RNA cargos in extracellular vesicles derived from blood serum in pancreas associated conditions

Senthil R. Kumar1,2,3*, Eric T. Kimchi2,3, Yariswamy Manjunath2,3, Saivaroon Gajagoon1, Alexei J. Stuckel3,4 & Jussuf T. Kaifi2,3

Exosomes are extracellular vesicles which are released from healthy and tumor cells into blood circulation. Unique biomolecular cargos such as RNA and protein are loaded in these vesicles. These molecules may have biological functions such as signaling, cell communications and have the potential to be analyzed as biomarkers. In this initial study, we describe the analysis of exosomes in the serum of healthy subjects, intraductal papillary mucosal neoplasms and pancreatic ductal adenocarcinoma including the characterization of their RNA cargos by next generation sequencing (EXO-NGS). Results indicate the presence of a wide variety of RNAs including mRNA, miRNA, lincRNA, tRNA and piRNA in these vesicles. Based on the differential mRNA expression observed upon EXO-NGS analysis, we independently evaluated two protein coding genes, matrix metalloproteinase-8 (MMP-8) and transcription factor T-Box 3 (TBX3) by qRT-PCR for selective expression in the serum samples. Results indicate a variable expression pattern of these genes across serum samples between different study groups. Further, qRT-PCR analysis with the same serum exosomes processed for EXO-NGS, we observed two long non-coding RNAs, malat-1 and CRNDE to be variably expressed. Overall, our observations emphasize the potential value of different exosome components in distinguishing between healthy, premalignant and malignant conditions related to the pancreas.

Currently, only a small number of useful biomarkers for the clinical diagnosis of different types of cancer. These have been used for several years without any considerable changes. This results in patients undergoing unnecessary medical procedures. This is further compounded by the fact that a single cancer type may be heterogeneous, presumably due to disparate genetic defects in their tumors1. Recently, studies have been directed towards identification of biomarkers for cancer and other diseases, through non-invasive means utilizing components in blood such as circulating tumor cells (CTCs), cell free DNA (cfDNA) and very recently extracellular vesicles (EVs) which includes microvesicles and exosomes2–4. These liquid biopsy (noninvasive) based analysis present an alternate to conventional tumor biopsies (invasive) and may facilitate biomarker identification to detect early stages of a disease.

EVs such as exosomes are released into blood circulation and may have important functions in physiological and pathological conditions. These vesicles have recently invoked interest due to their potential in disease diagnosis and treatment5,6. They originate from a multivesicular body and upon fusing with the plasma membrane of the cell, they are released into the extracellular environment7. Exosomes range between 30–150 nm in size, are secreted by different cell types including tumor cells8, and are heterogeneous which could reflect the phenotypic state of the cell that generates them. The presence of exosomes has been reported in different biological fluids such as saliva, semen, urine, cerebrospinal fluid, breast milk and blood9–12. Due to the presence of exosomes in variety of pathological conditions, it has been investigated as a source of novel biomarkers based on analysis of their protein and RNA content. Nucleic acids such as mRNA, miRNA, transfer RNAs and long non-coding RNAs (lncRNAs) have been detected in exosomes13,14. Further, nucleic acid inside exosomes could be more resistant to RNA degrading enzymes such as RNases compared to those present in free circulation, which allows for a higher detection sensitivity and specificity12. The utility of exosomes and their cargos, especially the protein content has

1Veterinary Medicine & Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO, 65211, USA. 2Department of Surgery, School of Medicine, University of Missouri, Columbia, MO, 65212, USA. 3Harry S. Truman Veterans Hospital, 800 Hospital Drive, Columbia, MO, 65212, USA. 4Department of Medicine, Division of Gastroenterology and Hepatology; University of Missouri, Columbia, MO, 65212, USA. *email: kumars@missouri.edu
been previously reported for early detection of pancreatic cancer and predicting organotropic metastasis\textsuperscript{15,16}. However, the RNA elements in exosomes and their probable utility as biomarkers are less explored in this disease. PDAC is projected to become the second leading cause of cancer-related mortality in the United States by 2030\textsuperscript{17}. Due to the lack of screening imaging modalities and specific biomarkers, early diagnosis of this disease is difficult and about 50% of all patients are diagnosed at advanced stages\textsuperscript{18} and have limited therapeutic options. Unique challenges associated with PDAC include aggressive etiology and deep anatomic location. Currently, serum C19-9 is used as a circulating biomarker for PDAC, although its sensitivity and specificity are less than enough for its use as a diagnostic or early stage biomarker\textsuperscript{3}. Recently, exosome associated protein glypican has been reported as an early detection marker for PDAC\textsuperscript{15}. However, this was disputed by different studies which indicated that glypican may not be an ideal marker to detect early PDAC\textsuperscript{19,20} because of its presence in non-malignant conditions.

Profiling of RNAs in circulation, specifically miRNAs, has been reported to identify biomarkers for pathologies including cancer\textsuperscript{21–23}. A majority of these RNAs appears to be associated with exosomes. In this initial study, our underlying objective was the isolation and characterization of exosomes in blood serum along with the analysis of RNA cargos present in these vesicles by next generation sequencing (EXO-NGS). Further, we wanted to evaluate any variation in gene expression identified by EXO-NGS across healthy subjects, premalignant and malignant conditions associated with the pancreas. Such comparisons would offer a minimally invasive means of screening exosome RNA biomarkers that lay the groundwork in identifying potential biomarkers for early disease detection.

