Polyphosphate Expression by Cancer Cell Extracellular Vesicles Mediates Binding of Factor XII and Contact Activation

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Abstract:
Extracellular vesicles (EV) have been implicated in diverse biological processes, including intracellular communication, transport of nucleic acids, and regulation of vascular function. Levels of EV are elevated in cancer, and studies suggest that EV may stimulate thrombosis in cancer patients through expression of tissue factor. However, limited data also implicates EV in activation of the contact pathway of coagulation through activation of factor XII (FXII) to factor XIIa (FXIIa). To better define the ability of EV to initiate contact activation, we compared the ability of EV derived from different cancer cell lines to activate FXII. EV from all cell lines activated FXII, with those derived from pancreatic and lung cancer cell lines demonstrating the most potent activity. Concordant with activation of FXII, EV induced the cleavage of high molecular weight kininogen to cleaved kininogen. We also observed that EV from cancer patients stimulated FXII activation and HK cleavage. To define the mechanisms of FXII activation by EV, EV were treated with calf intestinal alkaline phosphatase or E. coli exopolyphosphatase to degrade polyphosphate; this treatment blocked binding of FXII to EV and the ability of EV to mediate FXII activation. In vivo, EV induced pulmonary thrombosis in wild-type mice, with protection conferred by deficiency of FXII, HK, or prekallikrein. Moreover, pretreatment of EV with calf intestinal alkaline phosphatase inhibited their prothrombotic effect. These results indicate that polyphosphate mediates binding of contact factors to EV, and that EV-associated polyphosphate may contribute to the prothrombotic effects of EV in cancer.

Conflict of interest: COI declared - see note

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Polyphosphate Expression by Cancer Cell Extracellular Vesicles Mediates Binding of Factor XII and Contact Activation

Short title: Cancer cell polyphosphate activates FXII

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KEY POINTS

- Cleaved high molecular weight kininogen is observed in many patients with cancer, suggesting activation of the contact system.

- Extracellular vesicles from cancer cell lines or patients with cancer express polyphosphate, bind and activate FXII, and are prothrombotic.
Abstract

Extracellular vesicles (EV) have been implicated in diverse biological processes, including intracellular communication, transport of nucleic acids, and regulation of vascular function. Levels of EV are elevated in cancer, and studies suggest that EV may stimulate thrombosis in cancer patients through expression of tissue factor. However, limited data also implicates EV in activation of the contact pathway of coagulation through activation of factor XII (FXII) to factor XIIa (FXIIa). To better define the ability of EV to initiate contact activation, we compared the ability of EV derived from different cancer cell lines to activate FXII. EV from all cell lines activated FXII, with those derived from pancreatic and lung cancer cell lines demonstrating the most potent activity. Concordant with activation of FXII, EV induced the cleavage of high molecular weight kininogen to cleaved kininogen. We also observed that EV from cancer patients stimulated FXII activation and HK cleavage. To define the mechanisms of FXII activation by EV, EV were treated with calf intestinal alkaline phosphatase or E. coli exopolypophosphatase to degrade polyphosphate; this treatment blocked binding of FXII to EV and the ability of EV to mediate FXII activation. In vivo, EV induced pulmonary thrombosis in wild-type mice, with protection conferred by deficiency of FXII, HK, or prekallikrein. Moreover, pretreatment of EV with calf intestinal alkaline phosphatase inhibited their prothrombotic effect. These results indicate that polyphosphate mediates binding of contact factors to EV, and that EV-associated polyphosphate may contribute to the prothrombotic effects of EV in cancer.
Introduction

Circulating extracellular vesicles (EV) are comprised of a heterogeneous mixture of vesicles of divergent size and origin. Elevated levels of EV are present in plasma from patients with cancer, and may be derived from cancer and other cells in the tumor microenvironment. Characterization of EV by analyzing their content suggests that EV may be useful cancer biomarkers.

Thrombosis is a common complication of cancer, affecting up to 20% of individuals with certain cancer types. Patients with cancer-associated thrombosis (CAT) have increased mortality compared to patients who remain thrombosis-free. Several mechanisms may underlie the development of CAT, including the prothrombotic effects of EV. Although studies are confounded by different methods of EV isolation and alternative approaches to assessing tissue factor antigen and/or activity, evidence suggests that EV tissue factor activity is associated with thrombosis in pancreatic, but not other cancer types.

In addition to tissue factor-mediated activation of coagulation, the contact pathway may initiate and amplify coagulation reactions. Classically, this pathway is initiated through auto-activation of FXII to FXIIa following binding to specific surfaces. Activation of FXII leads to activation of plasma prekallikrein (PK) to plasma kallikrein (PKa), which activates additional FXII. FXIIa also activates factor XI (FXI), leading to activation of the intrinsic coagulation pathway. High molecular weight kininogen (HK) is a critical co-factor for PK and FXI activation by FXIIa, and is converted to cleaved HK (cHK) by PKa. Inorganic polyphosphate (polyP) released from platelet alpha granules and other sources may initiate contact activation and amplify coagulation reactions through its ability to activate FXII, enhance activation of FXI by thrombin, promote prothrombin cleavage and modulate fibrin structure.
There is little information available concerning the role of contact activation in CAT. One study demonstrated that EV derived from prostate cancer cells (prostasomes) activated FXII through effects of polyP\textsuperscript{33}. Prostasomes also induced pulmonary emboli in mice in a manner inhibited by an anti-FXIIa monoclonal antibody, and stimulated thrombin generation in normal plasma.

