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Exosomal AZGP1 as a New Diagnostic Marker Candidate for Pancreatic Cancer

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ABSTRACT

Objective: Pancreatic cancer diagnosis is difficult because it usually doesn't have symptoms until cancer migration. So, it was called the 'King of cancer'. To diagnose pancreatic cancer, commonly CA 19-9 was used, but CA 19-9 has some problems that make it difficult to use solely to diagnose pancreatic cancer. Therefore, new biomarkers for prognosis and diagnosis of pancreatic cancer were urgently required.

Design: To find exosomal biomarkers, screening was performed using qRT-PCR with exosomal protein coding 7145 genes. Among the candidates, AZGP1 of cell pellets and exosomes was detected by western blot. To check whether exosomal AZGP1 affects cancer migration, the transmembrane assay was performed.

Result: The gene expression of AZGP1 is only detected in LNCaP and AsPC-1 among tested samples. Interestingly, AZGP1 was detected in the entire pancreatic cancer cell lines tested with different 2 types in the exosome. SNU pancreatic cancer cell lines showed only one type (lower type) of AZGP1. According to transmembrane assay, a lower form of exosomal AZGP1 could induce cancer migration better than higher type of AZGP1 and negative control.

Conclusion: AZGP1 can detect all of the pancreatic cancer cell lines. According to proteomics data, exosomal AZGP1 expression and pattern were different between pancreatic cancer patients and healthy people. Exosomal AZGP1 should be a good candidate to diagnose pancreatic cancer. Moreover, the lower type of exosomal AZGP1 can induce cancer migration better than other types of AZGP1, and this suggested that Korean-derived cancer cell lines might migrate faster than others.

Keywords: Pancreatic cancer; Exosome; Prognosis; Diagnostic marker; Migration

INTRODUCTION

Pancreatic cancer is the most lethal malignant cancer with an overall 5-year survival rate of 11% [1]. Pancreatic cancer is also a major concern in the West world [2]. The United States Surveillance, Epidemiology, and End Results Program researched from 1973 to 2014, the age-standardized occurrence rate of pancreatic cancer increased by 1.03% per year. It can eventually be predicted that it will become the second most deadly cancer in the United States by 2030 year [3,4]. Pancreatic cancer is caused by damage to the DNA of the pancreas, which should promote abnormal growth of cells. Symptoms of pancreatic cancer appear after several stages and are initially confused with other diseases to the extent that it is difficult to think of it as a serious disease [5]. Tumours within the pancreas are made up of small lumps, which are difficult to detect with current imaging techniques [6]. For these reasons, when pancreatic

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cancer is diagnosed, 80%-90% of patients are diagnosed with advanced stages, and treatment is impossible at the time of diagnosis. In addition, patients who are possible for surgical resection also show a short survival rate [7].

MRI, CT, and ultrasound imaging tests are commonly used to diagnose pancreatic cancer, but are still insufficient to detect small sizes of cancers [8,9]. In addition, endoscopic ultrasound-guided fine needle aspiration allows for preoperative biopsy with various complications [10,11]. Therefore, carbohydrate antigen 19-9 is currently being used for the diagnosis of pancreatic cancer. This became known in the 1980s with the development of the N19-9 monoclonal antibody [12]. At first, it was developed for the diagnosis of colorectal cancer [13], but it has been found that it is easier to diagnose pancreatic cancer or biliary tract cancer [14]. The sensitivity of the CA 19-9 test was reported to be 79% and specificity to 82% [15]. However, it is also increased in gastric cancer, lung cancer, thyroid cancer, ovarian cancer, liver cancer and various benign diseases [16-20]. In addition, since 5% of the total population is known as Lews a-b-genotype, even if a malignant tumor appears, it is difficult to diagnose pancreatic cancer with CA19-9 alone [21]. Therefore, new biomarkers that can diagnose pancreatic cancer are needed.

