EVCD, TARGETING BETA SHEET RICHNESS OF TUMOR DERIVED EXTRACELLULAR VESICLES FOR PANCREATIC CANCER SCREENING

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Pancreatic cancer is a deadly disease and a relatively uncommon form of cancer. However, it is projected to be the second leading cause of cancer deaths in the US by 2040. The 5-year survival rate of pancreatic cancer patients is 10 percent. Currently, there are no effective screening methods available. Extracellular vesicles are nanoparticles secreted by all cells and play versatile roles in human health. EVs can be used as a non-invasive biomarker for pancreatic cancer screening since they can be isolated from bodily fluids. Currently, single molecule biomarkers have been proposed for pancreatic cancer screening. They lack sensitivity and specificity. We studied the 'collective attribute' of protein secondary structures of EVs from two cancer (MiaPaCa2 and PANC-1) and one healthy cell line (HPNE). Protein secondary structures of EVs were studied using circular dichroism spectroscopy. We found that cancerous EVs contain more beta sheet rich proteins than non-cancerous EVs.

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LIST OF ABBREVIATIONS

EV .....Extracellular Vesicles.

PDAC.....Pancreatic Ductal Adenocarcinoma.

KRAS.....Kirsten Rat Sarcoma Viral Oncogene.

TP53.....Tumor Protein 53.

CDKN2A .....Cyclin Dependent Kinase Inhibitor 2A.

DNA.....Deoxy Ribonucleic Acid.

RNA .....Ribonucleic Acid.

miR/miRNA.....Micro RNA.

CNS.....Central Nervous System.

MAPK.....Mitogen-Activated Protein Kinase.

ERK.....Extracellular Signal-Regulated Kinases.

PTEN.....Phosphatase and Tensin Homolog.

PI3Kγ .....Phosphoinositide 3-Kinase Gamma.

EMT .....Epithelial to Mesenchymal Transition.

CD82 .....Cluster of Differentiation 82.

CLDN4.....Claudin 4.

EPCAM.....Epithelial Cell Adhesion Molecule.

CD151 .....Cluster of Differentiation 151.

LGALS3BP.....Galectin 3 Binding Protein.

HIST2H2BE.....Histone H2B Family Member E.

HIST2H2BF.....Histone H2B Family Member F.

MHC .....Major Histocompatibility Complex.

EGF .....Epidermal Growth Factor.

MFG-E8 .....Milk Fat Globule-EGF Factor 8 Protein.

Rab .....Ras Associated Binding Protein.

Arf .....ADP-Ribosylation Factor.

GAPDH.....Glyceraldehyde-3-Phosphate Dehydrogenase.

TSG101 .....Tumor Susceptibility 101.

ALIX .....ALG-2-Interacting Protein X.

HSC70.....Heat Shock Cognate 70.

HSP90 .....Heat Shock Protein 90.

TGF- β.....Transforming Growth Factor Beta.

IL .....Interleukin.

TNF- α.....Tumor Necrosis Factor Alpha.

LAMP .....Lysosome-Associated Membrane Glycoproteins.

TfR .....Transferrin Receptor.

FTIR.....Fourier Transform Infrared.

NMR .....Nuclear Magnetic Resonance.

CD .....Circular Dichroism.

mg .....Milligram.

mL .....Milliliter.

µL.....Microliter.

µg .....Microgram.

ng.....Nanogram.

CO_2.....Carbon Dioxide.

C .....Celsius.

pH.....Potential of Hydrogen.

U.....Enzyme Units.

PMSF .....Phenylmethylsulfonyl Fluoride.

KDa.....Kilo Dalton.

L.....Liters.

cm.....Centimeter.

ng.....Nanogram.

mDeg.....Milli Degrees.

s.e.m.....Standard Error of the Mean.

CA 19-9.....Carbohydrate Antigen 19-9.

hTERT.....Human Telomerase Reverse Transcriptase.

INTRODUCTION

Pancreatic Cancer

Pancreatic cancer is a highly fatal disease and its causes remain unknown[1]. It is hypothesized that they might be initiated by different gene mutations such as KRAS, TP53 and CDKN2A[2]. By 2040, it is estimated that pancreatic cancer will be the second most common cause of cancer-related deaths in the United States[3] and there will be a 61.7% increase in the total number of pancreatic cancer cases worldwide[4]. As of 2020, the five-year survival rate of pancreatic cancer patients is about 10% in the United States[5]. Pancreatic cancer has poor prognosis because of the poor diagnosis. Patients usually do not show symptoms until the disease had reached metastasis stage at which point surgery, radiation and chemotherapy may extend the survival but does not cure the disease[6]. In the past decade, several advancements have been made in diagnostic approaches, radio and systemic therapies. However, these advancements only have less impact on patient outcomes[7]. At present, effective screening methods do not exist. There is a dire need for new strategy and technology for the early detection of pancreatic cancer.