To further define interactions of EV polyphosphate from cancer cells and cancer patients with the contact system, we examined cell lines derived from pancreas, colon, and lung cancers, as well as plasma from cancer patients. EV derived from these cells bound FXII in a polyP-dependent manner and stimulated FXII activation with relative activity proportional to the thrombotic risk associated with each of these tumors\textsuperscript{34}. These results suggest that polyP-mediated contact activation by EV may contribute to thrombosis in cancer.
Materials and Methods

Cell culture

Human dermal fibroblasts (HDF, ATCC) and pancreatic cancer cells (L3.6)\textsuperscript{35} were cultured in Dulbecco’s modified Eagle’s medium (DMEM). Non-small cell lung cancer (H1975, ATCC), colorectal cancer (HT29, ATCC), and lymphoma (U937, ATCC) cells were maintained in RPMI 1640. All media was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Isolation of extracellular vesicles (EV)

Cells at 70% confluency were washed with phosphate-buffered saline (PBS) and cultured in serum-free medium for 40 hours. EV were isolated using protocols outlined by the extracellular RNA research portal (www.exRNA.org/resources/protocols). Briefly, conditioned medium was centrifuged at 450 g for 10 minutes, the supernatant re-centrifuged at 2,200 g for 12 minutes, and the second supernatant centrifuged at 15,000 g for 30 minutes then concentrated to 400 µl using a 50 kDa molecular weight cut-off filter (Amicon Ultra-15). The EV-containing concentrate was loaded onto a qEV size exclusion column (SP1, IZON) equilibrated with HBS (HEPES buffered saline; 20 mM Hepes, pH 7.4, 100 mM NaCl). Twenty-eight 500 µl fractions were collected and the concentration of EV in each was measured using a ZetaView NTA PMX-120 (Particle Metrix). Protein concentration in each fraction was measured using a DC Protein Assay (Bio-Rad), and fractions with the highest particle numbers and lowest protein concentrations (Fractions 7-10) were pooled and concentrated. The EV concentration, size distribution, and protein concentration of the pooled preparation was determined.

Isolated EV were further characterized by immunoblotting using antibodies to CD63 (sc-5275, 1:500), CD81 (sc-23962, 1:500) and CD9 (sc-13118, 1:500). Bound antibodies were detected
using an HRP-conjugated secondary antibody (rat anti-mouse IgG1-HRP, 1144-05, Southern Biotech) and developed using SuperSignal West Pico ECL solution.

**Electron microscopy (EM)**

EV (10 μl) were deposited on formvar-carbon coated grids (Electron Microscopy Sciences, Hatfield, PA) for 1 hour. Grids were fixed with 4% paraformaldehyde, pH 7.4 for 10 minutes, washed and stained with 1% uranyl acetate, then contrasted with 1.8% methylcellulose containing 0.1% uranyl acetate and air-dried. Transmission electron microscopy was performed using an FEI Tecnai G2 Spirit BioTWIN (FEI, Hillsboro, OR) at 100 kV. Digital images were obtained using an Orius 832 CCD 11-megapixel Camera, (Gatan, Pleasanton, CA).

**Plasma preparation**

All human studies (procurement of plasma samples) were conducted after informed written consent under an approved Cleveland Clinic IRB protocol. Blood from healthy donors or patients with cancer was collected into vacutainer tubes containing 3.8% sodium citrate (BD Biosciences, Franklin Lakes, NJ). Platelet poor normal human plasma (NHP) was obtained by centrifugation at 200 g for 20 minutes, followed by centrifugation at 2,500 g for 20 minutes, and was stored at -80°C.

**FXII activation**

Pooled NHP (100 μl) was added to a mixture containing 50 μl of substrate S-2302 (0.33 mM) and 50 μl of HBS with or without EV derived from cancer (L3.6, H1975, HT29, and U937) or HDF cells, in 96-well microplates (07-200-568, Corning™ 3632). Dextran sulfate (DS) (10 μg/ml) was used as positive control for FXII activation. Samples were incubated for 160 min at 37°C, and FXIIa generation was assessed by measuring hydrolysis of S-2302 at 405 nm in a
microplate reader (Bio-Tek, Winooski, VT). For relative FXIIa quantification, various concentrations of human FXIIa (Enzyme Research Laboratories) were incubated in NHP containing S-2302 to establish a standard curve. FXII-deficient plasma was obtained from a patient with inherited FXII deficiency and no detectable FXII.

To assess direct activation of FXII by EV, 60 µl of purified human FXII (375 nM, Enzyme Research Laboratories) was incubated with HBS, L3.6-EVs, and DS in 60 µl of reaction buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% polyethyleneglycol 8000, 10 mM ZnCl₂) containing S-2302 (330 mM). Changes in optical density at 405 nm were monitored on a microplate reader for 180 minutes. FXII cleavage was analyzed in parallel by immunoblotting using a goat anti-human FXII polyclonal antibody (GAFXII, Affinity Biologicals).

**Cleavage of HK**

Cleavage of HK as a marker of contact activation [27-29] was assessed by immunoblot. Briefly, 0.25 µl of NHP pretreated with or without cancer cell-derived EV preincubated in the absence or presence of calf intestinal alkaline phosphatase (CIP) or corn trypsin inhibitor (CTI) was analyzed using 10% SDS-PAGE under reduced conditions. After transfer to PVDF, membranes were blotted using affinity-purified rabbit antibodies raised against a peptide corresponding to either a sequence in domain 5 of the human HK light chain (DHGKHKHGKGKHKNGK) that recognize intact HK (~120 kDa) and the free cHK light chain (54 kDa, 47 kDa), or antibodies raised to an epitope (CQPLGMISLMK) in the c-terminus of the HK heavy chain exposed after cleavage of HK to cHK (62 kDa) (Figure S1). Bound antibodies were detected using a 1:15,000 dilution of IRDye 800CW-conjugated goat anti-rabbit IgG (LI-COR Biosciences). The membrane was scanned using an Odyssey CLx.
imager and relative amounts of intact and cleaved HK were quantified using Image Studio Lite 5.2.

**EV polyphosphate detection**

To characterize EV-associated polyP, EV were pre-incubated with either HBS, DNase I, RNase A, or CIP at 37°C for 1 hour, solubilized, and analyzed on a Tris-Borate-EDTA (TBE) gel (EC62252, Thermo Fisher Scientific)\(^3\). The gel was stained in buffer (2.5% Ficoll-400, 10 mM EDTA, 3.3 mM Tris-HCl) containing 50 µg/ml DAPI, and destained with the same buffer not containing DAPI. Long-, medium-, and short-chain polyP (a gift from Dr. James Morrissey\(^3\)) were used to estimate the chain length of EV-associated polyP.

