Nanoplasmonic quantification of tumour-derived extracellular vesicles in plasma microsamples for diagnosis and treatment monitoring

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Tumour-derived extracellular vesicles (EVs) are of increasing interest as a resource of diagnostic biomarkers. However, most EV assays require large samples and are time-consuming, low-throughput and costly, and thus impractical for clinical use. Here, we describe a rapid, ultrasensitive and inexpensive nanoplasmon-enhanced scattering (nPES) assay that directly quantifies tumour-derived EVs from as little as 1 μl of plasma. The assay uses the binding of antibody-conjugated gold nanoparticles and nanorods to EVs captured by EV-specific antibodies on a sensor chip to produce a local plasmon effect that enhances tumour-derived EV detection sensitivity and specificity. We identified a pancreatic cancer EV biomarker, ephrin type-A receptor 2 (EphA2), and demonstrate that an nPES assay for EphA2-EVs distinguishes pancreatic cancer patients from pancreatitis patients and healthy subjects. EphA2-EVs were also informative in staging tumour progression and in detecting early responses to neoadjuvant therapy, with better performance than a conventional enzyme-linked immunosorbent assay. The nPES assay can be easily refined for clinical use, and readily adapted for diagnosis and monitoring of other conditions with disease-specific EV biomarkers.

Extracellular vesicles (EVs), including exosomes and other membraneous vesicles, are abundantly secreted into the extracellular space by most cells, from where they can ultimately accumulate in the circulation. EVs actively participate in tumour initiation, progression and metastasis, shuttling signalling molecules (proteins and nucleic acids) that reflect their parental cell and tissue origins. Circulating tumour-derived EVs thus hold great potential as biomarkers for noninvasive cancer detection. However, translating tumour EVs into cancer biomarkers has been challenging due to the lack of simple methods for EV analysis and of biomarkers that distinguish tumour-derived EVs from normal EVs. Conventional detection technologies require time-consuming and labour-intensive isolation and purification procedures (for example, ultracentrifugation, immunomagnetic enrichment or microfluidic-based separation), followed by EV quantification and/or analyses of EV-carrying molecular contents (such as mRNA, microRNA and proteins). These techniques are impractical for clinical and research use since they require relatively large sample volumes, are complex, low-throughput and expensive, and have long turnaround times. Sample requirements are a particular barrier to animal-based research studies, since the blood volume available from common mouse models of human disease is very limited and precludes longitudinal studies. EV analyses are present on EVs derived from most cell types, but currently very few EV proteins have been proposed to represent cancer-associated biomarkers. Recently, methods have been developed to isolate tumour-derived EVs by capturing candidate tumour-regulated markers on the EV membrane, but the clinical utility of EVs as cancer biomarkers is still very limited since most proposed methods require time-consuming EV isolation steps before the actual analysis.

Assays useful in clinical settings generally share several features. Most are rapid, highly sensitive and specific, require minimal processing, and are usually amenable to automation. To address these issues, we developed a rapid nanoparticle-based EV assay in which EVs present in small volumes of unprocessed plasma samples were captured by an EV-specific antibody on the surface of a sensor chip, and then hybridized with two antibody-conjugated nanoparticle probes. Dual binding of EVs by the two nanoparticle probes produced a local plasmon to increase scattering intensity and shift its wavelength, resulting in a marked increase in the sensitivity and specificity of EV detection. Comparative and quantitative proteomic analyses of EVs derived from normal and tumour-derived pancreatic cell lines identified candidate biomarkers that were selectively enriched on EVs of pancreatic cancer cells — including ephrin type-A receptor 2 (EphA2), which is enriched on several tumours and plays critical roles in cancer progression, metastasis and...
EphA2 overexpression is reported to increase in vitro invasiveness and anoikis resistance of pancreatic adenocarcinoma cell lines, whereas EphA2 knockdown has the opposite effect, and EphA2 siRNA treatment decreases mouse pancreatic tumour growth and metastases in parallel with increased tumour-associated apoptosis. Substituting an EphA2-specific nanoparticle for one of the two EV-specific nanoparticles in our nanoplasmon-enhanced scattering (nPES) method produced a blood-based EphA2-EV nPES assay that demonstrated strong diagnostic sensitivity and specificity for pancreatic cancer patients in a pilot study performed with cohorts of normal healthy control subjects, pancreaticitis patients and pancreatic cancer patients with stage I–III cancer. Changes in EphA2-EV blood levels pre- to post-therapy also corresponded with therapy responses, suggesting that EphA2-EV blood levels could be used to non-invasively monitor treatment responses. This study thus indicates that the nPES platform can be used as a rapid, low-cost, high-throughput, sensitive and specific method for the detection and quantitation of EVs in microsample volumes from a variety of sample types. This approach can be readily customized to detect specific disease-derived EV populations for diagnostic-assay development, as indicated by our proof-of-concept studies in pancreatic cancer patients.

**Design of a purification-free nPES platform for EV detection**

Gold nanoparticles (GNPs) scatter light at characteristic wavelengths according to their size and shape. For example, 50 nm gold nanospheres (AuS) scatter green light, and 25 × 60 nm gold nanorods (AuR) scatter red light; however, when the distance between AuS and AuR particles is <200 nm, their scattering is coupled, forming a plasmon that shifts the spectra of scattered light to yellow while also markedly increasing in scattering intensity (Fig. 1a–f). We applied this principle to design a simple, purification-free nPES assay platform to directly detect EVs in microsamples of different specimen types, including culture media and plasma.