**Results and Discussion**

**Isolation and characterization of serum exosomes.** Several commercial kits are available to isolate exosomes from biological fluids which typically consists of polymeric additives to induce precipitation of exosomes\textsuperscript{24}. Use of these kits along with a standard centrifugation process (10,000 $\times$ g) allows for a faster separation of exosomes compared to the gold-standard technique of ultracentrifugation which is both laborious and time-consuming. Moreover, studies have reported that the yields and purity using these kits were higher compared to ultracentrifugation\textsuperscript{25,26}. While several options are available to isolate exosomes, depending on the source of exosomes and the purpose of the study, the protocol needs to be optimized to obtain a reasonably high yield and purity of these vesicles for downstream applications. We obtained exosomes (Fig. 1) from different serum samples within the size range (30–150 nm) which is in keeping with the previous studies\textsuperscript{27}.
In our study, exosomes isolated from the serum of healthy subjects and different pancreatic conditions were analyzed for their size distribution and quantity using Nanosight NS300 nanoparticle tracking analysis (NTA). Instruments such as dynamic light scattering and NTA have been used previously to measure the size of these vesicles. NTA instrument in addition to size distribution also measures the nanoparticle concentrations. The size distribution of exosomes analyzed by NTA between serum samples were different. The normal serum exosomes were predominantly 115 nm ± 20 nm in size while IPMN and PDAC serum exosomes exhibited sizes in the range of 94 nm ± 10 nm and 67 nm ± 25 nm, respectively (Fig. 1). While NTA was useful in analyzing exosome size and concentration the technique may have drawbacks. For instance, the size distributions obtained with this method may depend on set-up-parameters, and the concentration measurements based on refractive index of the material under examination. Further, underestimation of small size particles may be observed due to material-dependent lower detection limit. Hence more than one method may be necessary to analyze exosome morphology and size distribution. Further examination of purified vesicles by transmission electron microscopy (TEM) revealed that these vesicles possess varying size (35–100 nm) and morphology (Fig. 2a) resembling that of exosomes. The average size of serum exosomes as observed by EM was found to be 100 nm (range 90–120 nm) in healthy, 85 nm (range 62–95 nm) in IPMN and 59 nm (range 32–75 nm) in PDAC (Fig. 2b). There appears to be no consensus with regards to the minimal or maximal size range of exosomes in the literature. The lower size range reported varied between 30–50 nm and the upper size range varied between 100–150 nm. This discrepancy could be due to the various exosome isolation methods employed during the studies as well as their source of origin. Our own studies indicated that exosomes isolated from cell culture supernatants tend to be of relatively larger size (~125 nm) compared to liquid biopsy samples such as serum but well within the upper range of 150 nm (data not shown). Collectively, in our current study we observed that serum exosomes appear to be in the size range of 32–130 nm.

The concentration of exosomes was found to be different in the serum of healthy subjects and different pancreatic conditions were analyzed for their size distribution and quantity using Nanosight NS300 nanoparticle tracking analysis (NTA). Instruments such as dynamic light scattering and NTA have been used previously to measure the size of these vesicles. NTA instrument in addition to size distribution also measures the nanoparticle concentrations. The size distribution of exosomes analyzed by NTA between serum samples were different. The normal serum exosomes were predominantly 115 nm ± 20 nm in size while IPMN and PDAC serum exosomes exhibited sizes in the range of 94 nm ± 10 nm and 67 nm ± 25 nm, respectively (Fig. 1). While NTA was useful in analyzing exosome size and concentration the technique may have drawbacks. For instance, the size distributions obtained with this method may depend on set-up-parameters, and the concentration measurements based on refractive index of the material under examination. Further, underestimation of small size particles may be observed due to material-dependent lower detection limit. Hence more than one method may be necessary to analyze exosome morphology and size distribution. Further examination of purified vesicles by transmission electron microscopy (TEM) revealed that these vesicles possess varying size (35–100 nm) and morphology (Fig. 2a) resembling that of exosomes. The average size of serum exosomes as observed by EM was found to be 100 nm (range 90–120 nm) in healthy, 85 nm (range 62–95 nm) in IPMN and 59 nm (range 32–75 nm) in PDAC (Fig. 2b). There appears to be no consensus with regards to the minimal or maximal size range of exosomes in the literature. The lower size range reported varied between 30–50 nm and the upper size range varied between 100–150 nm. This discrepancy could be due to the various exosome isolation methods employed during the studies as well as their source of origin. Our own studies indicated that exosomes isolated from cell culture supernatants tend to be of relatively larger size (~125 nm) compared to liquid biopsy samples such as serum but well within the upper range of 150 nm (data not shown). Collectively, in our current study we observed that serum exosomes appear to be in the size range of 32–130 nm.