AZGP1 (2-zinc-glycoprotein) is a secreted glycoprotein with a size of 41 kDa. The exact mechanism of AZGP1 and its effect on tumor growth have not yet been elucidated, but its association with cancer is reported [22]. AZGP1 is produced by secretory epithelial cells and found in body fluids [23-25]. AZGP1 is related to cachexia of cancer, which may affect metastasis of cancer. According to proteomics analysis, AZGP1 is differently expressed in the plasma of pancreatic cancer patients compared to non-disease patients and chronic pancreatitis controls. Based on these results, we tested whether AZGP1 can be used as a pancreatic cancer biomarker and contribute to cancer metastasis.

METHODS AND MATERIALS

Cell Culture

Pancreatic cancer cell lines (AsPC-1, Panc-1, MIA PaCa2 and Capan-1), Prostate cancer cell lines (DU145, PC3 and LNCaP clone FGC), Pancreatic cancer SNU cell lines (SNU-213, 2466, 2469, 2485, 2491, 2543, 2570, 2571 and 2608), Keratinocyte cell line (HaCaT) were bought at Korean Cell Line Bank. Prostate normal cell line (RWPE-1) was bought at ATCC. All of the cells cultured at appropriate medium (DMEM, RPMI and Keratinocyte-SFM).

Quantitative Real-Time PCR (qRT-PCR)

The qRT-PCR was performed the same way as before [26]. Cells were seeded in 6 well plates and incubated for 24 hours. Medium was removed and 500 ul of Trizol (Life Technology, Thermo Fisher Scientific, USA) was directly added. cDNA was synthesized using 1 ug of total RNA. The primer sequences listed in **Table 1**.

Table 1: The primer list for Quantitative Real-Time PCR.

Gene	5'-3'	Primer sequence	Annealing temp. (C°)	Reference
AZGP1	Forward	AAA TAT CCT GGA CCG GCA AG	55.9	In this study
	Reverse	GAC TGG TAA GTG CCA TTT CC	54.5	
GAPDH	Forward	GTG AAG GTC GGA GTC AAC G	57.1	26
	Reverse	TGA GGT CAA TGA AGG GGT C	55.3	

Real-time PCR was performed using QGreen 2x SybrGreen qPCR Master Mix (CellSafe, Korea) with cDNA as a template. GAPDH was used as a quantitative control. Gene expression was calculated as the fold change value and indicated as delta-Cq.

Exosome Purification and Western Blot Analysis

Because of the difference of amount of exosome produced and variable doubling time of cells between the cancer cell types, the cell line was grown to 60% of the plate. For quantitative comparison for western blot analysis, the volume of medium was adjusted. Mediums were changed with serum free medium and incubated for 24 hours. Cells were centrifuged at 2000 grams for 30 minutes and 8 ml of supernatant was transferred. Exosome was isolated with the medium using Total Exosome Isolate (Invitrogen™, #4478359) based on the manufacturer's protocol. After purifying the exosome, exosomes were kept at -78°C before use.

Equal volume (30 µl) of exosomes was separated at 10%, and 12% acrylamide SDS-PAGE gel.

Total proteins extracted from cells at the time to extract exosome samples were used for the cell pellet control. First antibody [AZGP1 (#PA5-13580, Invitrogen), CD9 (#ab236630, abcam)] and secondary antibody [2nd anti-Rabbit (#ab6721, Abcam)] were used in this study.

Transmembrane Assay

The slightly modified transmembrane assay was used [27]. A549 cells (2 × 105 cell/ml) in the 750 µl of serum-free medium were added to the upper chamber with an exosome. In the down chamber, 1.5 ml of serum medium was added. After 24 hours of incubation, chambers were washed with PBS to remove the non-migrated cells. Migrated cells were stained with 1% crystal violet. After 15 minutes, the cells were observed using a micro-

scope (EVOS FL, Invitrogen™).