For a proof-of-principle demonstration, we used antibodies against CD81, CD63 and CD9, which are enriched on most EV membranes, to both capture and detect all EVs present in a sample. We first conjugated an anti-CD81 antibody to the silica surface of a sensor chip, so that all EVs that express this common EV marker are captured and enriched when the wells of this chip are loaded with samples containing EVs from any cell type. Bound EVs are detected by addition of anti-CD63-AuS and anti-CD9-AuR GNPs to these sample wells, so that dual binding of these two GNP species with immobilized EVs on the chip surface forms AuS-EV-AuR complexes (Fig. 1a). AuS and AuR signals are readily detectable by dark-field microscopy (DFM) (Fig. 1b,c), but AuS-EV-AuR complexes formed after addition of both GNPs produce nanoplasmons that markedly shift the spectra of the scattered light (Fig. 1d,e) and increase signal intensity (Fig. 1f), although this spectral shift is not always apparent due to significant variation in CD63/CD9 expression. Scanning electronic microscopy (SEM) performed to analyse AuS-EV-AuR, AuS-EV and AuR-EV binding and morphology on this sensor chip detected relatively uniform EV distribution, with readily detectable single and dual GNP binding events (Supplementary Fig. 1).

**Evaluation of the nPES platform for EV detection**

To characterize the EV detection performance of this nPES platform, EV samples revealing vesicle morphology and size distributions consistent with pure EV preparations (Supplementary Fig. 2) were added to EV-free plasma (Methods and Supplementary Fig. 3) to create EV plasma standards of known concentration, using nPES area ratios (area of nPES signal versus well area) to evaluate sample EV concentrations. Negative control assays performed with EV-free plasma revealed very low nPES area ratios (<0.02%), and similar results were found when 150 ng µl⁻¹ of the EV plasma standard was analysed on sensor chips without anti-CD81 capture antibody modification (Fig. 2a,b).

Experiments performed with only the AuS-anti-CD63 probe exhibited an nPES area ratio of 0.04% (Fig. 2a,b). Markedly different results were observed, however, when anti-CD81-conjugated sensor chips were incubated with this concentration standard and both of the two antibody-conjugated GNPs. Assays performed with both AuS-anti-CD63 and AuR-anti-CD9 exhibited area ratios >0.35% due to nPES signal enhancement (Fig. 2a,b). Reproducibly low nonspecific signal in negative control samples and AuS-EV assays, which exhibited only ~10% the signal of AuS-EV-AuR assays, strongly suggested that nPES area ratios should accurately reflect AuS-EV-AuR complex abundance, and thus plasma EV concentrations.

To confirm this hypothesis, we analysed the sensitivity and linearity of EV values from this nPES assay against those determined by a conventional enzyme-linked immunosorbent assay (ELISA). We found that nPES signal increased with EV concentration (Fig. 2c), and that there was a broad linear range (~10⁻¹–10⁻³ ng µl⁻¹) with a strong correlation (r² = 0.99) between calculated and known EV concentrations (Supplementary Fig. 4). This nPES assay revealed a 0.07 ng µl⁻¹ limit of blank and a 0.23 ng µl⁻¹ limit of detection, whereas ELISA (the standard quantification method for EVs) failed to detect EV concentrations lower than 10 ng µl⁻¹ (Fig. 2d). Notably, area ratios generated by AuS-EV-AuR labelling were significantly greater (P < 0.05) than those generated by single GNP labelling (AuS-EV) at all concentrations, and this difference progressively increased with EV concentration (slopes of 0.010 for AuS-EV, and 0.069 for AuS-EV-AuR), revealing a signal-amplification plasmon-coupling effect of double-GNP recognition (Fig. 2d). The nPES platform required very little plasma due to its extremely high sensitivity, and unprocessed plasma samples that fell above the assay’s linear range (estimated 50 µg µl⁻¹) could be diluted >40-fold and still generate signal within this range (Supplementary Fig. 5). We found that 1 µl of plasma was sufficient for these dilutions, performed as well as larger input volumes (Supplementary Table 1), and yielded sufficient material for >5 replicate wells using 5 µl of diluted plasma, which exhibited good intra- and inter-assay reproducibility in samples with low and high nPES signal (Supplementary Table 2). A comparable ELISA required a pre-purification procedure and a minimum of 50 µl of undiluted plasma for a single replicate well. These ELISAs also cost more than nPES assays ($1.65 versus $1.20 per well) due to their requirement for prior EV purification and use of larger wells, greater sample volumes and need for an additional enzyme-linked detection antibody and reagents (Table 1). Based on these results, these nPES assays offered multiple advantages over ELISA methods routinely used to measure EV concentrations (Table 1).

**Identification of pancreatic-cancer-associated EV markers**

Pancreatic cancer is a lethal disease characterized by aggressive local invasion, early metastasis and a high degree of treatment resistance. Despite its dire prognosis, there are currently no effective noninvasive biomarkers for pancreatic cancer diagnosis. Blood carbohydrate antigen 19-9 (CA19-9) level is the only clinically accepted pancreatic cancer marker but is of limited use as it is approved only to monitor pancreatic cancer progression or response to therapy. Most pancreatic cancer patients are diagnosed with advanced disease, which usually precludes complete resection to greatly reduce the odds of a favourable treatment outcome. Noninvasive biomarkers that can be effectively employed for early pancreatic cancer diagnosis, and discriminate between pancreatic cancer and chronic pancreatitis, are thus desperately needed to reduce cancer morbidity and mortality. Pancreatic-cancer-derived EVs represent a likely source for such a biomarker, since pancreatic cancer cells differentially express multiple factors, some of which should be present on stably circulating EVs secreted by pancreatic tumours, thereby enhancing their detection in the circulation during early stages of the cancer. Such tumour-derived EVs may represent a relatively small contribution to a complex
EV population derived from a wide variety of tissues, however, and could thus be difficult to detect with conventional methods, which would require EV purification and subsequent analysis to determine the relative abundance of candidate diagnosis marker in this population. Our nPES assay could theoretically be rapidly adapted to sensitively quantitate tumour-derived EVs directly from patient blood samples, by replacing one of the two EV-specific GNPs with one specific for a membrane protein that is selectively enriched on EVs secreted by tumour cells. Both the conventional and the nPES approach suffer from the same limitation, however: a lack of known tumour-specific EV markers.