**Effect of phosphatase treatment on ligand binding by EV**

EV were incubated with bacterial exopolyphosphatase (PPX, a gift from Dr. Thomas Renne\(^3\)) (200 µg/ml, 37°C, 1 hour), which degrades polyP, or with buffer alone. EV were then incubated with 10 µg/ml of an AlexaFluor488-labelled mutant PPX construct lacking catalytic domains 1 and 2 (PPXΔ12)\(^3\). EV-bound PPXΔ12-AF488 was measured in fluorescence mode with a 488 nm excitation laser using a ZetaView NTA PMX-120. Anti-human CD81-AF488 antibody (20 µg/ml, FAB4615G, R&D systems) was used as a control ligand.

Similar studies were performed using AF488-labeled FXII. Conjugation of AF488 to PPXΔ12 and FXII was performed according to manufacturer’s instructions (Alexa Fluor488 Labeling Kit, Invitrogen).

**In vivo thrombosis studies**

To assess the role of the contact system in thrombosis induced by EV, we used EV prepared from L3.6 cells, some of which had been pretreated with CIP and others in buffer alone. Briefly,
wild-type, f12+/−, klkb1+/−, or kgn1+/− mice were anesthetized and injected via the exposed inferior vena cava with 0.3-0.4 µg EV/gm body weight in 150 µl of PBS containing 0.6 µg epinephrine/gm body weight39. Following injections, animals were observed until death, at which time the lungs were exposed, the trachea dissected and both lungs infused with 1 ml of OCT (Tissue-Tek, Torrance CA)40. One lung was then fixed in 4% paraformaldehyde and the other frozen in OCT and preserved at -80°C. Paraformaldehyde-fixed tissues were cut into 10 µm sections, which were stained with hematoxylin and eosin (H&E), scanned at 40X on a Leica SCN 400 Slide Scanner equipped with a Hamamatsu line sensor color camera, and examined using a digital microscope at 10X magnification. The number of vessel occlusions per 10 random high-powered fields was determined from H&E staining. Deposition of platelets and fibrin in thrombosed vessels was assessed by immunofluorescence staining using a rat anti-mouse CD42c antibody (Emfret Analytics, Cat# M050-0) and a monoclonal antibody to murine fibrin (59D8) (a gift from Dr. Hartmut Weiler, Blood Research Institute41), followed by incubation with donkey anti-rat IgG-Alexa594 or goat anti-mouse IgG-Alexa 488 (Invitrogen), respectively.

**Capillary immunoassay of HK and cHK in plasma**

HK and cHK in plasma were quantified using the WES capillary immunoblotting system (ProteinSimple) and human monoclonal anti-HK (Molecular Innovations, KNG17A12). Standard curves were generated by adding known quantities of purified HK (Enzyme Research Laboratories) to HK deficient plasma (Affinity Biologics). Standard curves for cHK were generated by adding known quantities of purified cHK (Enzyme Research Laboratories) to normal plasma deactivated with Laemmli buffer and boiled for 10 minutes at 95°C. Diluted plasma samples were mixed 4:1 with Fluorescent Master Mix and heated for 5 minutes at 95°C.
Samples were loaded into a 24 well 12-230 kDa WES detection plate and analyzed. Peak areas of immunoreactivity and standard curves were used to quantify HK and cHK concentrations.

**FXII activation by EV from patients with cancer**

EVs from patients with cancer and normal healthy donors were isolated using a Pan-exosome isolation kit (MACS, Miltenyi Biotech). Citrated human plasma (300 µl) was centrifuged at 15,000 g at 4°C for 5 minutes, and the supernatant was incubated with pan-exosome beads conjugated with anti-human CD9/CD63/CD81. The beads were collected using a magnet. EV binding to beads was confirmed by flow cytometric analysis of the immunoprecipitated EV using FITC mouse anti-human CD9 (BD Biosciences) or PE mouse anti-human CD63 (BD Biosciences).

To assess the ability of the immunoprecipitated EV to activate FXII, control beads, or beads that had been incubated with plasma from healthy controls or cancer patients were added to aliquots of the same normal plasma. FXII activation assessed by hydrolysis of S-2302.

**Statistics**

Data are represented as means ± SEM. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). Significance was determined by either a two-tailed, unpaired t test or analysis of variance with multiple comparisons test with P values of less than 0.05 (*P < 0.05, **P < 0.01, ***P < 0.001). Time to event data were modelled using Cox proportional hazards regression models with continuous predictors included as 2 degree of freedom restricted cubic splines, which allow for non-linear relationships between log hazards and predictors, as implemented in the cph and rcs functions of the R package rms. Significance of predictors was assessed using likelihood ratio tests with P values less than 0.05 as the threshold. Event times were censored at death times when the primary event did not include
death. Violations of the proportional hazard assumptions were assessed using residuals plots (R function cox.zph).

Results

EV characterization

EV from all cell types eluted with a similar profile following size exclusion chromatography, with the highest particle numbers observed in fractions 7-10 (Figure 1A); plasma proteins eluted in fractions 18-22 (Figure 1B). EV from L3.6 and HDF cells that eluted in fractions 7-10 were analyzed for tetraspanin content by immunoblotting with antibodies to CD9, CD63 and CD 81 (Figure 1C). Tetraspanins were present in fractions 8 to 12, with peak expression in fractions 8 and 9. Nanoparticle tracking analysis of HDF and L3.6 EV within fractions 7-10 demonstrated a mean particle size of 99.3 and 97.6 nm (Figure 1D), respectively. EV demonstrated the expected bilamellar appearance by electron microscopy (Figure 1E).

Cancer cell-derived EV express polyphosphate

We assessed polyP content of EV derived from the cell lines under study\textsuperscript{43} (Figure 2A) using a standard curve prepared using long-chain polyP (Figure S2A). EV from L3.6 and H1975 cells expressed the greatest amount of polyP (9.35 ± 1.45 and 9.43 ± 1.17 ng/µg EV protein, respectively), with lesser amounts expressed by EV derived from HT29 and U937 cells (6.89 ± 0.59 and 6.11 ± 0.75 ng/µg, respectively). Cancer cell EV expressed more polyP than HDF EV (4.58 ± 0.43 ng/µg).
Using a non-denaturing TBE gel, we determined that cancer cell-derived EV expressed long-chain polyp, with a chain length of ~960 phosphate monomers\textsuperscript{33}. PolyP was removed from EV by treatment with CIP\textsuperscript{44}, as well as \textit{E. coli} exopolyphosphatase (PPX; not shown); DNase and RNase did not remove polyP expression (Figure 2B). EM studies demonstrated that EV remained intact following CIP treatment (Figure S2B).

