONCLUSION

In this study, I investigated the secretory proteomic profile of the pancreatic CSCs including GLRX3. GLRX3 was highly expressed in the tissue and blood of pancreatic cancer patients. GLRX3 was involved in cancer cell proliferation, migration, invasion, maintenance of CSC properties. These suggest that GLRX3 is a potential biomarker for pancreatic cancer as well as a therapeutic target of pancreatic CSCs.

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ABSTRACT (IN KOREAN)

췌장암 줄기 세포 특성에 기반한 췌장암 신규 분비 바이오마커로서의 GLRX3의 역할 규명

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정 문 재

췌장암은 전신항암약물치료나 방사선 요법과 같은 기존의 치료법에 반응을 보이지 않을 뿐만 아니라, 조기 진단이 어려워 현재 가장 치명적인 인체 암 중 하나로 받아들여지고 있다. 암 줄기 세포는 암의 발생, 암 진행 및 재발과 관련되어 있으며, 췌장암 줄기 세포와 관련된 바이오마커가 일부 보고되고는 있지만, 그 기능과 기전에 대해서는 명확하게 밝혀진 바가 없다. 이번 연구에서는 췌장암 줄기 세포의 새로운 바이오마커를 찾기 위한 방법으로 스피어 배양법(sphere culture method)을

이용하여 췌장암 줄기 세포를 농축하였다. 그리고 스피어세포와 일반세포의 배양 배지로부터의 세크리톰(secretome)을 2차원 겔 전기영동 및 MALDI-TOF 질량 분석 방법을 이용하여 분석하였다. 스피어세포와 대조군 암세포간에 2배 이상의 발현 차이를 보이는 200개의 단백질을 확인하였고, 이중 55개의 단백질이 스피어세포에서 발현이 증가 되어 있었다. 이전 보고들에서 암세포 또는 암 줄기 세포와 관련이 있는 것으로 알려져 있는 HSP90AB1, ALDH, vimentin, AKR, GLRX3 (Glutaredoxin3)의 발현이 실제로 췌장암 세포주에서 증가 되어 있음을 웨스턴블롯분석을 통해 확인할 수 있었다. 이렇듯 발현이 증가된 단백질 중 GLRX3를 새로운 췌장암 줄기 세포의 바이오마커로 선택하여 검증을 진행하였다. 췌장암 줄기 세포의 표지자로 이미 알려진 CD24+/CD44+/ESA+ 세포에서도 GLRX3의 과발현이 확인되었다. 또한 췌장암 환자의 조직, 혈액 및 여러 세포주에서도 GLRX3가 과발현됨을 입증할 수 있었다. 췌장암의 발생 및 암 줄기 세포의 표현형과 관련하여 GLRX3의 역할을 규명하기 위하여 췌장암 세포주에서 shRNA 와 siRNA transfection을 시행하였고, GLRX3의 발현을 억제함으로써

암세포 증식, 암세포 이동, 클론 생성 및 스피어세포 형성 등이 감소됨을 확인할 수 있었다. 또한 GLRX3 발현 억제를 통해 SCID 마우스에서 종양의 형성과 성장이 억제됨을 확인할 수 있었다. 전신항암약물치료 감수성에 대한 영향과 관련하여, GLRX3 발현 억제를 통해 췌장암 세포의 gemcitabine 약제에 대한 감수성이 증가 되는 것으로 확인 되었다.

이상의 결과를 바탕으로, 분비 단백질인 GLRX3가 췌장암 환자에서 예후와 재발을 예측할 수 있는 새로운 바이오마커로서 가능성이 있음을 확인하였고, 향후 췌장암 줄기 세포를 억제하는 새로운 치료 전략으로서 GLRX3의 발현 억제 약물에 대한 연구가 필요할 것으로 생각된다.

핵심 되는 말: 췌장암, 암 줄기 세포, 바이오마커, Glutaredoxin3, 항암약물치료