Statistics

All experiments were repeated more than three times independently. Results are indicated as mean values ± standard deviation. Statistically significant differences were analyzed using a GraphPad Prism. When only two groups were compared, the student's t-test was used (***: P<0.01).

RESULTS

General Schemes for Candidate Identification

Exosomal protein coding 7145 genes of different types of cancer were extracted from Extracellular vesicles database; Vesiclepedia (http://microvesicles.org/). Except reported protein as a biomarker, primers for candidate genes were made to check mRNA expression using Quantitative Real-Time PCR [28].

The Expression of AZGP1 was Detected in Pancreatic Cancers

The expression of AZGP1 mRNA of cancer cells was tested with Quantitative Real-Time PCR. ΔCq values of AZGP1 mRNA were 0.004 at LNCaP clone FGC, and 0.006 at AsPC-1 cell line (Figure 1). Even though AZGP1 is considered a diagnostic marker for prostate cancer [29,30], AZGP1 mRNA expression of AsPC-1 was higher than that of LNCaP clone FGC. We suggest that AZGP1 should be better to diagnose pancreatic cancer.

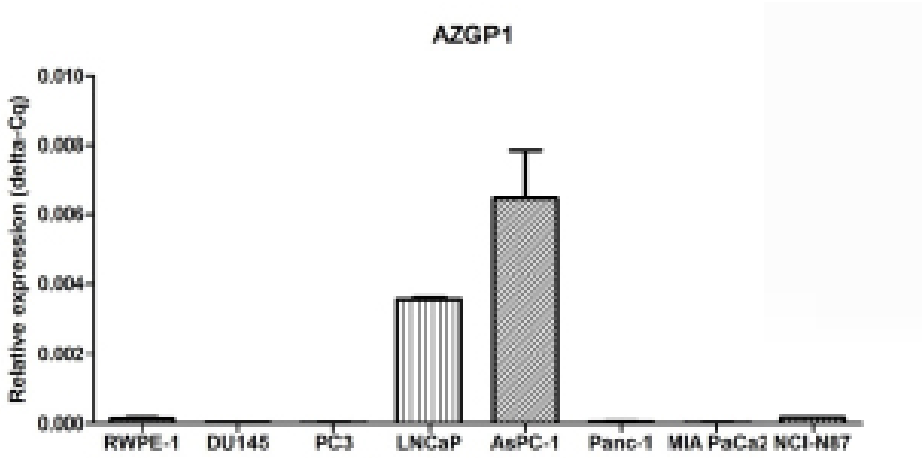

Figure 1: mRNA of AZGP1 only expressed in AsPC-1 and LNCaP cell line, AZGP1 of various cell lines was detected by Quantitative Real-Time PCR. GAPDH was used as innate reference for gene expression.

Interestingly, all of the pancreatic cancer cell lines showed similar expression of AZGP1 in the cell pellet sample (Figure 2). In the previous proteomic research, human plasma samples from patients with pancreatic cancer compared with healthy and chronic pancreatic control samples showed that a higher amount of AZGP1 was detected in patients [31]. So, we used an exosome to detect exosomal AZGP1. All of the cell lines showed the protein band at 48 kDa, except AsPC-1 and DU145 (lower than 48 kDa band). CD9 was used as an exosome marker (Figure 3).

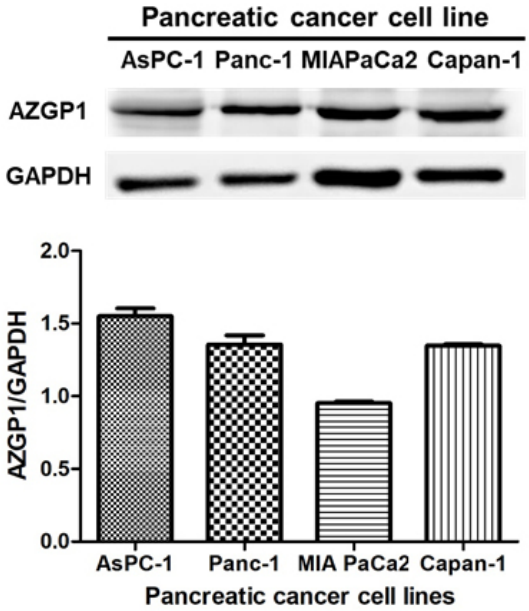

Figure 2: All of the pancreatic cancer cell lines have AZGP1 band, AZGP1 expression of pancreatic cancer cells was detected by western blot assay. Cell pellets that were extracted at the time of preparing the exosome were used. GAPDH was used as an innate control. The bands were quantified using Image J