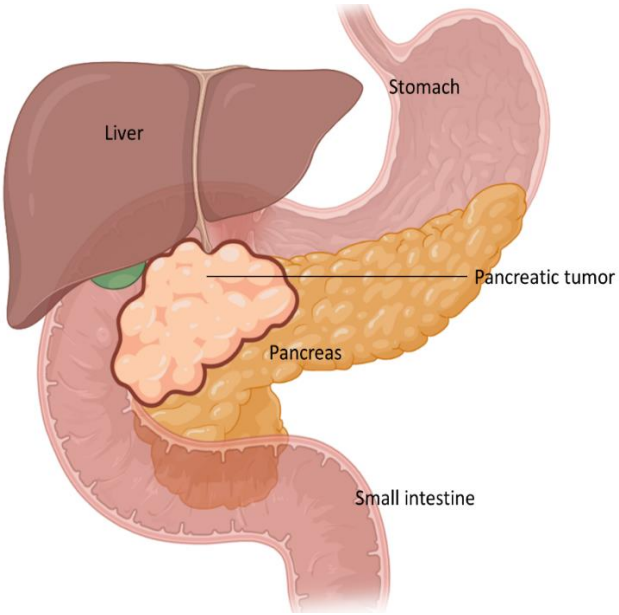

Figure 1. Illustration of pancreatic cancer.

Extra Cellular Vesicles (EV)

Multi-cellular organisms have the ability to communicate with neighboring cells or distant cells. This intercellular communication is carried out via direct cell-cell contact, exchange of secreted molecules, and intercellular transfer of extracellular vesicles (EVs). These EVs can range in size from 30 – 1000nm[8]. Similar to a cell, Extracellular vesicles are composed of a lipid bilayer. There are several transmembrane and cytosolic proteins along with DNA/RNA enclosed in these extra cellular vesicles[9]. They are broadly classified into two groups based on their origin. EVs that originate through the budding from the plasma membrane of cells are referred to as microvesicles, ectosomes, or microparticles. EVs that are of endosomal origin and formed inside the cells through multivesicular bodies are called as exosomes (Figure 2A). Extracellular vesicles can be isolated from different bodily fluids such as urine, blood, breast milk, saliva, amniotic fluid, ascites, semen, bile and cerebrospinal fluid[10].

Extracellular vesicles play versatile roles in intercellular communication[11], blood coagulation[12], immune system[13], cancer[14], neurologic & urologic diseases[15], bacterial infections[16] and clearance of toxic drugs & cell fragments[17,18](Figure 2B). Generally, EVs are targeted towards specific recipient cell which receives critical biochemical molecules such as proteins, lipids and nucleic acids from a donor cell. These exchanged biochemicals play vital role in intercellular communication[19]. EVs have been found to be beneficial as well as detrimental in human health. For example, in immune system, it has been shown that Foxp3+ T regulatory cells transfer miRNA to T helper cells. This miRNA transfer hinders cell proliferation and IFN-γ secretion, which in turn causes suppression of systemic disease[20]. However, EVs are also responsible for immunosuppression such as T-cell apoptosis[21,22]. Similarly, EVs play beneficial role in Central Nervous System (CNS) by sustaining neuronal integrity[23]. Contrastingly, EVs have also been

shown to be detrimental by exchanging pathogenic proteins like prions[24], β-Amyloid[25] and aggregation of Alpha-synuclein[26].

Recently, the implications of extracellular vesicles on cancer has been studied extensively. EVs play major role in several different types of cancers such as melanoma, breast, prostate, gastric, lung, colon and pancreatic cancer[27]. They influence all aspects of cancer from initiation & proliferation to invasion & metastasis[28]. Exosomes, a subset of EVs have been shown to influence pathogenesis and progression of several pancreatic precancerous conditions such as pancreatitis and pancreatic fibrosis[29]. A recent study has demonstrated the procancerous role of exosomes in PDAC as well as from cultured pancreatic cancer cells. Such exosomes isolated from the serum of PDAC patients are enriched with survivin, a cell survival protein[30]. Interestingly, microRNAs from exosomes have been shown to suppress MAPK/ERK pathway. These biochemical pathways are crucial for tumor formation[31]. Cell proliferation and migration is increased in pancreatic cancer by exosomal microRNA-182 which directly targets beta-transducin repeat-containing proteins[32]. MicroRNA-182 has also been shown to influence pancreatic cancer progression[31]. Another type of miRNA, known as miR-301a-3p mediates M2 macrophage polarization. The polarization happens through PTEN/PI3Kγ pathway, which in turn promotes pancreatic cancer progression[33]. Apart from the above-mentioned functions, exosomes play crucial roles in angiogenesis in pancreatic cancer and Epithelial-Mesenchymal Transition (EMT), which is an important event in pancreatic cancer development[34,35]. Similar to other disease conditions, EVs play both beneficial and detrimental role in cancer. Tumor-derived EVs help cancer cells to determine their microenvironment enabling cancer progression. On the other hand, EVs from non-tumor cells help in suppression of cancer progression[36].