We examined the ability of polyP to mediate ligand binding to EV, initially using an Alexa Fluor 488 (AF488)-labeled construct of PPX in which the catalytic domains were deleted (PPX-Δ12)\textsuperscript{38}. Binding of PPX-Δ12-AF488 to EV before and after treatment with PPX was calculated by comparing the difference in fluorescent particle number as determined by measurement of the area under each binding curve (AUC). We observed that not all EV bound PPX-Δ12-AF488, suggesting heterogeneous polyP expression (Figure 2C). Pretreatment of EV with PPX reduced the binding of PPX-Δ12-AF488 by 73\%, demonstrating a key role for polyP in binding (Figure 2C). We did not observe any changes in the binding of AF488-labeled anti-CD81 antibody to EV after PPX treatment (Figure 2D).

Long-chain polyP is a potent activator of FXII\textsuperscript{31,32}. Therefore, we also examined the role of polyP in binding of FXII to EV. L3.6-derived EV bound FXII-AF488 in a manner similar to PPX-Δ12 (Figure 2E); pretreatment of EV with PPX reduced binding of AF488-FXII by 46\%.

**Cancer cell-derived EVs activate FXII in human plasma**

Using a chromogenic assay based on hydrolysis of S-2302, we initially observed that EV from L3.6, H1975 and HT29 cells induced FXII activation in NHP in a time-dependent manner (Figure 3A). In subsequent studies, the relative amounts of FXIIa generated was estimated by comparison to a standard curve prepared by adding known amounts of FXIIa to NHP (Figure S3). The relative amount of FXII activation caused by EV was dependent on EV concentration, with
little activation caused by HDF or U937 EV (Figure 3B). The ability of EV to activate FXII was inhibited by pretreatment with CIP, but not DNase or RNase (Figure 3C).

To further demonstrate the activation of FXII by EV, we assessed the ability of L3.6 EV to cause hydrolysis of S-2302 in FXII-deficient plasma, as well as in the presence of corn trypsin inhibitor (CTI), a FXIIa inhibitor (Figure 3D). As expected, FXII activation by L3.6 EV was not detectable in FXII-deficient plasma. CTI also significantly reduced FXII activation in NHP.

To determine whether plasma cofactors were required for activation of FXII by EV, we assessed the ability of L3.6 EV to activate purified FXII. Incubation of EV with FXII enhanced FXIIa generation, as measured by S-2302 hydrolysis (Figure 3E) and cleavage of FXII (Figure 3F). Heating EV to 100° C to destroy protease activity did not block their ability to activate FXII (Figure S4).

**EV-induced activation of FXII correlates with cleavage of high molecular weight kininogen**

Since HK cleavage is a marker of contact activation\textsuperscript{27-29}, we determined whether EV caused cleavage of HK in parallel with S-2302 hydrolysis using immunoblotting. EV from L3.6 and H1975 cells caused generation of cHK in NHP in a concentration-dependent manner (Figure 4A); using LI-COR-based quantification, we determined that the ratio of cHK to HK increased from 8% to 16%, 6% to 17%, and 5% to 11% when EV at concentrations of 0.5 and 1 μg/ml from L3.6, H1975 and HT29 cells, respectively, were analyzed (Figure 4B). EV from HDF or U937 cells caused minimal cleavage of HK.

Pretreatment of EV with CIP, but not DNase I or RNase A, inhibited EV-induced cHK generation (Figure 4C, D). Likewise, CTI inhibited the ability of L3.6 EV to generate cHK (Figure 4E).
EV from L3.6 pancreatic cancer cells induce pulmonary emboli through a contact activation-dependent pathway

EV derived from L3.6 cells were injected into the inferior vena cava (IVC) of wild-type (WT), FXII-deficient (f12<sup>−/−</sup>), high molecular weight kininogen-deficient (kng<sub>1</sub><sup>−/−</sup>) or prekallikrein-deficient (klkb1<sup>−/−</sup>) mice. Pulmonary emboli developed in all WT mice (Figure 5A), with thrombi demonstrating extensive platelet and fibrin deposition (Figure 5B, C). However, the extent of thrombus varied among different strains, with the largest thrombotic burden observed in WT mice, the lowest in f12<sup>−/−</sup> mice, and intermediate amounts in kng<sub>1</sub><sup>−/−</sup> and klkb1<sup>−/−</sup> mice (Figure 5D). The thrombus burden was significantly reduced compared to WT mice in all the contact factor-deficient strains.

We also treated L3.6 EV with CIP prior to infusion into WT mice. Compared to PBS-treated EV, the number of pulmonary emboli in mice that received phosphatase-treated EV was significantly reduced (Figure 5E). These mice also demonstrated a prolonged time to death (28±6 min) compared to mice that received untreated EV (16.8±1.8 min; P < 0.008) (Figure 5F).

Elevated levels of cHK in patients with cancer

To examine the clinical relevance of our findings, we measured levels of cHK in cancer patients. Analysis of plasma from five patients with pancreatic cancer by immunoblotting demonstrated increased cHK, detected using antibodies to HK domain 5 (Figure 6A), or an HK heavy chain-specific antibody (Figure 6B).

We next analyzed a larger sample of pancreatic cancer patients using quantitative capillary immunoblotting. Samples were obtained before the advent of any thrombotic events. This analysis confirmed the presence of elevated levels of cHK in patients with pancreatic cancer.
(Figure 6C; P < 0.0001). However, only 6 of the 23 patients in this cohort developed thrombosis, and given the small sample size, we were unable to demonstrate a statistically-significant increase in cHK in these patients.

We used the same approach to analyze plasma obtained from patients presenting to our cancer thrombosis clinic with signs and/or symptoms of venous thromboembolism (VTE) (Figure 6D). We observed higher levels of cHK in cancer patients than normal controls (P < 0.0001). However, again due to the small sample size that included only 6 patients with thrombosis, we could not demonstrate that cHK/total HK differentiated patients with VTE from those without.