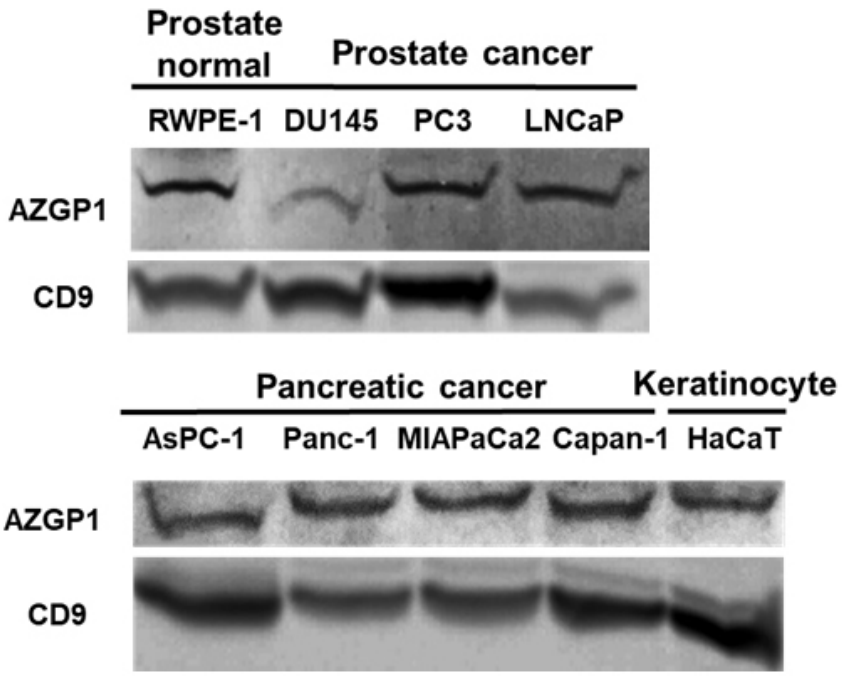

Figure 3: Exosomal AZGP1 was detected two types in cancer cell line; The exosome was isolated from the cell culture medium. When cells were grown 60% of plates, the media was changed to serum free. After 24 hours, the media was prepped and the exosome was isolated from the media. CD9 was used as an exosome marker.

Only Lower Form of AZGP1 was detected in SNU Pancreatic Cancer Cell Line

SNU (Seoul National University) cell lines have been made from Korean cancer patients since 1982 [32]. In the SNU cell lines of pancreatic cancer, AZGP1 was detected at the same size (**Figure 4A**). To compare the band size of **Figure 4A**, the exosomes of SNU-2469, SNU-2491, AsPC-1 and Panc-1 were analysed. The band of AZGP1 of only Panc-1 showed at the 48 kDa but the band of SNU-2469, SNU-2491 and AsPC-1 showed lower than that (**Figure 4B**).