We therefore attempted to identify EV membrane markers to test the ability of a modified nPES assay to detect and quantify tumour-derived EVs. We chose pancreatic cancer as our model system, and used a liquid chromatography–mass spectrometry (LC–MS)/MS-based proteomics and bioinformatics approach to identify transmembrane proteins on EVs derived from human pancreatic carcinoma (PANC-1 and MIA PaCa-2) and pancreatic ductal adenocarcinoma (BxPC-3) cell lines. This approach identified 128 membrane proteins (Supplementary Table 3), of which only 26 were expressed on the EVs of at least 2 of the 3 pancreatic cancer cell lines. Of these 26 membrane proteins, only EphA2 exhibited significantly higher expression, according to the Oncomine database (www.oncomine.org), in human pancreatic cancer tissue samples than chronic pancreatitis or normal pancreatic tissue samples (Supplementary Fig. 6).

EphA2 was also of particular interest due to its reported strong association with cancer progression, metastasis and prognosis. Correspondingly, we found that EphA2 was significantly expressed by EVs from pancreatic cancer cell lines but not by EVs from a non-transformed human pancreas cell line (HPNE) (Fig. 3a). Based on these results, we selected EphA2 as a candidate marker for detection of pancreatic-cancer-derived EVs (EphA2-EVs).

Figure 1 | Design of an nPES platform for EV detection. a, Schematic overview of the nPES assay for specific detection of EVs. b–d, Dark-field microscope (DFM) images of AuS-anti-CD63 (green), AuR-anti-CD9 (red) and AuS-EV-AuR complexes, which are detectable as bright yellow dots. Scale bars: main images, 2 μm; magnified images, 100 nm. e, f, Scattering spectra (e) and intensities of AuS-anti-CD63 (ref. 48), AuR-anti-CD9 and AuS-EV-AuR complexes (f). The scattering spectra and related intensities were recorded from 10 randomly selected particles for each complex by a spectrograph CCD equipped with a monochromator (CASCADE 512B, Roper Scientific). Data represent mean ± SEM; n = 10 replicates per sample.
CD9 were expressed on EVs of all pancreatic cell lines analysed in this study (Supplementary Table 3). We therefore established a modified nPES detection system incorporating one capture antibody (anti-CD81) and two antibody-conjugated GNP probes (anti-EphA2-AuS and anti-CD9-AuR), where pancreatic-cancer-derived EVs were quantified by recognition of CD9/EphA2 double-positive EVs. To evaluate the specificity of the CD81-EphA2-CD9 system for pancreatic-cancer-derived EVs, supernatants of cell lines derived from normal (HPNE) or tumour (PANC-1) tissue were analysed at progressive tissue-culture time points. EphA2-EV signal was significantly lower in HPNE than in PANC-1 cell culture supernatants at all time points and did not significantly increase with culture duration, unlike PANC-1 EphA2-EV signal and total EV signal from HPNE and PANC-1 cells, which increased linearly with culture duration (Fig. 3b,c). Plasma samples from normal healthy controls (NC), and pancreatitis and pancreatic cancer patients were also tested to determine the pancreatic cancer specificity of the EphA2-EV (CD81-EphA2-CD9) and of general-EV (CD81-CD63-CD9) detection and quantification systems. EphA2-EV signal was significantly higher in plasma samples of pancreatic cancer than in samples from pancreatitis patients or NC subjects (P < 0.001), who had similar EphA2-EV signals (Fig. 3d), whereas general-EV signal was not significantly different among these groups, nor were EV-CD9 ELISA values. EphA2-EV increases were also observed in these samples when we employed a custom EphA2-EV ELISA, although this assay required EV pre-isolation and detected much smaller differences (Fig. 3d). Calculations based on NanoSight,
ELISA and nPES assay data (Supplementary Table 4) determined that EphA2-EVs represented approximately 0.15% and 0.26% of total plasma EVs in NC and pancreatitis samples, respectively, and 5.93% of plasma EVs in pancreatic cancer patient samples. To address the ability of the nPES CD81-EphA2-CD9 system to detect EphA2-EVs during pancreatic tumour development, athymic nude mice permissive for tumour growth were injected with PANC-1 cells and analysed for EphA2-EV blood levels every 10 days post-injection. EphA2-EV plasma levels remained stable in control mice (P > 0.5 for a difference at any time point), but increased with time in mice injected with pancreatic tumour cells, significantly diverging from both baseline and control mice by 20 days post-injection (Fig. 3f). EphA2-EV plasma levels highly correlated (r² > 0.60) with tumour size (Supplementary Fig. 7).