Finally, we prospectively analyzed the cohort of 21 patients seen in the cancer thrombosis clinic who did not have thrombosis at the time of presentation for the subsequent development of VTE. The log relative hazard for death and/or developing VTE, or for just developing VTE, as a function of the cHK/total HK ratio is depicted in Figure 6E and 6F, respectively. Due to the small sample sizes (13/21 with VTE or death and 6/21 with VTE only), these analyses were unable to demonstrate a statistically-significant increased risk of future events in patients with elevated cHK/HK (P = 0.11, 0.39, respectively), though a clear trend toward increased risk is seen in the range of cHK values from 0.075 to 0.105.

Though these studies demonstrate activation of the contact system in cancer, they do not prove that circulating EV contribute. To address this question, we used beads coated with antibodies to CD81, CD63 and CD9 to immunoprecipitate EV from a normal individual and three patients, two with pancreatic and one with colorectal cancer. The ability of immunoprecipitated EV to activate FXII was then assessed. As shown in Figure 6G, a small amount of FXIIa generation was caused by EV from the normal donor, but greater activation was induced by EV from each of the cancer patients.
Discussion

Our findings demonstrate that long-chain polyphosphate is expressed on EV derived from cancer and non-transformed cells. On the EV surface, polyP mediates binding of FXII. As a consequence of FXII binding, EV activate FXII to FXIIa, leading to activation of the contact system and intrinsic coagulation pathway.

Thrombosis is a common complication in patients with cancer\textsuperscript{12,13}. Multiple mechanisms for the development of thrombosis have been proposed, with significant interest in the role of tissue-factor expressing EV\textsuperscript{18-20,45}. Levels of tissue factor-positive EV correlate with the development of thrombosis in patients with pancreatic cancer\textsuperscript{22,46}, although such a relationship has not been demonstrated for other cancer types\textsuperscript{22,23,47-49}. There has been comparatively little study of the intrinsic, FXII-mediated pathway in cancer. Our studies demonstrate that activation of this pathway occurs commonly in cancer patients (Figure 6A-D). Moreover, the ability of EV from different types of cancer cells to cause FXII activation varies and correlates with the strength of the association of the parental cancer cells with thrombosis\textsuperscript{50}. These findings suggest that cHK may potentially serve as a biomarker of thrombosis in cancer, although addressing this question will require larger studies.

PolyP-expressing EV induced pulmonary emboli in mice, while pretreatment of EV with phosphatase impaired their prothrombotic activity. Moreover, the ability of polyP expressing EV
to induce pulmonary emboli was significantly reduced in mice deficient in FXII, prekallikrein or HK; these findings implicate a critical role of the contact activation system in EV-induced thrombosis. In contrast to the studies of Nickel et al\textsuperscript{33}, we found that \textit{kllb1/\textendash} mice were also protected from development of EV-induced thrombi in vivo; the reasons for this discrepancy will require additional investigation.

There is little information available concerning the role of polyP in cancer cells, the mechanisms by which polyP becomes associated with cancer cell-derived EV, and why only a subpopulation of EV express polyP. Though the biosynthetic pathway of polyP in mammalian cells is not well understood, the cellular content of polyP may depend upon the activity of inositol hexakisphosphate kinase 1 (IP6K1)\textsuperscript{51}. Mammalian cells lacking IP6K1 demonstrate impaired dynein-dependent trafficking, including endosomal sorting and vesicle movement\textsuperscript{52}, which may lead to decreased exosome release\textsuperscript{53}; genetic ablation of \textit{Ip6k1} in mice results in reduced platelet polyP\textsuperscript{54}. Data from the Human Protein Atlas suggests higher expression of IP6K1 in colorectal, pancreatic and prostate\textsuperscript{33} cancer; these findings may account, in part, for the elevated levels of polyP on pancreatic cancer cell-derived EV, as well as by prostasomes\textsuperscript{33}.

Our manuscript has several limitations, most importantly the fact that this study focused on polyP and contact activation but did not address other potential prothrombotic mechanisms of cancer cell EV, such as tissue factor. However, one study has demonstrated that prostasomes may enhance both tissue factor and intrinsic pathway-mediated coagulation activation\textsuperscript{33,39}, and this is likely the case with EV used in this study as well; indeed, tissue factor antigen expression by L3.6, but not the other cell lines, was detected by immunoblotting (Figure S5). Nevertheless, our clinical studies demonstrate activation of the contact system in a high proportion of cancer patients, and confirm that patient-derived EV activate FXII in plasma. While our clinical cohort
lacks the power to demonstrate that cHK is a biomarker for thrombosis, this limitation will be addressed in future studies.

In summary, our studies suggest that EV from patients with cancer, and cancer cells, express increased amounts of polyP compared to healthy individuals or non-transformed cell lines, and that these polyP-expressing EV bind FXII and activate the contact system. These EV induce thrombosis in animal models, and are less effective in doing so after treatment with phosphatase or in mice lacking key proteins of the contact system. These findings suggest a role for polyP expressing EV in cancer-associated thrombosis, and raise the possibility that polyP inhibitors may prevent thrombosis in cancer patients without increasing bleeding risk.
Data Sharing

For original data or protocols please contact mccraek@ccf.org.

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Author contributions

Young Jun-Shim and Victor Chatterjee planned and executed experiments. Shadi Swaidani and Ravi Kumar Alluri assisted with fluorescent ligand binding experiments. Suman Kundu assisted with cell culture. Dewen You performed experiments. Samantha Whitney and Edward Feener designed and conducted ProteinSimple assays for HK and cHK, and edited the manuscript. John Barnard performed biostatistical analyses. Alvin Schmaier designed and analyzed the murine pulmonary emboli studies, and assisted with editing of the manuscript. Alok Khorana provided clinical samples and assisted with experimental planning and editing of the manuscript. Keith McCrae developed concepts, organized data, and wrote and edited the manuscript.