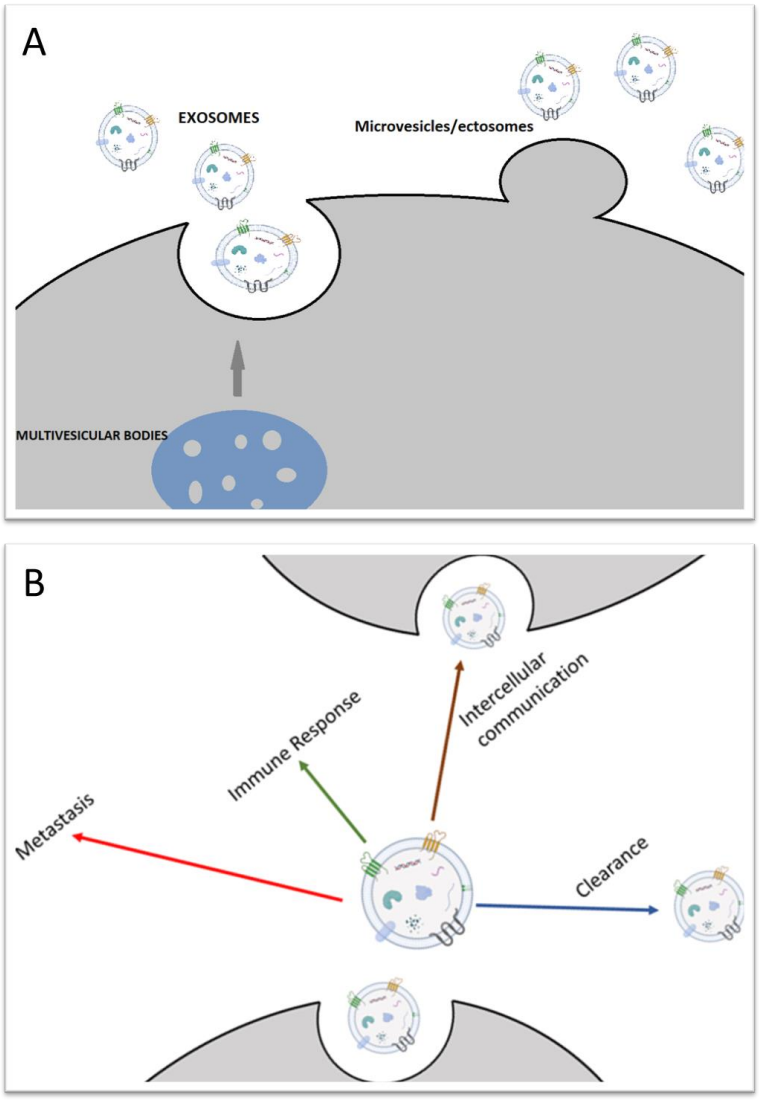

Figure 2. EV types and their roles.
(A) Exosomes and Microvesicles/Ectosomes. (B) Versatile roles of EVs in human health.

Collective Attribute of EVs

Extracellular Vesicles are a rich reservoir of important biomolecules such as proteins, lipids and nucleic acids. Since EVs are released into blood circulation, they are being investigated for their potential as a non-invasive cancer biomarker[37]. EVs are currently studied for the detection of various types of cancers such as breast[38], colorectal[39] and pancreatic cancer[40]. Different exosomal components such as lipid, proteins and nucleic acids are investigated for biomarkers in cancer. For example, exosomal protein such as CD82 has been proposed as a diagnostic biomarker in breast

cancer[41], CK18, PSA, PTEN, PSMA and several others for prostate cancer[42] and several EV protein biomarkers such as CLDN4, EPCAM, CD151, LGALS3BP, HIST2H2BE and HIST2H2BF have been studied for pancreatic cancer[43]. Recently, lipidomic characterization of EVs from human plasma was performed by mass spectrometry. Different fractions from ultracentrifugation revealed different types and abundance of lipid molecules such as Cholesterol ester, Ceramide, Diacylglyceride, Fatty acid and Triacyl glyceride. This lipidomic analysis provides insight into the lipid profile of EVs which can be used for diagnostic or prognostic purposes[44]. A shotgun lipidomic analysis was performed for exosomes isolated from colorectal cancer cell line LIM1215, which showed that the lipid content of the EVs were distinct from their parental cells[45]. Another lipidomic study conducted on the urinary exosomes isolated from prostate cancer patients revealed significant difference in phosphatidylserine and lactosylceramide compared to healthy samples, denoting the potential as cancer biomarker[46]. Similarly, nucleic acids such as exosomal microRNAs have also been proposed as potential biomarkers for cancer diagnosis. For example, miR-21 and miR-4454 for bladder cancer, miR-141 and miR-375 for prostate cancer and miR-4306, miR-1246, miR-3976 and miR-4644 for pancreatic cancer have been proposed as biomarkers[47].

The majority of these studies utilize single protein or RNA molecule as a biomarker which can be problematic leading to false negatives or false positives. Such usage of single molecules can also be masked by high background signals[48,49]. Another challenge in EV biomarker research is lack of simple methods available for analysis. Conventional techniques such as the ultracentrifugation and ultrafiltration require several steps and they are generally labor and time intensive[50]. After isolation, they require further steps such as protein/nucleic acid extraction before analyzing the biochemical contents. Apart from isolation and sample preparation, an important

factor hindering the use of EVs in clinical settings is the low sample yield[51]. In order to overcome these challenges, we studied the collective attribute of the EVs using the proteins. Table 1 summarizes the hallmark proteins present in the membrane and cytosol of EVs[19]. We investigated the collective secondary structure of the proteins present in the EVs to use them for pancreatic cancer screening. We employed a simple and effective circular dichroism spectroscopy technique to study the secondary structure of EV proteins.