The concentration of exosomes was found to be different in the serum of healthy subjects (7 × 10^8 particles/ml), patients with IPMN (7.06 × 10^10 particles/ml) and patients diagnosed with PDAC (4 × 10^11 particles/ml). Exosome concentrations in the serum from PDAC patients tend to be higher than healthy or IPMN samples. Previous reports indicate that exosome concentrations can vary between 1–3 × 10^12 particles per ml serum. To confirm the vesicles were indeed exosomes, protein markers for exosomes such as CD63 and CD9 were analyzed using immunoblot studies. Serum exosomes from different conditions exhibit both the presence of CD63 and CD9 (Fig. 1 inset), widely reported to function as exosome markers. Uncropped gel images are shown in supplementary (Fig. S1).

RNA recovery from exosomes. Total RNA was isolated from different serum exosomes and the RNAs were purified using a phenol-free lysis buffer and rapid spin columns. RNA from serum exosomes isolated from
each sample category was verified for integrity using a Bioanalyzer. A representative analysis of the small RNAs in serum samples are shown in supplementary (Fig. S2). Exosomes derived from serum typically yielded total RNA in the range of 2.4–12 ng/ml. RNA concentrations up to 10 ng were reported earlier in exosomes27. However, depending on the source of the starting material and the concentration of exosomes, the RNA concentrations could vary. Moreover, exosomes are a heterogeneous population of vesicles with widely varying cargo, in that some vesicles may have many RNAs while some carry only the protein cargo and no RNA.

Analysis of RNA in exosomes by sequencing. Two donors were used for each sample type. The library construction was made by ligating the adaptor sequences to the RNA in the samples. Subsequently, PCR amplification was performed34. Once the library construction was complete, the samples were subjected to sequencing using the NextSeq. 500/550 instrument in high output configuration. The adaptor sequences were trimmed after sequencing exosome libraries and single-end RNA reads were generated. An example of the number of reads pre and post quality filtering is depicted in supplementary (Fig. S3) for different samples. The total number of reads in millions (M) retained after quality assessment were approximately 10 M for healthy serum, 25 M for IPMN and 19 M for PDAC. Further, the reads retained or discarded after quality assessment is depicted in supplementary (Fig. S4). About 75–80% of the reads in different serum samples were of good quality reads. Our analysis also included spike-in-DNA controls35 to avoid any misinterpretations during data normalization, based on the presumption that different serum samples will not yield identical amounts of RNA for analysis.

Based on the mapped sequence read counts, the RNA content of different serum exosomes was quantified. The whole summary of reads mapping to gene annotation types are depicted in supplementary (Fig. S5). As illustrated in the pie chart graphs, serum exosomes contained very diverse RNA cargo. The following total gene types with varying abundance were present in exosomes from individual serum types: (mRNA (20,596), (lincRNA, 8660), ribosomal RNAs (rRNAs,3275), piwi interacting RNAs (piRNA, 2298), microRNAs (miRNA,1521) and transfer RNAs (tRNA/tRNA-like, 391). Also, other contents such as antisense elements to exons, introns, and small lncRNA, small Cajal body-specific RNAs (scaRNAs) including unannotated genes were also present in exosomes. Pie charts depicting the percentage of various RNAs between different serum exosomes are shown in Fig. 3. Overall, the abundance of these RNAs in exosomes are relatively high in PDAC serum compared to healthy and IPMN conditions. We will further focus only on three types of RNAs in this study (mRNA, piRNA and tRNA). Theoretically, it has been indicated that each exosome can accommodate 70–25,000 small RNA or protein molecules27. Given the size and high concentration of exosomes present in blood (10 10–1012 particles per ml of blood)31,36 it is not surprising that exosomes could hold a variety of biological molecules within its boundaries and be capable of evoking considerable biological effects in vivo.

Differentially expressed genes in serum exosomes. Total differentially expressed genes in exosomes between the serum types are shown as volcano plots in supplementary (Fig. S6). Overall, several genes were significantly up or down regulated in IPMN and PDAC compared to healthy serum exosomes. However, such differential expression of genes between two different healthy samples was very minimal. Often exosome studies involve reporting miRNA as biomarker candidates for cancer detection including that of the pancreas37. Other non-coding RNA types such as piRNA and tRNA are underrepresented and should be considered as an important source for differentiating the disease conditions independently or in combination with other RNA types. The ten differentially expressed mRNAs, piRNAs, and five tRNAs in different serum samples are listed in Tables 1–3. Heatmaps indicating the differences in various RNA expression are depicted in supplementary (Fig. S7). RNA-Seq raw data pertained to the depicted genes are shown in supplementary (File S1).

Among the different mRNA transcripts, MMP8 was high in PDAC exosomes. MMP8, a member of the matrix metalloproteinase family, has been implicated in several cancer types and reported to have conflicting roles in
cancer as a promoter and suppressor of metastasis. However, the role of MMP8 in pancreatic disease is less known. While MMP 8 has been implicated in acute pancreatitis its function in PDAC is unclear. A different study suggested that MMP8 could also function as a predictive biomarker in serum for colorectal cancer.