Conflict of Interest Statement

Dr. Khorana reports personal fees and non-financial support from Janssen, Bayer, Pfizer, Seattle Genetics, and Bristol-Myers Squibb; personal fees from Parexel, Pharmacypicals, Pharmacyte, Medscape; and grants to his institution from Merck, Array, Bristol Myers Squibb, Leap, and Seattle Genetics all outside the submitted work. The other authors report no relevant conflicts of interest.
Figure legends

**Figure 1. Characterization of EVs.** Particle numbers (A) and relative protein concentrations (B) in fractions eluted following qEV size exclusion chromatography. Bars represent mean ± SEM. (C) Tetraspanin (CD9, CD63, and CD81) content of purified EV were analyzed by immunoblotting in indicated elution fractions. (D) Size distribution histograms of pooled fractions 7-10 from HDF and L3.6 cells. (E) electron microscopic image of EV from HDF and L3.6 cells.

**Figure 2. Polyphosphate (polyP) on EV derived from cancer cells mediates ligand binding.** (A) The amount of polyP associated with cancer cell-derived EVs was estimated by comparison to a standard curve (Figure S2A). (B) Detection of polyP on L3.6- derived EV following incubation with buffer (HBS), DNase I and RNase A (D/R), or calf intestinal alkaline phosphatase (CIP). PolyP of different chain lengths (L: long, M: medium, and S: short) was used to estimate the size of EV-associated polyP. (C, D, E) L3.6-derived EV were preincubated with buffer or *E. coli* exopolyphosphatase (PPX), and the binding of (C) PPX-Δ12-AF488, (D) anti-CD81-AF488, or (E) FXII-AF488 was measured. The number of total EV was determined in scatter mode, and the number of ligand-binding EV in fluorescence mode using a 488 nm excitation laser. The differences in fluorescent particle numbers were compared by estimating the area under each curve (AUC) using GraphPad Prism 8. Bars represent means ± SEM. ***P < 0.001 by one-way ANOVA with multiple comparisons.

**Figure 3. FXII activation by EV derived from cancer cell lines.** (A) Activation of FXII induced by EV from cancer (L3.6, H1975, HT29 and U937) and HDF cells. (B) FXIIa generation by cancer cell-derived EV. EV-induced S-2302 hydrolysis was estimated at 90 minutes after addition of the indicated amount of EV (determined by protein concentration), and
comparison to a FXIIa standard curve (Figure S3). (C) FXIIa generated by cancer cell-derived EV treated with DNase I (10 U/ml), RNase A (10 U/ml) and CIP (1, 10, and 20 U/ml). (D) Effect of CTI on FXII activation induced by L3.6 EV in NHP and FXII-deficient plasma. Hydrolysis of S-2302 in plasma incubated with EV in the presence or absence of CTI was compared. (E) Activation of purified FXII (375 nM) by EV. EV (20 or 40 µg protein) were incubated in the presence of FXII (375 nM) and S-2302 and A405 was monitored for 90 minutes. (F) FXII cleavage in the presence of L3.6 EV was assessed by immunoblotting using the reaction mixture from (D). The FXII heavy chain (HC) and light chain (LC) were detected under reducing conditions using goat anti-human FXII polyclonal antibody. DS = dextran sulfate; CTI = corn trypsin inhibitor. Bars represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA with multiple comparisons.

**Figure 4. Cleavage of high-molecular-weight kininogen (HK) caused by cancer cell-derived EV.** (A) HK cleavage in NHP incubated with cancer cell-derived EV (0.5 and 1 µg/ml) analyzed by immunoblotting. (B) HK cleavage by EV as shown in (A) expressed as the percentage of cHK relative to intact HK. (C) HK cleavage 60 minutes after the addition of L3.6 EV following treatment of EV with CIP, DNase I, or RNase A. Dextran sulfate (DS) was used as a positive control. (D) Effect of CIP, DNase I, and RNase A on the cleavage of HK by EV from cancer and HDF cells; the ratio of cHK to HK was determined by immunoblotting and infrared quantification. (E) Effect of CTI on L3.6 EV-induced cHK generation. L3.6 EV were incubated with NHP in the presence or absence of CTI (10 µg/ml) for 60 minutes. For HK cleavage analysis, a polyclonal rabbit anti-human HK antibody (D5, Figure S1) was used to detect HK and cHK. LC denotes light chain: LC1 (54kDa), LC2 (47kDa). Bars represent means ± SEM. ***p < 0.001 by two-way ANOVA with multiple comparisons.
Figure 5. Pulmonary emboli in mice treated with EV derived from L3.6 cells. (A) Paraffin section of lung from a wild-type mouse after L3.6 EV infusion into the IVC. H&E stain, 5X magnification. (B, C) Frozen sections of lung following EV infusion into the IVC, stained for platelet GP1b (CD42c, red) or fibrin (mAb 59D8, green) at 20X and 10X magnification, respectively. DAPI (blue) stains cell nuclei; scale bars = 500 μm for 5x, 200 μm for 10x, and 100 μm for 20x magnification. (D) Number of thrombi observed in a random 10X field following infusion of L3.6 EV into wild-type (WT) C57BL/6, Factor XII-deficient (fl2−/−), high molecular weight kininogen-deficient (kng1−/−), and plasma prekallikrein-deficient (klkb1−/−) mice. (E) Effect of preincubation of L3.6 EV with buffer alone (untreated) or CIP (treated) prior to IVC infusion on density of lung thrombi, measured per 10X field. (F) Effect of preincubation of L3.6 EV with buffer alone (untreated) or CIP (treated) on time to death after IVC infusion. Each point represents an individual animal. Data shown in graphs are 95% confidence intervals and comparison between groups was made by ANOVA and student t tests.