Table 1. Common proteins present in EVs.

Classification	Proteins
Antigen Presentation	MHC Class I, MHC Class II
Adhesion molecules	Tetraspanins: CD63, CD81, CD9, CD37, CD53, CD82 Integrins: α3, α4, αM, αL, β1, β2 MFG-E8
Membrane Trafficking	Annexins: I, II, IV, V, VI, VII, XI Syndecan-1 Rab 2, Rab 5c, Rab 10, Rab 7 Arf 3, Arf 6, Arf 5 Clathrin
Enzymes	Pyruvate Kinase, Alpha Enolase, GAPDH, Elongation factors
Signal Transduction	Syntenin-1
Lipid Raft	Flotillin-1, Flotillin-2, Caveolin-1
ESCRT Proteins	Alix, Tsg101
Heat-shock proteins	Hsc70, Hsp90
Cytoskeletal proteins	Actin, Cofilin 1, Tubulin, Moesin
Cytokines	TGF- β, IL, TNF- α, EGF
Other transmembrane proteins	LAMP, TfR,
Other cytosolic proteins	Histones, ribosomal proteins, proteasome

Circular Dichroism

There are several different analytical techniques for the study of protein secondary structures. Most commonly used techniques include Mass spectrometry (LC-MS)[52], FTIR[53], X-Ray crystallography and NMR spectroscopy[54], Cryo-Electron microscopy[55] and Circular Dichroism Spectroscopy[56]. Circular Dichroism is a type of absorbance spectroscopy which measures the differential absorption of left and right circularly polarized light by an analyte[57]. The main application of CD spectroscopy is to study protein secondary structure based on the electronic transitions in the far UV region (170 to 240 nm) and tertiary structure information using aromatic amino acid residues in the near UV region (260 to 300 nm)[58]. Unlike other conventional protein characterization techniques, CD has a simple construction that utilizes a monochromator, waveplate that generates left and right circularly polarized light and a photomultiplier tube.

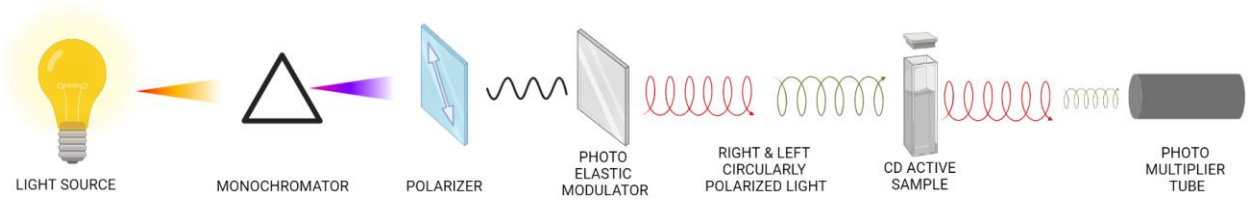

Figure 3. Parts of Circular Dichroism spectropolarimeter.

Most of the applications of CD spectroscopy is done on purified single protein analyte[59]. Employing CD spectroscopy for the study of membranous vesicles is under explored in the field of EV research. We extended the application of CD in studying the membranous vesicles and the secondary structure of their protein components as a whole (Figure 4) rather than studying individual proteins. A major advantage of the CD technique is its ability to measure protein quantities as low as 0.2 mg/ml[60]. Since EVs are biomolecules in the nanoscale sizes, they are secreted in low quantities. Hence, CD is an excellent technique for studying proteins in low

quantity EV yields. We studied EVs from two pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) and one non-cancerous pancreatic cell line (HPNE) using CD spectroscopy to investigate their collective protein secondary structure profiles.