Another coding transcript, TBX3 is also highly represented in PDAC exosome compared to healthy and IPMN serum. TBX3 protein product suppress E-cadherin and enhances melanoma invasiveness and is also correlated with advanced stages of gastric cancer. While these genes were studied previously in direct plasma or tissues, their presence in exosomes have not been reported. Interestingly, we also observed increased representation of the pancreatic and duodenal homeobox-1 (PDX1) transcript in IPMN and tumor exosomes. The protein product from this gene was reported to be involved in the transcriptional activation of insulin and has been implicated in the pathological and clinical features of invasive PDAC. Conversely, we also observed that some of the coding transcripts such as SIGLEC11 which is implicated in anti-inflammatory function and DCN which functions as a tumor suppressor and anti-metastatic protein were low in PDAC and IPMN exosomes. Also, KLRB1 (CD161) transcript appears to be low in IPMN and PDAC serum. KLRB1 transcript has been reported to be suppressed in lung tumors and esophageal squamous cell carcinoma although the exact biological function of this protein is unclear.

Like the protein coding transcripts, the presence of other small ncRNAs such as piRNA and tRNA transcripts were also observed in the exosomes. Previously, these ncRNAs were studied in either pancreatic tissues or cells but not in exosomes. For instance, tRNAs have been shown to interact with MEK2 in pancreatic carcinoma cells and alter cell behavior. Likewise, piRNAs may have tumorigenic or suppressive roles in cancer and are likely involved in regulation of DNA methylation. While piRNAs have been reported in variety of cancers, reports are sparsely available for pancreatic cancer. One study, however, indicated that piR-017061 was downregulated in pancreatic cancer tissues. We observed several piRNA transcripts increased or decreased in PDAC exosomes.
relative to healthy or IPMN conditions (Table 2). It is unclear regarding the significance of above mentioned ncR-
NAs in exosomes. However, their expression seems to vary in pathological conditions. We speculate that these
RNAs could be exchanged between the exosome target cells and may have functional significance.

Validation of differentially expressed exosome mRNAs by qRT-PCR analysis. To validate the
EXO-NGS sequencing results, the expression of candidate genes \(\text{MMP8} \) and \(\text{TBX3}\) were analyzed in the serum
exosomes under study. Consistent with NGS-EXO observations, both \(\text{MMP8} \) and \(\text{TBX3}\) were higher in tumor
exosomes compared to healthy or IPMN samples with Ct values ranging from 27.6–30.8 with a median Ct of 29.2
(\(\text{MMP8}\)), and Ct values (28.0–31.7) with a median Ct of 29.8 (\(\text{TBX3}\)) (Fig. 4a).

Both lncRNAs \(\text{malat1} \) and \(\text{CRNDE}\) have been reported to be increased in pancreatic cancer cells or tis-
ses\(^{50,51}\). However, their presence in exosomes are less known. Due to limitation in analysis of gene size during
EXO-NGS, lncRNA analysis in exosomes were conducted by qPCR analysis in exosomes isolated directly using
the serum samples under investigation. The differences in the expression of lncRNAs \(\text{malat1} \) and \(\text{CRNDE}\) in
serum exosomes are depicted in (Fig. 4b). Both lncRNAs were expressed higher in PDAC or IPMN vs healthy
samples. The Ct values ranges were between 27 and 31 with a median Ct of 28.7 for \(\text{malat1}\) and Ct values between
29–32 with a median Ct of 30.5 for CRNDE. The dissociation curves corresponding to each gene is depicted in
supplementary (Fig. S8).

Validation of differentially expressed exosome mRNAs by qRT-PCR analysis. To validate the
EXO-NGS sequencing results, the expression of candidate genes \(\text{MMP8} \) and \(\text{TBX3}\) were analyzed in the serum
exosomes under study. Consistent with NGS-EXO observations, both \(\text{MMP8} \) and \(\text{TBX3}\) were higher in tumor
exosomes compared to healthy or IPMN samples with Ct values ranging from 27.6–30.8 with a median Ct of 29.2
(\(\text{MMP8}\)), and Ct values (28.0–31.7) with a median Ct of 29.8 (\(\text{TBX3}\)) (Fig. 4a).

Both lncRNAs \(\text{malat1} \) and \(\text{CRNDE}\) have been reported to be increased in pancreatic cancer cells or tis-
ses\(^{50,51}\). However, their presence in exosomes are less known. Due to limitation in analysis of gene size during
EXO-NGS, lncRNA analysis in exosomes were conducted by qPCR analysis in exosomes isolated directly using
the serum samples under investigation. The differences in the expression of lncRNAs \(\text{malat1} \) and \(\text{CRNDE}\) in
serum exosomes are depicted in (Fig. 4b). Both lncRNAs were expressed higher in PDAC or IPMN vs healthy
samples. The Ct values ranges were between 27 and 31 with a median Ct of 28.7 for \(\text{malat1}\) and Ct values between
29–32 with a median Ct of 30.5 for CRNDE. The dissociation curves corresponding to each gene is depicted in
supplementary (Fig. S8).

While our studies indicate differences in various RNAs between serum types, the presence of exosome RNAs
could be differentially regulated in pancreatic tumor subtypes. For instance, previous RNA sequence analysis in
tissues from different pancreatic tumor subtypes\(^{52,53}\) which varied in their neoplastic cellularity\(^{53}\), indicated that an
individual mRNA could be differentially regulated (up or down) within these subtypes. Therefore, it is reasonable
to anticipate that exosomes and their components representing different cellular origins could likely mimic these
changes. For instance, as observed in this study the exosome mRNA transcripts \(\text{PDX1} \) and \(\text{CTSL} \) (\(\text{CTSL1}\)) both
were found to be high in PDAC. Previous studies\(^{52}\) indicated the presence of these genes in pancreatic tumor
tissues but high in progenitor and immunogenic subtypes compared to the basal type. Alternately, the
\(\text{DCN}\) transcript which we observed to be low in PDAC serum exosomes was also absent or decreased in most tumor
subtypes except immunogenic subtype. The mRNA distribution of these genes in PDAC serum exosomes from
the present study, compared to tumor tissues of different subtypes reported earlier\(^{52}\) are depicted in Table 4.