EphA2-EV nPES in pancreatic cancer diagnosis and monitoring. To investigate whether nPES EphA2-EV detects early pancreatic cancer cases, we analysed the EphA2-EV signal in plasma samples from a larger cohort, which included pancreatic cancer patients with early-stage disease (pancreatic cancer stages I and II) who could still potentially benefit from curative surgical resection (Supplementary Table 4). None of these groups significantly differed by age or gender, while plasma levels of the non-diagnostic pancreatic cancer biomarker CA19-9 differed between pancreatic...
Figure 4 | EphA2-EV detection and clinical performance. a, Comparison of EphA2-EV levels in plasma samples from normal control (n = 48), chronic pancreatitis (n = 48) and pancreatic cancer (n = 49: 8 stage I, 29 stage II and 12 stage III) patients. b–e, ROC curves of the ability of plasma EphA2-EV (black) and CA19-9 (red) levels to distinguish pancreatic cancer cases from normal control (b) and pancreatic cancer from pancreatitis cases (c), and stage I/II pancreatic cancer from normal control (d) and stage I/II pancreatic cancer from pancreatitis cases (e). ROC area under the curve (AUC) values are depicted on each graph and described in Table 2. f–h, EphA2-EV levels before and after neoadjuvant therapy in pancreatic cancer patients who revealed good/partial (n = 13; f) or poor responses (n = 10; g) to therapy, and the relative pre-to-post therapy differences of these groups (h). Data represent mean ± SEM; n = 3 replicates per sample, *P < 0.05 by two-sided t-test (f) or paired t-test (h). Researchers performing these analyses were not blinded to sample identity.
Table 2 | AUCs, sensitivities and specificities of EphA2-EV and CA19-9 for pancreatic cancer diagnosis.

| Comparison                                | AUC (95% CI) | Cutoff | Sensitivity (%) (95% CI) | Specificity (%) (95% CI) |
|-------------------------------------------|--------------|--------|--------------------------|--------------------------|
| Pancreatic cancer versus normal control   | CA19-9       | 0.81*** (0.72–0.90) | >20.60                  | 81% (67–91)              | 77 (62–88) |
|                                           | EphA2-EV     | 0.96 (0.93–0.99)     | >0.1668                 | 94 (85–98)               | 85 (72–93) |
| Pancreatic cancer versus pancreatitis     | CA19-9       | 0.74*** (0.64–0.84)  | >44.04                  | 61%*** (46–74)           | 81 (66–91) |
|                                           | EphA2-EV     | 0.94 (0.89–0.98)     | >0.1724                 | 89 (79–96)               | 85 (72–93) |
| Pancreatic cancer (stages I + II) versus normal control | CA19-9       | 0.81*** (0.69–0.91)  | >20.60                  | 80 (62–92)               | 77 (62–88) |
|                                           | EphA2-EV     | 0.96 (0.91–0.99)     | >0.1668                 | 91 (78–98)               | 85 (72–93) |
| Pancreatic cancer (stages I + II) versus pancreatitis | CA19-9       | 0.72*** (0.59–0.84)  | >0.1724                 | 81 (62–92)               | 56*** (39–70) |
|                                           | EphA2-EV     | 0.93 (0.87–0.97)     | >0.1724                 | 86 (71–95)               | 85 (72–93) |

*P < 0.05 and ***P < 0.001 for comparisons between CA19-9 and EphA2-EV. AUC, area under curve; CI, confidence interval.

cancer versus NC and pancreatitis patients, but not when these patients were segregated by early (stages I + II) and late (stage III) tumour stage (Supplementary Table 5). Similarly to previous results, plasma EphA2-EV levels were significantly higher in pancreatic cancer than in pancreatitis and NC cases (Supplementary Fig. 8, P < 0.001). Further, EphA2-EV levels of early-stage pancreatic cancer patients (stages I and II) were also significantly higher than those of the pancreatitis (P < 0.001) and NC cases (P < 0.001) (Fig. 4a and Supplementary Fig. 9). These results indicate a strong association between circulating EphA2-EV and pancreatic cancer, including early-stage pancreatic cancer, suggesting the potential utility of EphA2-EV as an early detection marker. Similar comparisons were also performed for the plasma levels of CA19-9, which is clinically approved as a means of monitoring a patient’s disease progression and therapy response, but not for pancreatic cancer diagnosis or staging. CA19-9 levels were significantly increased in pancreatic cancer versus pancreatitis and NC plasma samples, but not in early-stage samples (Supplementary Figs 10 and 11), although there was extensive overlap across all these groups that would reduce the discriminatory power of this assay for pancreatic cancer diagnosis. Receiver operating characteristic (ROC) curves indicated that plasma EphA2-EV levels are an excellent classifier for differentiating pancreatic cancer cases (including early-stage cancer) from pancreatitis and NC cases (area under the curve, AUC, 0.93–0.96; Fig. 4b–e and Table 2), performing significantly better than CA19-9 (P < 0.001). Plasma EphA2-EV sensitivities for pancreatic cancer versus NC (94%) or pancreatitis (89%) cases were significantly better than CA19-9 (81% and 61%, respectively). Notably, the EphA2-EV discriminatory sensitivity was only modestly diminished for comparisons of early-stage (stage I–II) pancreatic cancer versus NC (91%) or pancreatitis (86%), further demonstrating the potential of EphA2-EV as a promising early-detection marker for pancreatic cancer (Table 2).

Pancreatic cancer cases are frequently characterized by high rates of therapy resistance, and improved means of monitoring therapy responses are urgently needed to allow rapid modification of personalized treatment regimens in order to improve patient outcomes. We thus investigated whether plasma EphA2-EV levels reflected pancreatic tumour responses to neoadjuvant therapy. Plasma samples were collected from 23 pancreatic cancer patients before and after neoadjuvant chemotherapy and/or chemoradiation, and stratified according to patient responses to neoadjuvant therapy (Supplementary Table 6). Post-therapy EphA2-EV levels significantly decreased in patients with good or partial therapy responses (<50% viable tumour cells post-therapy)36, but not in patients with poor responses (>50% viable tumour cells post-therapy) (Fig. 4f,g), while CA19-9 levels in these samples did not significantly differ by treatment response (Supplementary Fig. 12). Changes in EphA2-EV levels are thus strongly associated with treatment response (Fig. 4h), and perform better than CA19-9 levels, which are sometimes used for the clinical evaluation of pancreatic cancer treatment responses. EphA2-EV levels may thus be a useful independent indicator to monitor pancreatic cancer patient responses to therapy.