Figure 6. Increased levels of cHK in plasma from cancer patients. (A) HK cleavage in plasma from pancreatic cancer patients compared to those from healthy donors (normal) by immunoblotting using antibody to HK domain 5. (B) Same plasmas analyzed using an antibody specific for the C-terminus of the free HK heavy chain (HC) after reduction (HKa1, Figure S1). (C) Ratio of cHK to HK in plasma from normal individuals and pancreatic cancer patients (n=26), determined by WES capillary immunoblotting. (D) ratio of cHK to HK in normal individuals and patients presenting to the cancer thrombosis clinic (N = 21) with symptoms of venous thromboembolism, determined by WES capillary immunoblotting. Bars represent means ± SEM. ***p < 0.001 by unpaired t test with Welch’s corrections. (E) Log relative hazard of VTE or death versus cHK/total HK ratio from the Cox proportional hazard modeling of mixed
cancer patients; a red dot indicates an VTE or death event, a black dot indicates a censored event and the shaded area is the 95% confidence interval for the log hazard. (F) Log relative hazard of VTE only (censored at death) versus cHK/total HK ratio from the Cox proportional hazard modeling of mixed cancer patients; a red dot indicates an VTE event, a black dot indicates a censored event and the shaded area is the 95% confidence interval for the log hazard. (G) FXII activation by EV immunopurified from a normal donor, or from patients with pancreatic or colon cancer. Bars represent means ± SEM. *p < 0.05, ***p < 0.001 by one-way ANOVA with unpaired t test with multiple comparisons.
References

1. Yáñez-Mó M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015;4:27066.
2. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PloS One. 2009;4(4):e5219.
3. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci. 2010;123(Pt 10):1603-1611.
4. Rahbarghazi R, Jabbari N, Sani NA, et al. Tumor-derived extracellular vesicles: reliable tools for Cancer diagnosis and clinical applications. Cell Commun Signal. 2019;17(1):73.
5. Rak J. Extracellular vesicles - biomarkers and effectors of the cellular interactome in cancer. Front Pharmacol. 2013;4:21.
6. Osti D, Del Bene M, Rappa G, et al. Clinical Significance of Extracellular Vesicles in Plasma from Glioblastoma Patients. Clin Cancer Res. 2019;25(1):266-276.
7. Kalluri R. The biology and function of exosomes in cancer. J Clin Invest. 2016;126(4):1208-1215.
8. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science. 2020;367(6478).
9. Zarà M, Guidetti GF, Camera M, et al. Biology and Role of Extracellular Vesicles (EVs) in the Pathogenesis of Thrombosis. Int J Mol Sci. 2019;20(11).
10. Huang T, Deng CX. Current Progresses of Exosomes as Cancer Diagnostic and Prognostic Biomarkers. Int J Biol Sci. 2019;15(1):1-11.
11. Whiteside TL. Validation of plasma-derived small extracellular vesicles as cancer biomarkers. Nat Rev Clin Oncol. 2020;17(12):719-720.
12. Khorana AA, McCrae KR. Risk stratification strategies for cancer-associated thrombosis: an update. Thromb Res. 2014;133 Suppl 2:S35-S38.
13. Khorana AA, Francis CW, Culakova E, Kuderer NM, Lyman GH. Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. J Thromb Haemost. 2007;5(3):632-634.
14. Kim AS, Khorana AA, McCrae KR. Mechanisms and biomarkers of cancer-associated thrombosis. Transl Res. 2020.
15. Abdol Razak NB, Jones G, Bhandari M, Berndt MC, Metharom P. Cancer-Associated Thrombosis: An Overview of Mechanisms, Risk Factors, and Treatment. Cancers (Basel). 2018;10(10).
16. Stark K, Schubert I, Joshi U, et al. Distinct Pathogenesis of Pancreatic Cancer Microvesicle-Associated Venous Thrombosis Identifies New Antithrombotic Targets In Vivo. Arterioscler Thromb Vasc Biol. 2018;38(4):772-786.
17. Dvorak HF, Quay SC, Orenstein NS, et al. Tumor shedding and coagulation. Science. 1981;212(4497):923-924.
18. Bharthuar A, Khorana AA, Hutson A, et al. Circulating microparticle tissue factor, thromboembolism and survival in pancreaticobiliary cancers. Thromb Res. 2013;132(2):180-184.
19. Geddings JE, Mackman N. Tumor-derived tissue factor-positive microparticles and venous thrombosis in cancer patients. Blood. 2013;122(11):1873-1880.
20. Almeida VH, Rondon AMR, Gomes T, Monteiro RQ. Novel Aspects of Extracellular Vesicles as Mediators of Cancer-Associated Thrombosis. *Cells.* 2019;8(7).

21. Nieuwland R, Gardiner C, Dignat-George F, et al. Toward standardization of assays measuring extracellular vesicle-associated tissue factor activity. *J Thromb Haemost.* 2019;17(8):1261-1264.

22. van Es N, Hisada Y, Di Nisio M, et al. Extracellular vesicles exposing tissue factor for the prediction of venous thromboembolism in patients with cancer: A prospective cohort study. *Thromb Res.* 2018;166:54-59.

23. Hisada Y, Mackman N. Cancer cell-derived tissue factor-positive extracellular vesicles: biomarkers of thrombosis and survival. *Curr Opin Hematol.* 2019;26(5):349-356.

24. Wu Y. Contact pathway of coagulation and inflammation. *Thromb J.* 2015;13:17.

25. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost.* 2016;14(1):28-39.

26. Naudin C, Burillo E, Blankenberg S, Butler L, Renné T. Factor XII Contact Activation. *Semin Thromb Hemost.* 2017;43(8):814-826.

27. Berrettini M, Lammle B, White T, et al. Detection of in vitro and in vivo cleavage of high molecular weight kininogen in human plasma by immunoblotting with monoclonal antibodies. *Blood.* 1986;68:455-462.

28. Hofman ZLM, de Maat S, Suffritti C, et al. Cleaved kininogen as a biomarker for bradykinin release in hereditary angioedema. *J Allergy Clin Immunol.* 2017.

29. Kerbiriou D, Griffin JH. Human high molecular weight kininogen. Studies of structure-function relationships and of proteolysis of the molecule occurring during contact activation of plasma. *Journal of Biological Chemistry.* 1979;254:12020-12027.

30. Choi SH, Smith SA, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. *Blood.* 2011;118(26):6963-6970.

31. Morrissey JH, Choi SH, Smith SA. Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation. *Blood.* 2012;119(25):5972-5979.

32. Puy C, Tucker EI, Wong ZC, et al. Factor XII promotes blood coagulation independent of factor XI in the presence of long chain polyphosphate. *J Thromb Haemost.* 2013:10.

33. Nickel KF, Ronquist G, Langer F, et al. The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis. *Blood.* 2015;126(11):1379-1389.