In the current study, we were able to investigate the RNAs in the patient serum exosomes, however, a limi-
tation in this study is the exosome isolation technique. While various methods have been reported in the liter-
ature regarding exosome isolation\(^{54}\), each has its own advantages and disadvantages. Thus far, the established
methods appear to lack robustness in obtaining pure exosome population. However, a previous report indicate
that both ultracentrifugation and commercially available total exosome isolation reagents have been shown to
recover exosomes in a comparable manner\(^{55}\). Another study, indicated that techniques such as precipitation

| mRNA –Gene ID | PDAC serum exosomes | PDAC Tumor subtypes* | | | |
|---------------|------------------|-----------------|---|---|---|
|               |                  | Basal | Classical/Progenitor | ADEX | Immunogenic | |
| \(\text{PDX1}\) | Up               | Down  | Up                   | Up   | Down        | |
| \(\text{CTSL}\) | Up               | None  | Down                 | None | Up          | |
| \(\text{DCN}\) | Down             | None  | Down                 | None | Up          | |

Table 4. Gene expression comparison between exosomes and tumor subtypes. The transcripts expressed in exosomes of PDAC serum in the current study was compared with previously reported results obtained in tissues from different PDAC tumor subtypes. The data for PDAC tumor subtypes* are from ref. \(^{52}\). The transcripts in exosomes appear to indicate the most likely PDAC tumor subtypes a particular gene may be up or down regulated.

While our studies indicate differences in various RNAs between serum types, the presence of exosome RNAs
could be differentially regulated in pancreatic tumor subtypes. For instance, previous RNA sequence analysis in
tissues from different pancreatic tumor subtypes\(^{52,53}\) which varied in their neoplastic cellularity\(^{53}\), indicated that an
individual mRNA could be differentially regulated (up or down) within these subtypes. Therefore, it is reasonable
to anticipate that exosomes and their components representing different cellular origins could likely mimic these
changes. For instance, as observed in this study the exosome mRNA transcripts \(\text{PDX1} \) and \(\text{CTSL} \) (\(\text{CTSL1}\)) both
were found to be high in PDAC. Previous studies\(^{52}\) indicated the presence of these genes in pancreatic tumor
tissues but high in progenitor and immunogenic subtypes compared to the basal type. Alternately, the
\(\text{DCN}\) transcript which we observed to be low in PDAC serum exosomes was also absent or decreased in most tumor
subtypes except immunogenic subtype. The mRNA distribution of these genes in PDAC serum exosomes from
the present study, compared to tumor tissues of different subtypes reported earlier\(^{52}\) are depicted in Table 4.

In the current study, we were able to investigate the RNAs in the patient serum exosomes, however, a limi-
tation in this study is the exosome isolation technique. While various methods have been reported in the liter-
ature regarding exosome isolation\(^{54}\), each has its own advantages and disadvantages. Thus far, the established
methods appear to lack robustness in obtaining pure exosome population. However, a previous report indicate
that both ultracentrifugation and commercially available total exosome isolation reagents have been shown to
recover exosomes in a comparable manner\(^{55}\). Another study, indicated that techniques such as precipitation

Figure 4. qRT-PCR analysis for RNA expression. Gene expression for \(\text{MMP8} \) and \(\text{TBX3}\) (a) including \(\text{malat-1} \) and \(\text{CRNDE}\) IncRNA (b) were analyzed by qRT-PCR in the same serum exosomes used in EXO-NGS studies. Two biological replicates were used for each normal, IPMN and PDAC samples and the experiments were performed in duplicates. The dissociation curves for the qRT-PCR are shown in supplementary (Fig. 8).
RNA concentration and integrity of each sample, 1 RNA Purification kit (System Biosciences) according to the manufacturer's instructions. Subsequently, to measure detectable levels for further validation as biomarkers in larger cohorts using routinely used technology platforms between patients. Also, any specific and abundantly expressed RNA transcripts in exosomes should exist within larger sample cohorts. Investigations of these biomolecules as markers should also consider biological diversity such as IPMN. Further validation of these biomolecules needs screening for more specific disease category and in different pancreatic tumor subtypes and could form the basis for investigating further in precursor lesions between patients. Also, any specific and abundantly expressed RNA transcripts in exosomes should exist within detectable levels for further validation as biomarkers in larger cohorts using routinely used technology platforms (e.g. qRT-PCR).