Discussion

Conventional protein biomarkers are often subject to variable regulation by non-specific factors, such as blood hydrolases, that can significantly impact their levels in the circulation37,38. Biomarker detection can also be impaired by non-specific competition from abundant proteins and peptides in blood, particularly in early stages of diseases when biomarker levels are low39,40. EV-based assays may be less affected by these confounding influences, since membrane-bound or membrane-enclosed EV biomarkers are likely to be at least partially protected from hydrolysis and more easily separated from abundant non-specific proteins than target proteins that are not physically constrained by a vesicle membrane. Such advantages may be particularly important for the detection of early disease stages when low biomarker levels are likely to be more susceptible to degradation or masking interactions.

EVs are increasingly recognized as a potentially valuable source of new diagnostic biomarkers, but current EV analysis techniques require complex and lengthy isolation procedures, and their volume requirements limit their use with common mouse models of human disease and preclude longitudinal studies. Sensitive detection and quantification of EVs associated with specific disease states, without the need for a separate pre-purification step, is highly desirable for both research and clinical applications. For example, a recent report described a surface-plasmon-resonance-sensor method that can distinguish healthy and pancreatic cancer patients based on exosomal microRNAs isolated from patient blood samples41; however, this method required both EV and RNA isolation before sample analysis and so is unsuitable for high-throughput clinical analyses.

Our nPES platform integrates EV capture and detection, using a plasmon-coupling effect to achieve dual increases in detection sensitivity and specificity to allow rapid, ultrasensitive biomarker quantification in small sample volumes. Notably, we achieved robust assay reproducibility using 1 μl of plasma samples (Supplementary Tables 1 and 2). This ability to use small sample volumes may be particularly valuable in clinical or research settings where samples are subject to volume constraints and are often required for multiple analyses. For example, the microsample volumes required for this assay permit multi-sample mouse time-course studies, which have not previously been feasible due to the large volume requirements of conventional EV analysis methods. This should greatly benefit studies using mouse models to monitor EV changes associated with tumour or disease progression and corresponding therapy responses.

The EV nPES platform that we have described should be generalizable to any disease state associated with a specific EV marker.
We selected pancreatic cancer as a proof-of-principle model in this study, using proteomics and bioinformatics to identify a candidate pancreatic-cancer-specific EV membrane biomarker for use as the disease-specific probe in a customized nPES assay. We found that EphA2 was highly enriched on EVs of pancreatic cancer cells but essentially absent on EVs of normal pancreas cells, and therefore chose EphA2 as a pancreatic cancer selective EV biomarker for our assay. EphA2 overexpression is not restricted to pancreatic tumours, however, as it is also overexpressed in the early stages of colorectal cancer and non-small-cell lung cancer, suggesting its potential as a target for early cancer detection, since it accumulates during tumour progression, interacting with downstream cancer-associated signalling pathways to promote malignant cell growth and invasiveness. Results of the present study illustrate the early diagnostic power of EphA2-EV for pancreatic cancer, and its potential to detect other forms of cancer should be addressed by future studies. If necessary, the cellular origin of EphA2-EVs, or other disease-associated EVs, could be addressed by replacing the EV-specific anti-CD9-AuR probe with a cell-specific AuR probe. Similarly, EV-markers associated with a particular mutation or phenotype could be assessed by replacing the anti-CD9-AuR probe or by parallel assays using an additional set of probes.

Studies were performed with pancreatic cancer patients, since this disease is characterized by aggressive local invasion, early metastasis and high rates of therapy resistance, and there are currently no FDA-approved non-invasive assays for pancreatic cancer diagnosis, and only one relatively non-specific biomarker, CA19-9, for evaluation of patient responses to therapy. Due to its nonspecific symptoms, aggressive nature, and the lack of effective strategies for early detection, 80–85% of pancreatic cancer patients are diagnosed with advanced disease, precluding surgical resection, which is the only available cure. We therefore evaluated the ability of our nPES EphA2-EV assay to discriminate pancreatic cancer patients with early disease (stages I and II, that is, patients who could still potentially benefit from curative surgical resection), from NC and pancreaticitits cases. Pre-therapy EphA2-EV blood levels accurately distinguished stage I/II pancreatic cancer patients from NC (AUC = 0.96) and pancreaticitits patients (AUC = 0.93), performing much better than similar comparisons using circulating CA19-9 levels, which are often used as an initial non-FDA-approved screening method in a pancreatic cancer diagnosis. We thus propose that an nPES EphA2-EV blood assay may have significant value as a pancreatic cancer screening test, since a rapid, accurate, non-invasive and inexpensive blood test for early pancreatic cancer diagnosis could improve early detection rates to improve patient outcomes. We do, however, acknowledge that this would require more imaging studies and/or biopsies to rule out false-positive results.

Low neoadjuvant therapy response rates are a major factor in the poor outcomes of pancreatic cancer patients. Circulating CA19-9 levels are sometimes used to monitor treatment responses, but more sensitive and specific non-invasive markers are needed to guide the design of more effective personalized therapy regimens. Our results suggest that an nPES EphA2-EV blood assay can be used to monitor therapy responses of pancreatic cancer patients, as EphA2-EV blood levels significantly decreased in patients that revealed good or partial but not poor therapy responses.