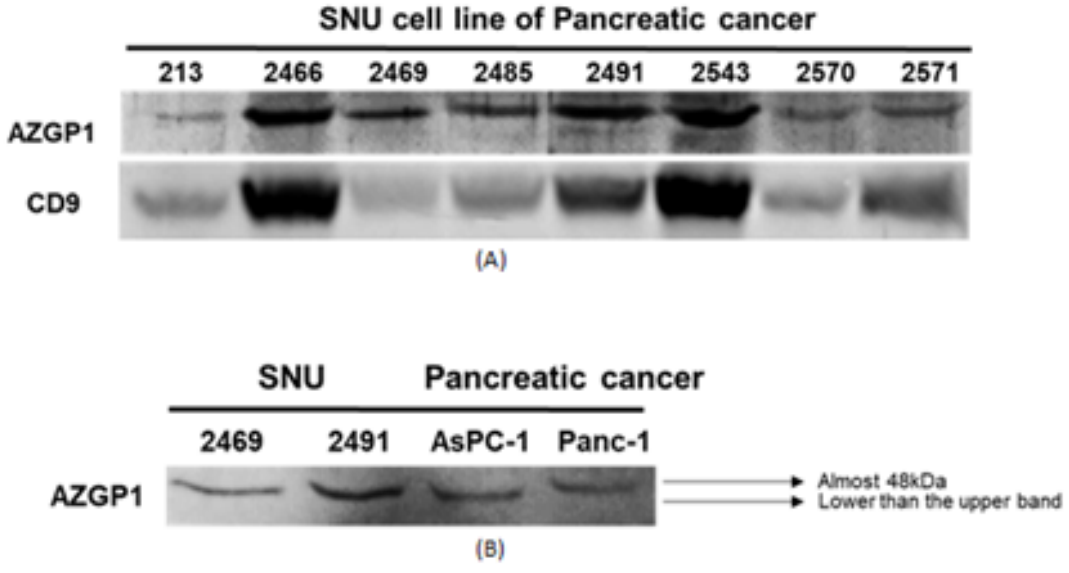

Figure 4: (A) SNU pancreatic cancer cell lines only have lower type of exosomal AZGP1; SNU pancreatic cancer cell lines have same size of AZGP1 Exosomes of SNU pancreatic cancer cell line were isolated and analysed with same way with previous experiment (B) SNU pancreatic cancer cell lines have lower band of AZGP1; To compare the size of exosomal AZGP1 derived from SNU pancreatic cancer cell line, western blot assay was performed. The sample is same with the previous experiment.

Lower Form of AZGP1 can Increase Cancer Cell Migration

To check whether exosomal AZGP1 can increase cancer migration, exosomes of DU145, RWPE-1, AsPC-1, Panc-1 and NCI-N87 were treated to A549, the lung cancer cell line. Cancer cell migration treated with exosomes of DU145 and AsPC-1 which showed lower form of AZGP1 were increased 4.28 and 3.71-fold, respectively compared to control that was not treated with exosome. Cancer cell migration treated with exosomes of Panc-1 and RWPE-1 which showed 48 kDa size of AZGP1 in-

creased 2.7 fold and similar extend, respectively with the control, and NCI-N87 which showed no AZGP1 band increased similar extend with the control (Figure 5).