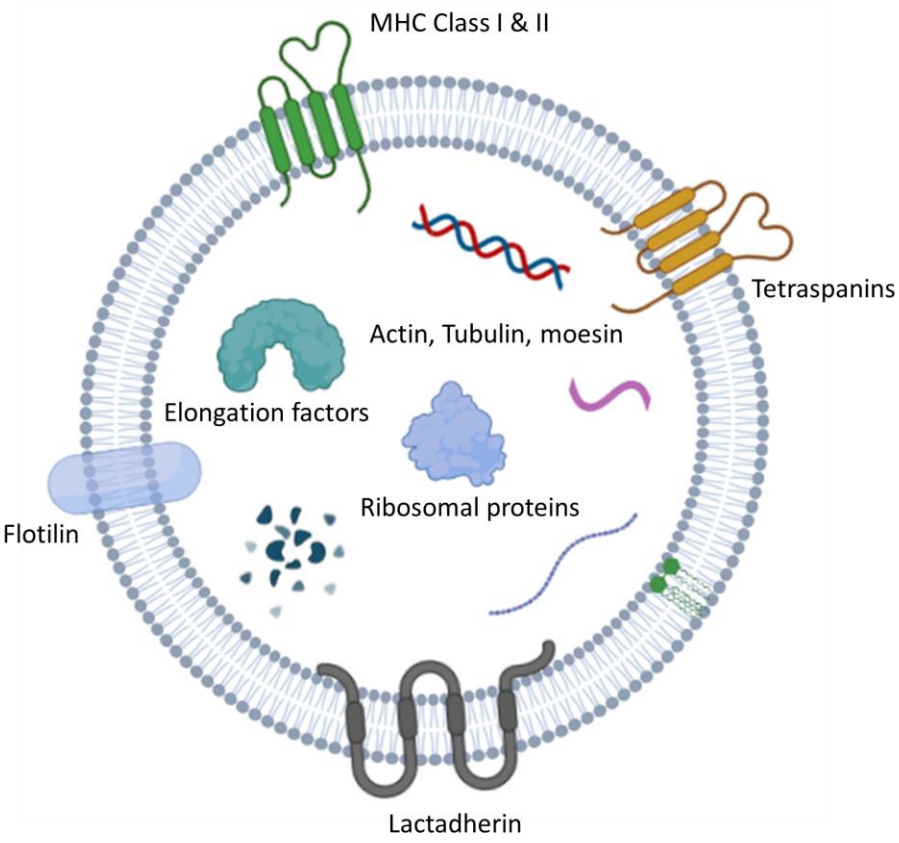

Figure 4. Illustration of different transmembrane and cytosolic proteins present in EVs.

MATERIALS AND METHODS

Cell Culture

MIA PaCa-2, PANC-1 and HPNE cells were seeded at a density of approximately 4 x 10^4 cells/mL on 100mm plates containing DMEM medium (Cytiva) supplemented with 10% FBS (Sigma Aldrich), 1U penicillin and 1 µg/mL streptomycin (Caisson labs). HPNE cells were supplemented with 0.l ng/ml rEGF (Novus Biologicals, USA). Cells were incubated for 24 hours at 37°C and 5 % CO_2. For collection media preparation, when the cells have reached 70% confluency, culture media was discarded, and the cells were washed thrice with warm PBS (pH 7.4). Upon washing, 10 mL of DMEM medium containing 10% exosomes depleted FBS (Fischer scientific) was added to the plates. The plates were then incubated at 37°C under 5% CO_2 for 48 hours.

EV Isolation

After the incubation period, conditioned medium was collected and filtered through 0.22 µm filter (Celltreat). Medium was then centrifuged at 10,000 x g for 30 minutes to remove any cells or debris. Supernatant was carefully transferred to polycarbonate ultracentrifugation tubes without disturbing any cell debris. Transferred media was ultra-centrifuged (Sorvall, Thermo scientific) at 200,000 × g for 70 minutes. Supernatant was carefully removed without disturbing the EV pellet. EVs were then suspended in 100 µL PBS (pH 7.4) and collected for further purification.

EV Purification

Isolated EVs were subjected to further purification by Proteinase K treatment followed by ultrafiltration. 1 µL Proteinase K (800 U/mL, New England Biolabs) was added to the EV samples and mixed thoroughly and incubated at 37°C for 30 minutes. After 30 minutes, the reaction was

stopped by adding 1 µL PMSF (100 mM, Chemimpex). After Proteinase K treatment, EV samples were subjected to ultrafiltration. Treated EV samples were loaded into a tangential flow ultrafiltration device with 50 KDa molecular weight cutoff filter (Amicon Ultra 0.5 centrifugal filter, Millipore Sigma) and centrifuged for 10 minutes. The flow through was discarded and the remaining EVs were collected and quantified using Nanodrop ND-1000 spectrophotometer (Thermo scientific) before CD analysis.

Circular Dichroism Spectroscopy

EV samples (MIA PaCa-2, PANC-1 and HPNE) were prepared in PBS (pH 7.4) at 0.2 mg/mL concentration. CD measurements were performed on a Jasco J-815 spectropolarimeter (Jasco, Japan). Nitrogen gas was used for purging at a flow rate of 5 L/min. 200 µl sample was loaded into a quartz cuvette with 0.1 cm pathlength and the samples were scanned from 200 to 260 nm wavelength region at 200 nm/min. PBS was measured as baseline and three sample scans were averaged and subtracted from baseline (PBS) for each spectrum. The data analysis and graphing were done in Origin Pro 2020.

RESULTS

CD Spectroscopy of EVs

Isolation of EVs without contaminating proteins is a challenge. Since circular dichroism is typically used for protein characterization, any contaminating proteins in the EV sample preparation might interfere with the CD results. We employed a series of steps to obtain purified EV samples for CD spectroscopy. We first used proteinase K treatment followed by ultrafiltration in order to improve both purity and yield of EVs. Proteinase K treatment was used to digest any contaminating proteins from the cells. However, a challenge with using proteinase K is the removal of digested proteins as well as the proteinase K enzyme after the treatment. Hence, we employed an effective ultrafiltration step. Ultrafiltration functions in two ways by removing proteinase K and also any remaining protein contaminants, thereby yielding purified EVs for further CD analysis.