34. Khorana AA, Connolly GC. Assessing risk of venous thromboembolism in the patient with cancer. *J Clin Oncol.* 2009;27(29):4839-4847.

35. Bruns CJ, Harbison MT, Kuniyasu H, Eue I, Fidler IJ. In vivo selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia.* 1999;1(1):50-62.

36. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* 2006;Chapter 3:Unit 3.22.

37. Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. *Proc Natl Acad Sci U S A.* 2006;103(4):903-908.

38. Labberton L, Kenne E, Long AT, et al. Neutralizing blood-borne polyphosphate in vivo provides safe thromboprotection. *Nat Commun.* 2016;7:12616.

39. Nickel KF, Labberton L, Long AT, et al. The polyphosphate/factor XII pathway in cancer-associated thrombosis: novel perspectives for safe anticoagulation in patients with malignancies. *Thromb Res.* 2016;141 Suppl 2:S4-7.

40. Zilberman-Rudenko J, Reitsma SE, Puy C, et al. Factor XII Activation Promotes Platelet Consumption in the Presence of Bacterial-Type Long-Chain Polyphosphate In Vitro and In Vivo. *Arterioscler Thromb Vasc Biol.* 2018;38(8):1748-1760.
41. Hui KY, Haber E, Matsueda GR. Monoclonal antibodies to a synthetic fibrin-like peptide bind to human fibrin but not fibrinogen. *Science*. 1983;222(4628):1129-1132.
42. Harrell FE, Jr. *rms: Regression Modeling Strategies*. R package version 5.1-4; 2019.
43. Aschar-Sobbi R, Abramov AY, Diao C, et al. High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *J Fluoresc*. 2008;18(5):859-866.
44. Lorenz B, Schröder HC. Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. *Biochim Biophys Acta*. 2001;1547(2):254-261.
45. Zwicker JI. Predictive value of tissue factor bearing microparticles in cancer associated thrombosis. *Thromb Res*. 2010;125 Suppl 2:S89-S91.
46. Tesselaar ME, Romijn FP, van der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost*. 2007;5(3):520-527.
47. Thaler J, Ay C, Mackman N, et al. Microparticle-associated tissue factor activity, venous thromboembolism and mortality in pancreatic, gastric, colorectal and brain cancer patients. *J Thromb Haemost*. 2012;10(7):1363-1370.
48. Cohen JG, Prendergast E, Geddings JE, et al. Evaluation of venous thrombosis and tissue factor in epithelial ovarian cancer. *Gynecol Oncol*. 2017;146(1):146-152.
49. Gezelius E, Flou Kristensen A, Bendahl PO, et al. Coagulation biomarkers and prediction of venous thromboembolism and survival in small cell lung cancer: A sub-study of RASTEN - A randomized trial with low molecular weight heparin. *PLoS One*. 2018;13(11):e0207387.
50. Connolly GC, Khorana AA. Risk stratification for cancer-associated venous thromboembolism. *Best Pract Res Clin Haematol*. 2009;22(1):35-47.
51. Mailer RKW, Hanel L, Allende M, Renne T. Polyphosphate as a Target for Interference With Inflammation and Thrombosis. *Front Med (Lausanne)*. 2019;6:76.
52. Chanduri M, Rai A, Malla AB, et al. Inositol hexakisphosphate kinase 1 (IP6K1) activity is required for cytoplasmic dynein-driven transport. *Biochem J*. 2016;473(19):3031-3047.
53. Kimura N, Inoue M, Okabayashi S, Ono F, Negishi T. Dynein dysfunction induces endocytic pathology accompanied by an increase in Rab GTPases: a potential mechanism underlying age-dependent endocytic dysfunction. *J Biol Chem*. 2009;284(35):31291-31302.
54. Ghosh S, Shukla D, Suman K, et al. Inositol hexakisphosphate kinase 1 maintains hemostasis in mice by regulating platelet polyphosphate levels. *Blood*. 2013;122(8):1478-1486.
55. Smith SA, Choi SH, Collins JN, Travers RJ, Cooley BC, Morrissey JH. Inhibition of polyphosphate as a novel strategy for preventing thrombosis and inflammation. *Blood*. 2012;120(26):5103-5110.
Figure 1

A

B

C

D

E

HDF

L3.6

Fraction#

100

70

55

25

25

CD63

CD81

CD9

HDF

L3.6

(99.3 nm)

(97.6 nm)

HDF

L3.6

[Image of graphs and images related to Figure 1]
Figure 2

A

B

C

D

E

polyP (ng/µg)

1,000 bps (~964mer)

L M S

HBS D/R CIP

Particle conc. (10^9/ml)

Area under the curve (AUC)

Particle conc. (10^9/ml)

Area under the curve (AUC)
Figure 4

A

|         | HDF | L3.6 | H1975 | HT29 | U937 |
|---------|-----|------|-------|------|------|
| (kDa) HBS DS | 0.5 | 1    | 0.5   | 1    | 0.5  |
| (µg)      |     |      |       |      |      |

B

HK cleavage (%)

C

|         | HDF | L3.6 |
|---------|-----|------|
| EV      | +   | +    |
| CIP     | +   | +    |
| DNase I | -   | +    |
| RNase A | +   | +    |
| DS      | +   | +    |

D

chHKHK (fold change)

E

|         | HDF | L3.6 | U937 |
|---------|-----|------|------|
| CTI     | -   | +    | +    |
Figure 5
Figure 6

Panel A shows Western blot analysis of HK protein levels in normal and pancreatic cancer tissues. The blot reveals bands at approximately 55 and 35 kDa, labeled as HK, LC1, and LC2. Panel B illustrates the same analysis with a different set of samples, highlighting bands labeled as HC.

Panel C presents a scatter plot comparing cHK/HK ratios in controls and pancreatic cancer patients (PANC). The x-axis represents the cHK/total HK ratio, and the y-axis shows the log relative hazard. Panel D displays a similar comparison but for controls and colon cancer (CAT) patients.

Panel E and F depict the log relative hazard as a function of the cHK/total HK ratio, with shaded areas indicating the range of values.

Panel G illustrates the FXa (nM) levels across different samples: beads, normal, pancreatic, and colon. The graph includes error bars and statistical significance markers (*, **, ***).