Conclusion
Our studies indicate the presence of exosome vesicles of varying size in different serum samples. While NTA method may be used in analyzing exosomes, additional methods such as EM will be necessary to analyze exosomes. Unlike NTA, EM not only allows to visualize exosome morphology but enables to measure even small size vesicles below 40 nm. By performing EXO-NGS analysis we identified several RNA transcripts which have not been previously reported in exosomes associated with different pancreatic conditions. While most previous RNA studies have been done with pancreatic tumor cells or tissues, liquid biopsy sources such as exosomes would facilitate the noninvasive discovery of useful biomarkers for early detection. The presence of RNAs such as piRNA and tRNA, hitherto sparsely reported in pancreatic exosomes, suggested the need for further studies to gain insights into their biological effects on target cells. While our studies involve limited sample analysis, validation in large prospective cohorts which include more cases of premalignant lesions will be extremely useful in detecting the early disease. Our future studies are directed toward such validation efforts in patients to identify prognostic or predictive biomarkers using RNA signatures.

Methods
Collection of serum samples. After approval of the Institutional Review Board (IRB), Office of Research, University of Missouri, and informed consent from the human subjects for study participation, blood samples without anticoagulants from healthy individuals, intraductal papillary mucinous neoplasms (IPMN) and PDAC (stage III-IV) were collected. An hour after the blood collection, serum was separated by centrifugation (2300 rpm, 15 min) and used immediately for isolation of exosomes or stored at −80 °C for long term. If stored, 500 μl of serum was centrifuged at 1500 × g for 30 min to remove residual cells and debris. The supernatant was transferred to a new 1.5 ml Eppendorf tube before exosome isolation. All methods were performed in accordance with relevant guidelines and regulations.

Exosome isolation from serum. ExoQuick Ultra (System Biosciences, Palo Alto, CA) was added to the supernatant from the above step at a 1:4 ratio, mixed gently, and incubated for 30 min at 4 °C. After the incubation, the admixture was centrifuged at 1500 × g for 30 min to recover exosomes for total RNA isolation. For gel purification, serum was diluted with PBS (two-fold) and processed using qEV size exclusion columns (IZON, Medford, MA) according to manufacturer's instructions.

Exosome particle size analysis. Exosomes obtained from serum were diluted in PBS (dilution depending on the concentration of exosomes) and subsequently analyzed using a NanoSight NS300. The instrument is equipped with a 405 nm laser (NanoSight, Amesbury, UK), and an electron multiplying charge-coupled device (EMCCD) was used to determine the histogram of particle size by tracking the Brownian motion of single particles at 25 °C for size and concentration determination. Videos were recorded for 60 seconds during which the movement of the nanoparticles were tracked by NTA software (version 2.3, NanoSight) with low refractive index corresponding to serum derived vesicles. The Stokes-Einstein equation was employed to determine the size distribution and number of particles within the sample.

Transmission electron microscopy. Exosomes isolated from the serum were processed for transmission electron microscopy (TEM) and negative staining. All specimen preparation was performed at the Electron Microscopy Core Facility, University of Missouri. Exosomes were suspended in PBS (pH 7.2), and placed on a negatively charged carbon coated copper grid and allowed to settle at room temperature for 20 minutes. Excess sample was removed by wicking with filter paper, then fixed for two minutes in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) solution. Grids were rinsed with three consecutive washes of distilled water and negative staining was performed by placing 5 μL of Nano Tungsten (Nanoprobes, Inc.) on each grid for two minutes prior to wicking to dryness with filter paper and allowed to dry at room temperature. Images were acquired with a JEOL JEM 1400 transmission electron microscope (JEOL, Peabody, MA) at 120 kV on a Gatan Ultrascan 1000 CCD (Gatan, Inc, Pleasanton, CA).

Total RNA isolation from exosomes. Total RNA from exosomes was isolated using the SeraMir Exosome RNA Purification kit (System Biosciences) according to the manufacturer's instructions. Subsequently, to measure RNA concentration and integrity of each sample, 1 μl of the final RNA eluate was used for measurement of small
RNA concentration by Agilent Bioanalyzer Small RNA Assay using Bioanalyzer 2100 Expert instrument (Agilent Technologies, Santa Clara, CA).

**Immunoblotting.** Exosomes isolated from serum were suspended in 50 μl M-PER reagent (Thermo Scientific) with HALT protease inhibitor cocktail (Thermo Scientific). After determining the protein concentration using Bradford analysis, the samples were fractionated on a 4–12% gradient SDS-PAGE gels (Invitrogen, Carlsbad, CA). Subsequently, the gels were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was incubated with anti-CD63 (H5C6) (Novus Biologicals, Centennial, CO) or anti-CD9 (D3H4P) antibodies (Cell Signaling Technology, Danvers, MA) overnight at 4°C, followed by incubation with peroxidase conjugated anti-mouse/-rabbit antibody. The proteins were visualized using an Immobilon Forte Western HRP blotting substrate (Millipore, Burlington, MA).

**NGS Library generation and sequencing.** Small RNA libraries were constructed with the Clean Tag Small RNA Library Preparation Kit (TriLink, Cat# L-3206) according to the manufacturer's protocol. The final purified library was quantified with High Sensitivity DNA Reagents (Agilent Technologies, PO# G2933-85004) and High Sensitivity DNA Chips (Agilent Technologies, PO# 5067–4626). The libraries were pooled, and the 140 bp to 300 bp region was size selected on an 8% TBE gel (Invitrogen, Life Technologies). The size selected library was quantified with High Sensitivity DNA 1000 Screen Tape (Agilent Technologies, PO # 5067–5584), High Sensitivity D1000 reagents (Agilent Technologies, PO# 5067–5585), and the TailorMix HT1 qPCR assay (SeqMatic, Cat# TM-505), followed by a NextSeq High Output single-end sequencing run at SR75 using NextSeq 500/550 High Output v2 kit (Cat #FC-404-2005, Illumina, San Diego, CA) according to the manufacturer’s instructions.