Circular dichroism measures the unequal absorption of left and right circularly polarized light. When the two circularly polarized components are absorbed differentially, the resultant is an elliptically polarized light[61] which is expressed as ellipticity in millidegrees (Figure 5A). Using CD, we studied the secondary structures of EV proteins from three different cell lines, an hTERT immortalized human pancreatic nestin expressing cell line (HPNE) and two pancreatic ductal adenocarcinoma cell lines (MIA PaCa-2 & PANC-1). We found that tumor derived EVs (Malignant) contained more beta sheet rich proteins compared to their healthy counterparts (Non-malignant). Spectral profiles obtained from circular dichroism spectroscopy reveal information about different secondary structural elements (Figure 5C) such as alpha helix, beta sheets and random coils[56] as the chromophore is the peptide bond.

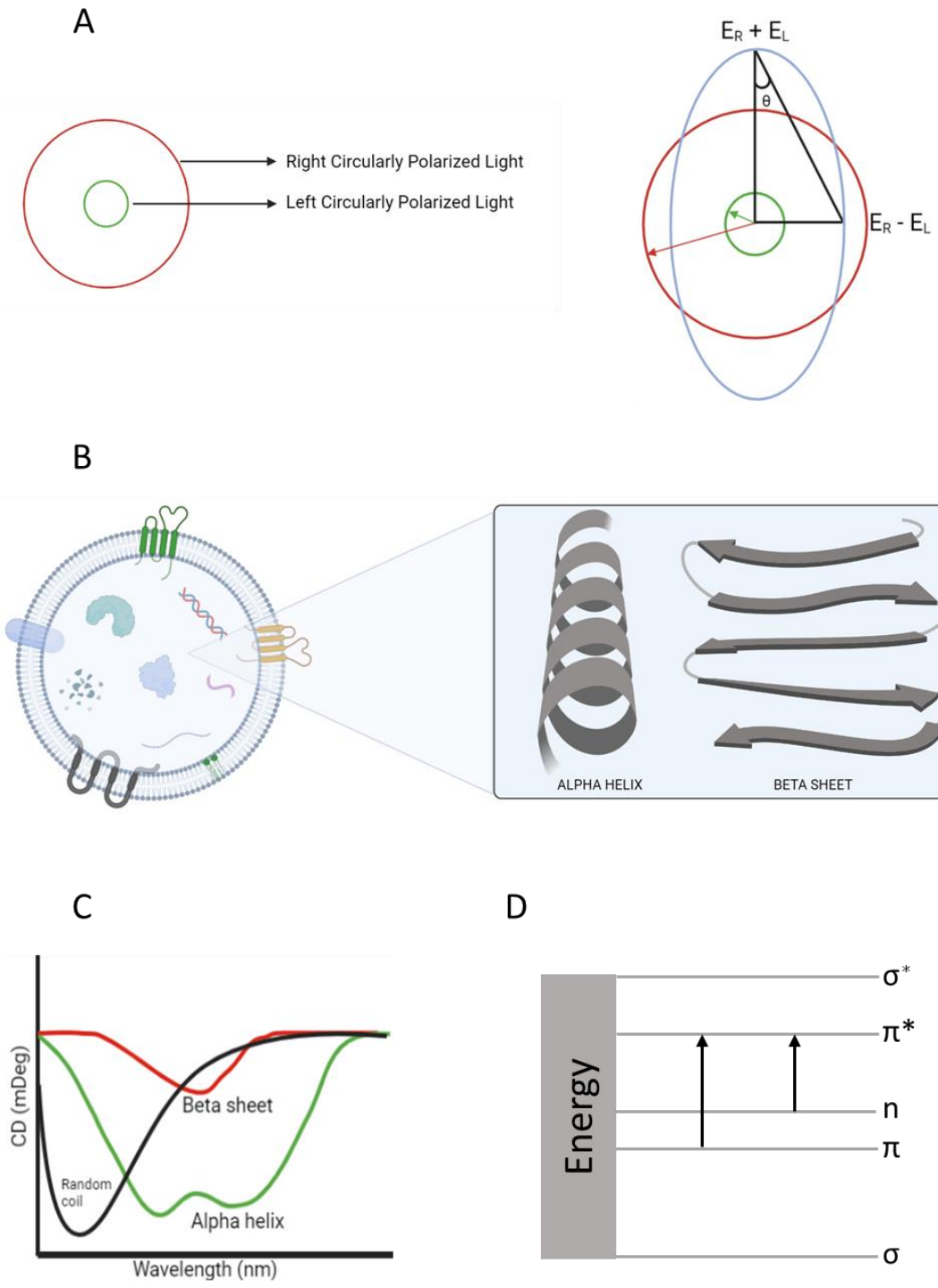

Figure 5. CD and protein secondary structure characteristics.
(A) Differential absorption of left and right circularly polarized components and the resultant elliptically polarized light. (B) Cartoon representation of alpha helix and beta sheet, major secondary structure components of proteins. (C) Typical CD profiles for alpha helix, beta sheets and random coil proteins. (D) Electronic transitions in carbonyl group of proteins during CD at 208 nm (π to π*) and at 222 nm (n to π*).