The nPES approach described here offers an attractive means for the rapid, purification-free and ultrasensitive measurement of circulating EVs in small sample volumes. Our proof-of-concept study has promising translational implications, and opens important potential avenues for future research. Noninvasive nPES EphA2-EV analyses could improve early pancreatic cancer detection and treatment monitoring, but larger prospective studies are required to validate these results. The nPES platform could also be readily customized to diagnose and monitor other cancers and infections by replacing one or both probes with disease- or cell-type-specific EV markers. Further, although the image-capture and analysis aspects of this assay have already been automated, and can be directly translated to a clinical setting, minor changes to increase assay capacity and automation would be needed for high-throughput detection.

**Methods**

**Experimental design.** This translational study was designed to establish and validate a rapid, purification-free three-probe EV quantification assay that could be modified by addition of a pancreatic-cancer-specific EV probe to allow high-sensitivity and high-specificity diagnosis of early- and late-stage pancreatic cancer from small blood samples. Cancer-derived EVs are of great interest as potential diagnostic markers, but few cancer-specific EV markers have been identified and most current EV assays are labour-intensive and low-throughput, thus limiting them impractical for clinical use. We therefore attempted to develop a three-probe capture and detection system, where a capture antibody recognizing an EV membrane protein (anti-CD81) was used to enrich EVs within a sensor well, while antibody-conjugated AuR and AuS recognizing two additional EV membrane proteins served as EV probes. This approach was designed so that binding of the different gold nanoparticles to the article-specific protein on an EV would shift the wavelength and increase the intensity of scattered light under dark-field illumination to improve the sensitivity and specificity of EV detection. We next used comparative proteomics to identify membrane proteins with known cancer associations that were selectively enriched on EVs of human pancreatic carcinoma or ductal adenocarcinoma cell lines as candidates for a pancreatic cancer specific EV probe. In order to demonstrate the feasibility of this approach, a probe against this marker was included in our three-probe assay, which was then tested for its sensitivity and specificity for pancreatic cancer EVs in cell culture supernatants; pre-treatment blood samples of a small cohort of 10 pancreatic cancer, 10 pancreaticitits and 10 control patients; or longitudinal blood samples of mice injected with a human pancreatic cancer cell line. We next examined the nPES assay performance in a larger, independent cohort of 59 pancreatic cancer patients, 48 pancreaticitits and 48 control patients, its ability to distinguish early pancreatic cancer disease stages from pancreaticitits and control patients, and its performance against another commonly pancreatic cancer marker, CA19-9, which is not FDA approved for pancreatic cancer diagnosis or cancer staging. Finally, we analysed whether assay values reflected pancreatic tumour volume and response to neoadjuvant therapy using blood samples collected from 23 pancreatic cancer patients before and after neoadjuvant therapy, and stratified according to patient responses to treatment, comparing results to CA19-9 expression to assess assay non-inferiority.

**Cell culture.** The human pancreatic cancer cell lines PANC-1, Mia PaCa-2, and the human pancreas cell line HPNE were obtained from the American Type Culture Collection (Manassas, Virginia). PANC-1 and BxPC-3 cells were cultured in RPMI-1640 medium (Hyclone, GE Healthcare Life Sciences), Mia PaCa-2 cells were cultured in DMEM/high-glucose medium (Hyclone, GE Healthcare Life Sciences), and HPNE cells were cultured in minimal essential medium (MEM, Hyclone, GE Healthcare Life Sciences) with 10% FBS. All cultures were supplemented with 10% fetal bovine serum (FBS) from Biofluids (primary technology, Thermo Scientific Inc.), penicillin (1 U) and streptomycin (1 mg ml⁻¹) and incubated at 37 °C in a humidified 5% CO₂ incubator. All cell lines were cultured in triplicate under the same conditions and then harvested to collect independent EV samples.

**Clinical samples.** Pancreatic cancer, pancreaticitits and NC subject plasma samples from a small trial cohort (n = 10 per group) and a larger validation cohort (n = 48–49 per group) were collected at the time of diagnosis by the department of pathology and genomic medicine at Houston Methodist Hospital after approval by the Institutional Review Board (IRB0213-0011). Based on data from the trial cohort, chi-square power analysis (PASS V8.0,3, Kaysville, Utah) indicated that we required at least 47 clinical specimens per group to detect an effect size of 30%, with α = 0.05 and 90% power. Pancreatic cancer samples used for treatment evaluation, and demographic information, treatment history and responses to therapy, were obtained from 23 pancreatic cancer patients undergoing treatment at MD Anderson Cancer Center. Plasma samples were collected from these pancreatic cancer patients 1–2 months before and after neoadjuvant chemotherapy and/or chemoradiation. All patients gave written informed consent for study participation (IRB PA11-0670 and IRB PA14-0646). Treatment response was assessed by pathologists at MD Anderson as part of the routine diagnostic evaluation, using a grading system based on previously proposed criteria to evaluate the extent of residual tumour. Demographic information is listed in Supplementary Tables 2 and 3, including age, gender and cancer stage. The investigators were not blinded to the group identities of the clinical samples during sample analysis.

**Preparation of EV concentration standards.** Plasma samples were centrifuged at 110,000g overnight, and supernatants were collected as EV-free plasma. Standard EV samples of known mass isolated from pooled human serum (System Biosciences Inc.) were dissolved in EV-free plasma to a final concentration of 1 μg μl⁻¹, and further diluted to required concentrations (30,000, 15,000, 6000, 900, 300, 100, 20, 10, 2 μg μl⁻¹)
7,500, 3,750, 1,870, 938, 469 and 234 μg ml⁻¹) by 2-fold dilution with EV-free plasma at time of use.