**Bioinformatic analysis.** Raw reads filtering. The Exosome RNA-seq analysis initiates with a data quality check of the input sequence using FastQC, an open-source quality control (QC) tool for high-throughput sequence data56. FastQC runs analyses of the uploaded raw sequence reads that reveals the quality of the data and inform the subsequent preprocessing steps in the analysis. Following the initial assessment, Bowtie257 was used to map the spike-in DNA before the trimming and filtering steps where RNA-seq reads are preprocessed to improve the quality of data input for read mapping. The open-source tools used for trimming of adapters are FastqMcf56 and Cutadapt58, while PRINSEQ59 was used in the quality filtering step.

Reads mapping. Sequencing adapters were trimmed from reads, and filtered for quality and length in the preprocessing steps. FastQC is then re-run to analyze the trimmed reads, allowing for a before and after comparison. The processed set of input data was used in the subsequent read mapping step. The improved set of sequence reads are merged, if needed, using SeqPrep60 then mapped to the reference genome assembly (GRCh37-hg19) using Bowtie257. Using the open-source software BEDTools61 and SAMTools62, read alignment and read coverage tracks are generated and deployed to the genome browser.

RNA abundance analysis. Abundance determination and differential expression analysis was performed by DEseq63. Abundance levels for ncRNAs (miRNAs, tRNAs, rRNAs, lincRNAs, piRNAs, snoRNAs), antisense transcripts, coding genes and repeat elements (LTR, LINE, SINE, and tandem repeats) were determined. A summary of reads overlapping each of these annotations in the reference genome is created using SAM tools.

cDNA synthesis and quantitative real-time PCR (qRT-PCR). Exosome RNA was converted to cDNA using a High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) with RNSae inhibitor treatment according to manufacturer’s protocol. qRT-PCR was performed using SYBR Green assay for exosome mRNA expression (n = 3 each sample) of matrix metalloproteinase-8 (MMP-8), TBX3 and long non-coding RNAs, malat-1 and CRNDE. β-actin was used as internal control. Relative quantification (fold change) was determined using 2−ΔΔCt method. Following primers were used in this study: MMP8, F-CCTGGTGGCCTTTGATGA, R- GTGGCATAG TTGGGATAG; TBX3, F- GTGACTGCATACCAGAAT, R- TTCTCTTCGGCCATTTC; Malat1, F- GTTGTCAAGCAAGAAG, R-TCCATA TGCGTGCTAAACA. Two replicate serum samples each from healthy, IPMN and PDAC were used. Analysis was done in duplicates.

Statistical analyses. Differences in gene expression levels were analyzed using either a paired t-test or nonparametric two-tailed Mann Whitney –U test. P value < 0.05 was considered significant.

**Data availability**

The data pertained to this manuscript are available to readers upon publication. Certain materials as a part of this data but not associated with this manuscript may not be shared due to either ongoing analysis or publication restrictions.

Received: 29 May 2019; Accepted: 30 January 2020;
Published online: 18 February 2020