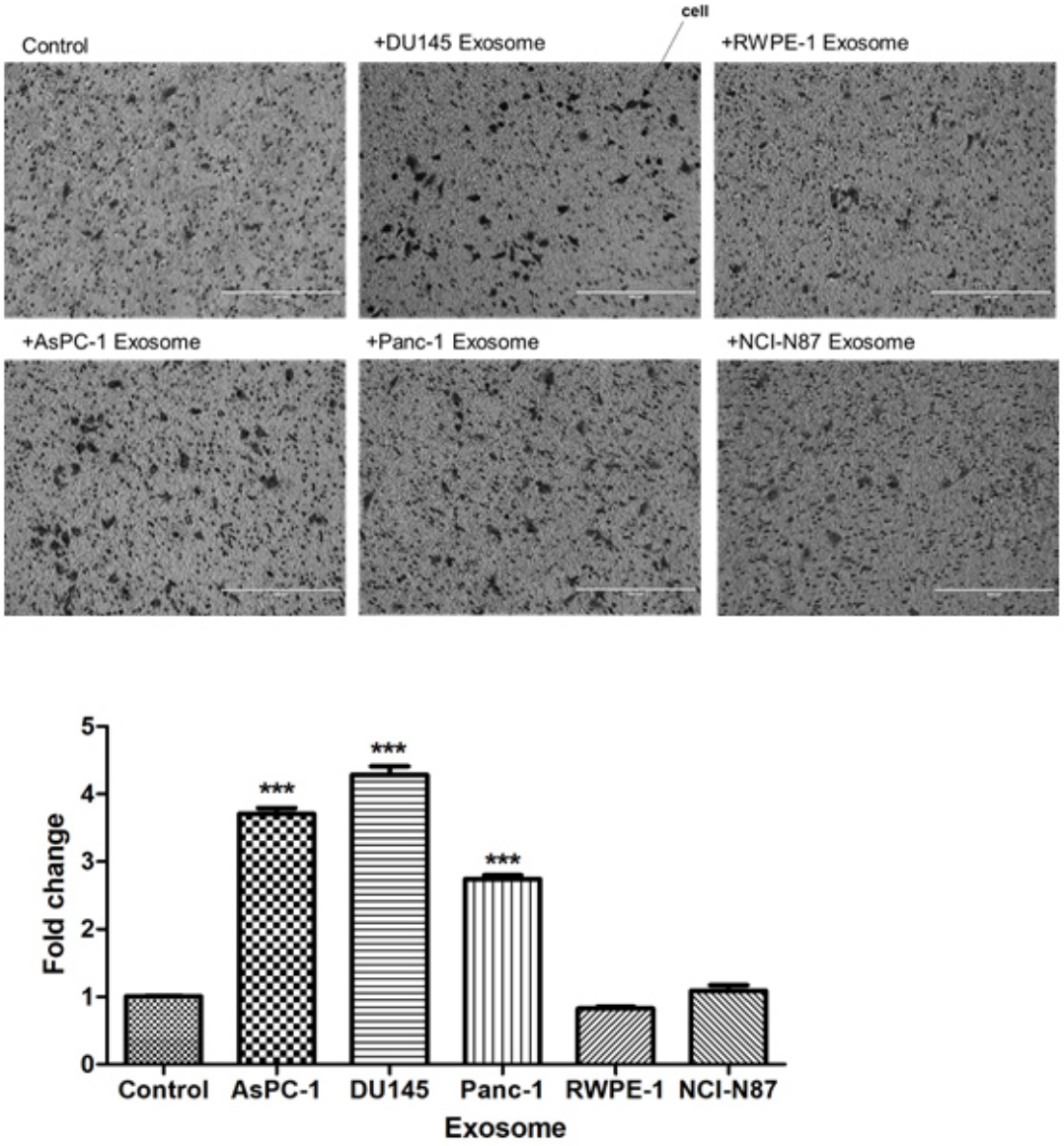

Figure 5: Lower type of exosomal AZGP1 induced cancer migration; Exosomes from cancer cells were isolated in the same way. A549 cells were treated with 200 µl of cancer derived exosome. After 24 hours, non-transferred cells were removed. 1% crystal violet was treated for staining and pictures were taken by microscope.

DISCUSSION

Pancreatic cancer is one of the most malignant cancers because of an overall 5-years survival rate of 11%. The only marker to diagnose pancreatic cancer, CA 19-9, can't detect the pancreatic cancer. Because CA 19-9 doesn't have specificity to pancreatic cancer perfectly, some pancreatic cancer patients could not be detected using CA 19-9. So, we tried to find a new biomarker candidate to diagnose pancreatic cancer more exactly.

After checking the mRNA expression level to find pancreatic cancer biomarkers among the exosomal proteins, AZGP1 was only detected in LNCaP clone FGC and AsPC-1. At the protein level, AZGP1 was detected in all of the pancreatic cancer cell lines. For convenient diagnosis, biomarkers must be detected in the blood. So, we checked whether AZGP1 can be detected in the exosome of pancreatic cancer cell lines or not.