EV isolation from culture media. Cells were grown in culture media with 10% FBS for at least 48 h, washed three times with phosphate-buffered saline (PBS) (pH 7.0), and then cultured for 48 h in serum-free media. Culture supernatants were then collected and centrifuged at 40g for 15 min to pellet cells, centrifuged at 4,000 rpm for 10 min, and treated with 100 μg ml⁻¹ of proteinase K for 1 h at 37 °C. After incubation, cells were washed with PBS (pH 7.0) and counted using a NanoSight LM10 instrument (Malvern Instruments) equipped with a C18 Pepmap 100 enrichment column (Thermo Scientific; 5 μm particle size, 100 μm i.d.) containing 0.1% trifluoroacetic acid and 0.5 μg ml⁻¹ of H₂O/acetonitrile (95:5), centrifuged at 10,000 rpm for 1 h at 37 °C, and then washed three times with 0.01% Tween-20 in PBS. For all the experiments, samples were subjected to LC-MS/MS analysis.

LC-MS/MS proteomics analyses. EV samples were incubated with M-PER mammalian protein extraction reagent (Thermo Scientific) for 30 min in an ice bath and extracted protein concentrations were measured with a bicinchoninic acid (BCA) assay (micro BCA Kit, Thermo Scientific). Protein extracts were diluted to 1 μg μl⁻¹ with 100 μM NH₄Cl, incubated with 10 μM diethyldithioctetol, and then centrifuged at 10,000 rpm for 1 h at 37 °C. Protein hydrolysis was arrested by addition of 0.1% trifluoroacetic acid and the peptide solutions were diluted to 0.25 μg μl⁻¹ with H₂O/acetonitrile (95:5), centrifuged at 21,000 g for 20 min, and supernatants directly subjected to LC-MS/MS analysis.

Peptides were separated using an Ultimate 3000 nano-LC (Thermo Scientific Co.) equipped with a C18 Pepmap 100 enrichment column (Thermo Scientific; 5 μm particle size, 100 μm i.d. × 5 mm) and a C18 Pepmap 100 analytical column (Thermo Scientific; 3 μm particle size, 100 μm pore size, 75 μm i.d. × 150 mm), using flow rates of 20 μl min⁻¹ and 300 nl min⁻¹ for loading and analytical columns, respectively. Eluted peptide fractions were analysed by a Velvo Pro Dual-Pressure Linear Ion Trap Mass Spectrometer (Thermo Scientific). One MS scan was followed by eight MS/MS scans. All of the MS/MS spectra were used to search Mascot 2.3.0 (Matrixscience) using a measurement tolerance of 0.5 Da.

Antibody modification of gold nanoparticles (GNPs). Modifications of gold AuS and AuR with different antibodies followed similar procedures. Briefly, 40 μl of carbonyl functionalized gold nanoparticles (GNPs; 9 × 10¹⁰ M, Ocean NanoTech) were mixed with 20 μl MES buffer (pH 4.7, Ocean NanoTech), then mixed with 2 μl of EDC/sulfo-NHS solution (Sigma-Aldrich; 2 μg ml⁻¹ 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 μg ml⁻¹ N-hydroxysuccinimidio (sulfo-NHS) in MES buffer), incubated for 10 min at room temperature, and then washed three times with 0.01% Tween-20 in PBS (Ocean NanoTech), then resuspended in 40 μl washing buffer and stored at 4 °C.

Construction of an nPES platform for EV quantification. Amino group-functionalized glass slides (NH₂-glass, Nanocs Inc.) were ultrasonically cleaned in methanol for 5 min, thoroughly flushed with deionized water, dried under an N₂ stream, and then the NH₂-functionalized glass was covered with a 50-μl polydimethylsiloxane membrane (CW-50R-1.0 CultureWell Gasket, Grace Bio-Labs Inc.). Slides were then filled with 10 μl per well of 12.5 μg ml⁻¹ anti-human CD81 antibody (R&D systems, clone no. 454720) in 5 mM EDC/PBS and incubated at room temperature for 2 h in a moist chamber, aspirated and filled with 10 μl per well of 5% BSA/PBS (pH 7.0) and incubated at room temperature for 4 h, then PBS (pH 7.0) washed and aspirated three times before loading these wells with analysis samples. Sample wells were filled with 5 μl per well of plasma samples (diluted x40 with pH 7.0 PBS) or cell culture EV samples, incubated at room temperature for 4 h in a moist chamber, washed three times with PBS, three times with PBS and then filled with 7 μl per well of AuS and AuR PBST probe solution (4 × 10⁻¹¹ M for each particle) and incubated at room temperature for 1 h, after which the PDMS cover was removed, the slide was washed three times with PBST and three times with PBS, then filled with a cover slip and imaged by dark-field microscopy (DFM).

DFM imaging and scattering spectroscopy measurements. DFM images were acquired on an inverted microscope (Olympus IX71, Olympus Co.) equipped with a 100 objective lens (NA = 0.8) and a dark-field condenser (0.8 < NA < 0.95). The scattered light from a 100 W halogen lamp was recorded by an Olympus DP70 digital camera to generate dark-field colour images and by a spectrograph CCD equipped with a monochromator (CASCADe 512B, Roper Scientific) to obtain scattering spectra (integrated over 10 s) of selected AuS, AuR and AuS-AuR particles in wells.

DFM images were processed with the National Institutes of Health (NIH) Image J software with the colour threshold as hue 0, saturation 0, and brightness 255. Image areas with brightness equal to 255 were software-selected, and the ratios of these areas to the total image areas were calculated by the software to give area ratios indicating specific nPES EV signal. Linear regression of nPES area ratio with log(c) EV concentration was used to generate the standard concentration curve for derivation of experimental EV concentrations.