**References**

1. van’t Veer, L. J. & Bernards, R. Enabling personalized cancer medicine through analysis of gene-expression patterns. Nature. 452, 564–570 (2008).
2. Gold, B., Cankovic, M., Furtado, L. V., Meier, F. & Gocke, C. D. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology. J. Mol. Diagn. 17, 209–224 (2015).
46. Pleshkan, V. V., Zinov’eva, M. V., Vinogradova, T. V. & Sverdlov, E. D. Transcription of the KLRB1 gene is suppressed in human cancer tissues. Eur. J. Cell Biol. 277, 116–126 (2009).
47. Nukarinen, E. et al. Association of Matrix Metalloproteinases -7, -8 and -9 and TIMP -1 with Disease Severity in Acute Pancreatitis. J. Clin. Biochem. 47, 1286–1292 (2015).
48. Rodriguez, M., Aladowicz, E., Lanfrancone, L. & Goding, C. R. Tbx3 represses E-cadherin expression and enhances melanoma cell invasion and migration. J. Extracell. Vesicles. 1, 11–18 (2013).
49. Zeringer, E. et al. Methods for the extraction and RNA profiling of exosomes. World J. Methodol. 3, 11–18 (2013).
50. Chen, K. et al. The Overlooked Fact: Fundamental Need for Spike-in Control for Virtually All Genome-Wide Analyses. Mol. Cell. Biochem. 36, 662–667 (2015).
51. Melo, S. A. et al. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer Cell. 26, 707–721 (2014).
52. Chronopoulos, Liebenthal, T. J. & del Río Hernández, A. E. Exosomes as a platform for ‘liquid biopsy’ in pancreatic cancer. Converg. Sci. Phys. Oncol. 3, 013005.
53. Thirkettle, S. et al. Matrix metalloproteinase 8 (collagenase 2) induces the expression of interleukins 6 and 8 in breast cancer cells. J. Biol. Chem. 288, 16282–16294 (2013).
54. Nukarinen, E. et al. Association of Matrix Metalloproteinases -7, -8 and -9 and TIMP -1 with Disease Severity in Acute Pancreatitis. A Cohort Study. PLoS One. 11, e0161480 (2016).
55. Böckelman, C. et al. Serum MMP-8 and TIMP-1 predict prognosis in colorectal cancer. BMC Cancer. 18, 679 (2018).
56. Rodrigue, M., Aladowicz, E., Lanfrancone, L. & Goding, C. R. Tbx3 represses E-cadherin expression and enhances melanoma invasiveness. Cancer Res. 68, 7872–7881 (2008).
57. Miao, Z. et al. Tbx3 overexpression in human gastric cancer is correlated with advanced tumor stage and nodal status and promotes cancer cell growth and invasion. Virchows Arch. 469, 505–513 (2016).
58. Quint, K. et al. The expression pattern of PD-1, SHIP, Patched and Gli-1 is associated with pathological and clinical features in human pancreatic cancer. Pancreatology. 9, 116–126 (2009).
59. Angata, T. et al. Cloning and characterization of human Siglec-11. A recently evolved signaling molecule that can interact with SHP-1 and SHP-2 and is expressed by tissue macrophages, including brain microglia. J. Biol. Chem. 277, 24466–24474 (2002).
60. Soeia Fugueia, D. D., Götte, M. & Viola, M. More than matric: the multifaceted role of decorin in cancer. Eur. J. Cell Biol. 92, 1–11 (2013).
61. Pleshkan, V. V., Zinov’eva, M. V., Vinogradova, T. V. & Sverdlov, E. D. Transcription of the KLRB1 gene is suppressed in human cancer tissues. Mol. Gen. Microbiol. Viral. 4, 3–7 (2007).
54. Batrakova, E. V. & Kim, M. S. Using exosomes, naturally-equipped nanocarriers, for drug delivery.
53. Wood, L. D. & Hruban, R. H. Pathology and molecular genetics of pancreatic neoplasms.
55. Schageman, J.
47. Müller, S. et al. Next-generation sequencing reveals novel differentially regulated mRNAs, lncRNAs, miRNAs, sdRNAs and a piRNA in pancreatic cancer. Mol. Cancer. 14, 94 (2015).
48. Wang, X. et al. Interaction of tRNA with MEK2 in pancreatic cancer cells. Sci. Rep. 6, 28260 (2016).
49. Weng, W., Li, H. & Goel, A. Piwi-interacting RNAs (piRNAs) and cancer: Emerging biological concepts and potential clinical implications. Biochim. Biophys. Acta Rev. Cancer. 1871, 160–169 (2019).
50. Zhang, Y., Yang, X., Shi, M., Wen, C. & Shen, B. MiR-216a decreases MALAT1 expression, induces G2/M arrest and apoptosis in pancreatic cancer cells. Biochem. Biophys. Res. Commun. 483, 816–822 (2017).
51. Wang, G., Pan, J., Zhang, L., Wei, Y. & Wang, C. Long non-coding RNA CRNDE sponges miR-384 to promote proliferation and metastasis of pancreatic cancer cells through upregulating IRS1. Cell Prolif. 50, e12389 (2017).
52. Raphael, B. J. et al. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. Cancer Cell. 32, 185–203 (2017).
53. Wood, L. D. & Hruban, R. H. Pathology and molecular genetics of pancreatic neoplasms. Cancer J. 18, 492–501 (2012).
54. Batrakova, E. V. & Kim, M. S. Using exosomes, naturally-equipped nanocarriers, for drug delivery. J. Control. Release. 219, 396–405 (2015).
55. Schageman, J. et al. The complete exosome workflow solution: from isolation to purification of RNA cargo. Biomed. Res. Int. 2013, 253957 (2013).
56. Andrews, S. FastQC. A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc (2009).
57. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).
58. Martin, M. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. EMBnet J. 17, 10–12 (2011).
59. Schmieder, R. & Edwards, R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 27, 863–864 (2011).
60. John, J. S. SeqPrep, https://github.com/jstjohn/SeqPrep (2011).
61. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics. 26, 841–842 (2010).
62. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 25, 2078–2079 (2009).
63. Anders, S. & Huber, W. Differential expression analysis for sequence count data. Genome Biology. 11, R106 (2010).

Acknowledgements
A core faculty grant from College of Veterinary Medicine to S.R.K. is kindly acknowledged. The authors are grateful for the help of Maren Fleer and Sydney Hackwell for their assistance in this study. S.R.K. receives support from a Grant #5UL1TR002345.

Author contributions
S.R.K. conceived the study and wrote the manuscript. E.T.K. coordinated patients for this study. S.G. performed the experiments along with S.R.K. Y.M. along with E.T.K. had direct contact with the patients to collect blood samples and subsequent processing for downstream studies. S.R.K. and A.J.S. performed the bioinformatic analysis. J.T.K. as the responsible IRB primary investigator coordinated patient recruitment and subjects’ consenting, blood sample acquisition and processing/analysis in the laboratory. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-59523-0.

Correspondence and requests for materials should be addressed to S.R.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020