Exosome is secreted in various forms with 30 nm-150 nm size and is one of the extracellular vesicles that originate from endosomes. Exosome can influence intracellular signaling. Exosomal AZGP1 was detected in pancreatic cancer and prostate cancer, but the size of exosomal AZGP1 of DU145 and AsPC-1 is differing from that of another cell line. AZGP1 could have 7 different sizes because of glycosylation [33].

CONCLUSION

SNU pancreatic cancer cell lines also showed the same lower size of AZGP1 with that of DU145 and AsPC-1. To check whether lower size of exosomal AZGP1 can increase cancer migration or not, we performed a transmembrane assay. Treatment of exosomal AZGP1 of DU145 and AsPC-1 that have lower size of AZGP1 increased cancer migration better than another exosomal AZGP1. This result suggested that SNU pancreatic cancer cell lines might migrate more easily and pancreatic cancer in Ko-

rean might migrate more easily. In conclusion, exosomal AZGP1 should be valuable to detect pancreatic cancer biomarker with or without CA 19-9, and can be used to predict whether cancer migration will be faster or not, but it is necessary to study in more detail why the different sizes of AZGP1 are made and how the lower size of AZGP1 affects cancer migration.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to report.

REFERENCES

1. Rawla P, Sunkara T, Gaduputi V (2019) Epidemiology of pancreatic cancer: Global trends, etiology and risk factors. World J Oncol. 10(1): 10–27.
2. McGuigan A, Kelly P, Turkington RC, Jones C, Coleman HG (2018) Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. World J Gastroenterol. 24(43): 4846-4861.
3. Siegel RL, Miller KD, Jemal A (2019) Cancer statistics, 2019. CA Cancer J Clin. 69(1): 7-34.
4. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM (2014) Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 74(11): 2913–2921.
5. Krech RL, Walsh D (1991) Symptoms of pancreatic cancer. J Pain Symptom Manag. 6(6): 360-367.
6. Iyengar D, Tatiparti K, Gavande NS, Sau S, Iyer AK (2022) Nanomedicine for overcoming therapeutic and diagnostic challenges associated with pancreatic cancer. Drug Discov Today. 27(6): 1554-1559.
7. Benassai G, Quarto G, Perrotta S, Furino E, Benassai GL, et al. (2015) Long-term survival after curative resection for pancreatic ductal adenocarcinoma-surgical treatment. Int J Surgy. 21(Suppl 1): S1–3.
8. Al-Hawary M (2016) Role of imaging in diagnosing and staging pancreatic cancer. J Natl Compr Canc Netw. 14(5 suppl): 678-680.
9. Kamisawa T, Wood LD, Itoi T, Takaori K (2016) Pancreatic cancer. Lancet. 388(10039): 73-85.
10. Berry W, Algar E, Kumar B, Desmond C, Swan M, et al. (2017) Endoscopic ultrasound-guided fine-needle aspirate-derived preclinical pancreatic cancer models reveal panitumumab sensitivity in KRAS wild-type tumors. Int J Cancer. 140(10): 2331-2343.
11. Katanuma A, Maguchi H, Hashigo S, Kaneko M, Kin T, et al. (2012) Tumor seeding after endoscopic ultrasound-guided fine-needle aspiration of cancer in the body of the pancreas. Endoscopy. 44(S 02): E160-E161.
12. Galli C, Basso D, Plebani M (2013) CA 19-9: Handle with care. Clin Chem Lab Med. 51: 1369-1383.
13. Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, et al. (1979) Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic Cell Genet. 5: 957-971.
14. Ballehaninna UK, Chamberlain RS (2011) Serum CA 19-9 as a biomarker for pancreatic cancer-a comprehensive review. Indian J Surg Oncol. 2(2): 88-100.