Mouse pancreatic cancer model. Six- to eight-week-old male nude mice (C6NENU F1) purchased from Charles River Laboratories (Wilmington, MA) were housed in Houston Methodist Research Institute (HMRI) animal facilities in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. All animal procedures followed HMRI policies and IACUC-approved protocols. Three mice were subcutaneously injected in the left flank with 2 × 10⁶ Panc-1 cells suspended in 100 μl of PBS to establish subcutaneous pancreatic tumours and compared to three healthy control mice that did not receive tumour cell injections. Mouse retro-orbital blood samples for analysis of EphA2-EV plasma levels and tumour size data were collected at 0, 10, 20, 30 and 40 days post-injection. Calipers were used to determine tumour length and width, and tumour volume was estimated using the modified ellipsoid volume formula (V = l × w × h⁰·⁵). Investigators were not blinded to the group identity of the animal samples during nPES analyses.

Western blots. Western blot analyses were performed with 10 μg EV protein lysate and precast Mini-PROTEAN TGX gels and polyvinylidene difluoride membranes (Bio-Rad) using standard methods.

Measurement of method repeatability. Assay reproducibility was assessed using two randomly selected samples that were analysed in 3 assays with 20 replicates that were performed on 3 separate days to generate 60 values per sample. Resulting values were used to calculate intra- and inter-assay means and coefficients of variation (% CV).

SEM image analysis. SEM images of GNP binding to EVs were generated using EVs purified from 50 μl of human plasma with ExoQuick kits to reduce EV artifacts. Purified EVs were immobilized on silicon chips and hybridized with anti-Cd63-AuS and anti-Cd9-AuR, as described above. Sensor chips were then washed (pH 7.0) washed, dried under gentle N₂ stream, then treated with a direct current for 60 s after coating approximate 1 μm layer of AuS-AuR functionalized gold nanoparticles (sulfo-NHS) in MES buffer, incubated at 37 °C for 1 h, then washed three times with 0.01% Tween-20 in PBS (Ocean NanoTech), then resuspended in 40 μl washing buffer (Ocean NanoTech), then resuspended in 40 μl washing buffer and stored at 4 °C.

EV concentrations in patient plasma samples. A NanoSight LM10 instrument and Nanoparticle Tracking Analysis software (Malvern Instruments) were used to measure total-EV concentrations in pooled patient plasma samples (10 pancreatic cancer, 10 pancreatitis and 10 NC samples). Estimates of total-EV protein μl⁻¹ plasma were determined by BCA assay of EVs isolated from pooled plasma samples with ExoQuick kits. The nPES assay standard curve equation (nPES signal intensity = 0.069 × log(c) (EphA2 EV concentration) - 0.09) was used to calculate the EphA2 EV concentration of each sample. The EphA2 EV percentage of total-EVs in each patient group was calculated as the ratio of EphA2-EV protein content for each group (EphA2 EV concentration / total-EV protein concentration). Total-EVs per assay well were calculated as the input volume of diluted plasma sample (5 μl of 40x diluted plasma) and EphA2-EVs per well as the fraction of EphA2-EVs/total-EVs.
Statistical analysis. GraphPad Prism version 5.0 (GraphPad Software) and MedCalc statistical software version 13.0 (MedCalc Software bvba) were used for all calculations. The MedCalc was used to create heat maps of patient nPES EphA2-EVs levels. Statistical analyses were performed using Student’s t-tests, one-way ANOVAs with Bonferroni’s post-test or Kruskal-Wallis one-way ANOVA with Dunn’s post-test as determined by sample distribution and variance. Differences with \( P < 0.05 \) were considered statistically significant. ROC curves were used to determine sensitivity and specificity, with the optimal cut point defined as the point of closest approach to the upper left axes according to the following criterion: \( \min(1\text{–sensitivity}) + (1\text{–specificity})^2 \). Figures were prepared using GraphPad Prism (GraphPad) and Origin software (OriginLab).

Data availability. The data underlying the figures are available in figshare with identifier http://dx.doi.org/10.6084/m9.figshare.2465030 (ref. 48). The authors declare that all other data supporting the findings of this study are available within the paper and its Supplementary Information.

Received 11 May 2016; accepted 14 December 2016; published 6 February 2017

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Acknowledgements
We thank P. Mcshane at Houston Methodist Hospital and M. W. Hurd at the University of Texas M.D. Anderson Cancer Center for organizing clinical samples in this study. The work was primarily supported by research funding provided by NIH grants R01AI113725, R01AI122932, U54CA143837 and 5P50CA126752-08, an NIH intramural research program, a John S. Dunn Foundation award, a PANCAN-AACR Career Development Award (14-20-25-KOAY), a Radiological Society of North America seed grant (RSD1429), and the Sheikh Ahmed Center for Pancreatic Cancer Research and the Center for Radiation Oncology Research at The University of Texas M.D. Anderson Cancer Center.

Author contributions
K.L., F.L., J.F. and Y.H. designed the research plan. K.L., F.L., J.F., D.S. and C.L. performed the experiments. E.J.K., D.W.B., K.Y. and M.H.K. collected the clinical samples and clinical data. K.L., F.L., C.J.L., Y.L., Z.Z. and Y.H. performed data analysis. K.L., F.L., C.J.L., Z.Z. and Y.H. wrote the manuscript, and all authors contributed to the revision of the manuscript.

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How to cite this article: Liang, K. et al. Nanoplasmonic quantification of tumour-derived extracellular vesicles in plasma microsamples for diagnosis and treatment monitoring. Nat. Biomed. Eng. 1, 0021 (2017).

Competing interests
The authors declare no competing interests.