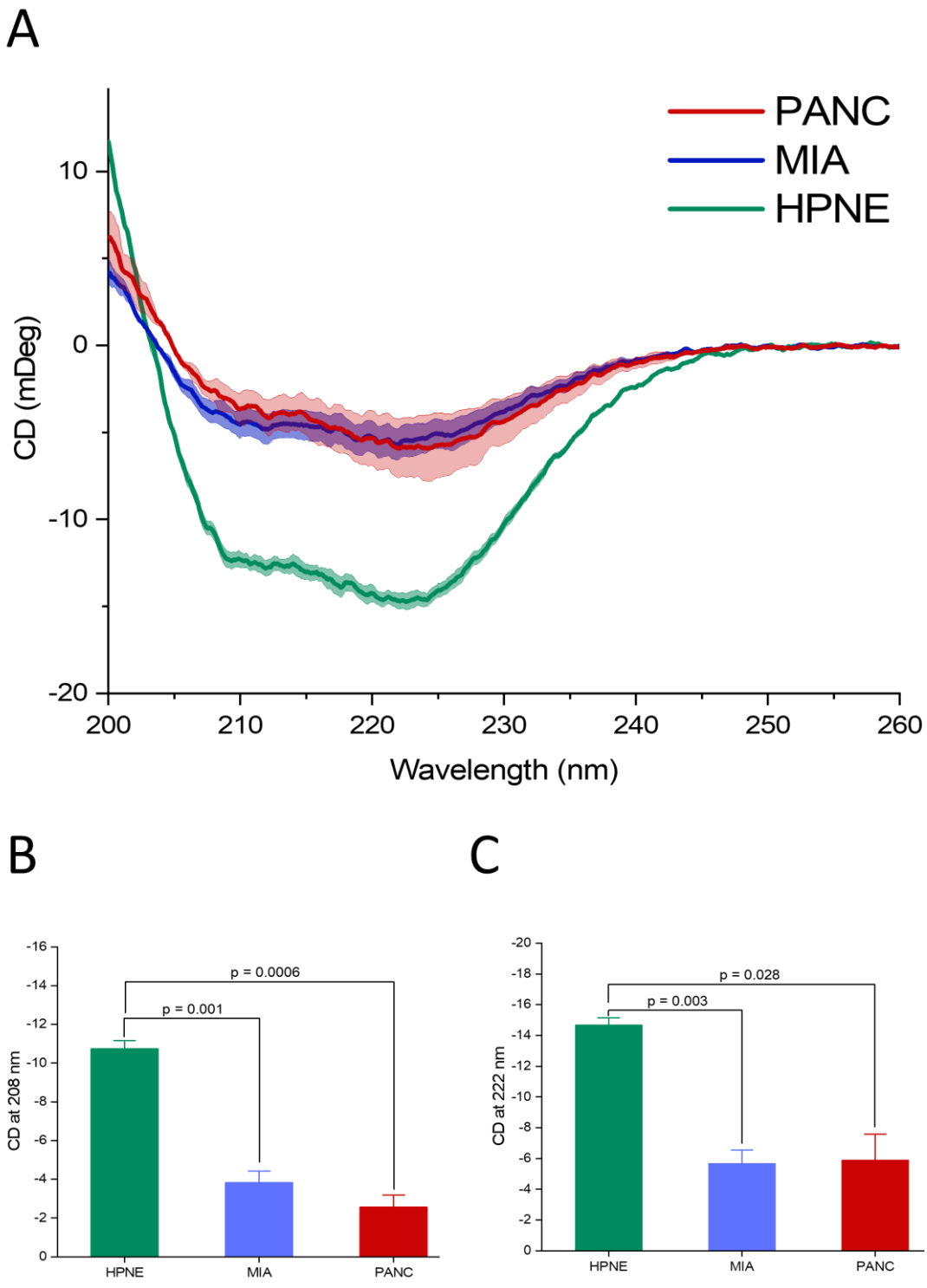

Figure 6. Beta sheet richness of Tumor derived EVs.
(A) CD spectra of PANC-1, MIA PaCa-2 and HPNE EVs, (B) Peak ellipticity of EV samples at 208 nm, (C) Peak ellipticity of EV samples at 222 nm. Error bars, mean ± s.e.m; n=3; p values were determined by unpaired two-tailed t-tests.

From CD spectroscopy, we obtained similar CD patterns for tumor derived EVs compared to EVs from healthy cells (Figure 6A). The obtained spectra indicate there is a difference in electronic transitions (far UV region) in proteins of cancerous and non-cancerous EVs, which shows that there is a difference in protein secondary structure components between healthy and cancerous EVs. During CD, two main electronic transitions take place at the peptide bond (Figure 5D), namely n to π^* around 220 nm and π to π^* around 207 nm[57]. When using CD, signals from beta sheets arising from weaker π to π^* can be masked by the signals from alpha helix if the concentration is low. Hence, we used a concentration of 0.2 mg/ml for better detection of beta sheet signals. Comparison of ellipticity at 208 nm (Figure 6A) and 222 nm (Figure 6B) of the three EVs revealed significant difference between HPNE & MIA PaCa-2 and HPNE & PANC-1. EVs from HPNE showed the highest positive peaks at 208 and 222 nm, indicative of typical alpha helix structure. EVs from MIA PaCa-2 & PANC-1 showed decreases in peaks at 208 and 222 nm, indicating the beta sheet protein richness. This difference in the peak values is attributed to the electronic transitions occurring during circular dichroism. Significant differences in the CD spectra and ellipticity at 208 and 222 nm conclude that the tumor derived EVs are enriched with beta sheet proteins when compared with healthy EVs.