15. Goonetilleke KS, Siriwardena AK (2007) Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. Eur J Surg Oncol. 33: 266-270.
16. Steinberg W (1990) The clinical utility of the CA 19-9 tumor-associated antigen. Am J Gastroenterol. 85: 350-355.
17. Kim BJ, Lee KT, Moon TG, Kang P, Lee JK, et al. (2009) How do we interpret an elevated carbohydrate antigen 19-9 level in asymptomatic subjects? Dig Liver Dis. 41(5): 364-369.
18. Tsao KC, Hong JH, Wu TL, Chang PY, Sun CF, et al. (2007) Elevation of CA 19-9 and chromogranin A, in addition to CA 125, are detectable in benign tumors in leiomyomas and endometriosis. J Clin Lab Anal. 21(3): 193-196.
19. Parra JL, Kaplan S, Barkin JS (2005) Elevated CA 19-9 caused by hashimoto's thyroiditis: Review of the benign causes of increased CA 19-9 level. Dig Dis Sci. 50(4): 694-695.
20. Mann DV, Edwards R, Ho S, Lau WY, Glazer G (2000) Elevated tumour marker CA19-9: Clinical interpretation and influence of obstructive jaundice. Eur J Surg Oncol. 26(5): 474-479.
21. Kim MS, Jeon TJ, Park JY, Choi J, Shin WC, et al. (2017) Clinical interpretation of elevated CA 19-9 levels in obstructive jaundice following benign and malignant pancreatobiliary disease. Korean J Gastroenterol. 70(2): 96-102.
22. Liu X, Li Z, Ge W, Wen F, Zheng S, et al. (2022) High expression of AZGP1 is associated with poor prognosis in colorectal cancer.
23. Frenette G, Dube JY, Lazure C, Paradis G, Chretien M (1987) The major 40-kDa glycoprotein in human prostatic fluid is identical to Zn-alpha 2-glycoprotein. Prostate. 11(3): 257–270.
24. Sanchez LM, Lopez-Otin C, Bjorkman PJ (1997) Biochemical characterization and crystalization of human Zn-alpha2-glycoprotein, a soluble class I major histocompatibility complex homolog. Proc Natl Acad Sci. 94(9): 4626–4630.
25. Ohkubo I, Niwa M, Takashima A, Nishikimi N, Gasa S, et al. (1990) Human seminal plasma Zn-alpha 2-glycoprotein: Its purification and properties as compared with human plasma Zn-alpha 2-glycoprotein. Biochim Biophys Acta. 1034(2): 152–156.
26. Nguyen AT, Kim KY (2020) Inhibition of proinflammatory cytokines in Cutibacterium acnes-induced inflammation in HaCaT cells by using Buddleja davidii aqueous extract. Int J Inflammation.
27. Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, et al. (2013) *In vitro* cell migration and invasion assays. 752(1): 1.
28. Kim YE, Nguyen AT, Min J, Kim M, Kim KY, et al. (2022) Rtn3 (reticulon 3) as a novel prostate cancer-specific biomarker

derived from exosome. J Biomark. 8(8): 150.

29. Yip PY, Kench JG, Rasiah KK, Benito RP, Lee CS, et al. (2011) Low AZGP1 expression predicts for recurrence in margin-positive, localized prostate cancer. Prostate. 71(15): 1638-1645.

30. Cao R, Ke M, Wu Q, Tian Q, Liu L, et al. (2019) AZGP1 is androgen responsive and involved in AR-induced prostate cancer cell proliferation and metastasis. J Cell Physiol. 234(10): 17444 17458.

31. Pan S, Brentnall TA, Chen R (2015) Proteomics analysis of bodily fluids in pancreatic cancer. Proteomics. 15(15): 2705-2715.

32. Ku JL, Park JG (2005) Biology of SNU cell lines. Cancer Res Treat. 37(1): 1-19.

33. Liu S, Jiang X, Shang Z, Wang YH, Wang Z (2020) N-glycan structures of target cancer biomarker characterized by two-dimensional gel electrophoresis and mass spectrometry. Anal Chim Acta. 1123: 18-27.