DISCUSSION

At present, there are no effective early diagnostic methods for pancreatic cancer screening. Some biomarkers such as the serum carbohydrate antigen 19-9 have been proposed for pancreatic cancer but they suffer from lacking sensitivity and specificity for early detection[62]. Liquid biopsy has been an important topic in cancer research. Liquid biopsies involve the collection of critical biomolecules such as nucleic acids, proteins and EVs from body fluids and analyzing them for screening a disease[63,64]. Owing to their cargo property of carrying several biochemical molecules, the extracellular vesicles can be used as disease biomarkers. Since these EVs are found abundantly in blood and other bodily fluids they can be utilized for liquid biopsies, particularly in cancer[65]. These properties of EVs make them ideal biomarker candidates which can be used in screening assays. However, not all biomolecules from EVs can be utilized for screening purposes. Using lipids from EVs as a potential biomarker for disease diagnosis requires complex biochemical analysis such as mass spectrometry and it is a long road ahead until lipids are utilized as biomarkers in clinical settings. Several studies have been proposed that exosomal DNA can be used as a biomarker for detecting cancer[66]. However, there are studies which debate whether the exosomes contain any DNA or not[67]. This is still a debate in the field of EV research and more studies are required to use EV DNAs as biomarkers in the near future. EV Proteins on the other hand have been well studied now[10,30] and do not require complex analysis as lipids. Protein levels increase with different stages of diseases. Protein values are higher in cancer when compared to healthy exosomes[68]. Hence, studying EV proteins can give prognostic information. Several transmembrane proteins such as the tetraspanins are key components of the EV's membrane and are involved in their formation and function[69], making them more ubiquitous in EVs than nucleic acids. This makes them suitable for biomarker research over lipids and nucleic acids.

Circular Dichroism spectroscopy is a simple and effective technique for studying protein secondary structures. CD has also proven useful in several other biomedical applications such as drug discovery[70], drug binding studies[71], protein-ligand interaction studies[72], thermal stability and degradation of biomolecules[73–76]. Apart from studying the secondary structures of soluble proteins, an important aspect of CD spectroscopy is the ability to study membrane proteins. In general, studying transmembrane proteins is difficult since they have intracellular domain, phospholipid domain and extracellular domain. While the intercellular and extracellular domains of the transmembrane protein are hydrophilic, the phospholipid part of the transmembrane protein is hydrophobic in nature making it difficult to extract and analyze. While membrane proteins are difficult to purify, crystallize, and maintain its functional viability, it can still be studied using CD spectroscopy[71,77]. A key feature of CD is that it allows characterization of proteins in their native state in membranes and does not require extraction.

Most of the proposed pancreatic cancer screening biomarkers are single molecules which suffer from lacking sensitivity and specificity. A robust alternative approach away from single biomolecule approach is needed for developing effective screening methods. For example, as an alternative to the single biomarker approach, it was reported that a combination of miRNA-16a & miRNA-196a along with CA19-9 has shown a positive outcome for the detection of stage I pancreatic cancer[78]. Recently, a spectrophotometric analysis of the collective attribute of the nucleic acids and proteins ratio was proposed as an effective cancer screening technique[79]. We used a similar ‘collective attribute’ approach using CD to study EV proteins and found that tumor derived EVs rich in beta sheet proteins. This observation can be explained by the formation of beta sheets which require more energy than alpha helix. It has been shown that cancer cells have altered metabolism[80] and Otto Warburg proposed that cancer cells adopt a mode of increased glucose

import via aerobic glycolysis in order to meet their energy demand[81]. Beta sheet richness of cancerous EVs can be explained by the cancer cells' high energy requirements, which will enable the formation of more beta sheet rich proteins. In conclusion, we were able to collectively study the secondary structure of EV proteins including both cytosolic and transmembrane proteins and observed a difference in their secondary structure composition. This critical finding can be used in development of biomarker assays for pancreatic cancer using EVs.

CONCLUSION

Pancreatic cancer remains a deadly disease even to this day mainly because of the lack of effective early diagnosis. It is estimated that the pancreatic cancer related deaths will increase in the upcoming years. Effective screening strategies are needed to address this critical biomedical issue. Single biomarker assays suffer from lack of sensitivity, specificity and are subject to background noise. We used a different approach called as the 'collective attribute' rather than relying solely on a single biomolecule. This approach also eliminated the need for complex sample preparation and processing. EV protein structures can be studied with intact vesicles and does not need any extraction. Using CD spectroscopy technique, critical differences were observed in cancerous and non-cancerous EVs. We have shown that tumor derived EVs contain more beta sheet rich proteins than normal EVs. This finding will contribute to scientific literature in pancreatic cancer research and this phenomenon can also pave way for similar studies in other forms of cancer. Extracellular vesicles hold a promising future in disease diagnosis and this finding will help in development of biomarkers and screening assays which can be used in clinical settings in the near future. A drawback of CD is that it requires expertise in handling and nitrogen tanks for purging. Based on our finding, new biomedical devices focusing on the circular dichroism principle can be developed which can be used in clinical settings.

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