Original Research

X-inactive-specific transcript of peripheral blood cells is regulated by exosomal Jpx and acts as a biomarker for female patients with hepatocellular carcinoma

Xiang Ma, Tingdong Yuan, Chao Yang, Zusen Wang, Yunjin Zang, Liqun Wu and Likun Zhuang

Abstract

Background: Long noncoding ribonucleic acid (lncRNA) X-inactive-specific transcript (Xist) was reported to affect cell proliferation and metastasis in hepatocellular carcinoma (HCC). However, there are rare reports focusing on the diagnostic evaluation and regulatory mechanism of Xist expression from peripheral blood cells of patients with HCC.

Methods: In this study, a cohort of 206 female participants including healthy volunteers (HVs) and patients with chronic hepatitis B (CHB), cirrhosis and HCC was recruited. Coculture system was used to evaluate the effects of exosomal JPX transcript, XIST activator (Jpx) on Xist expression of blood cells.

Results: First, Xist expressions of both peripheral blood mononuclear cells and granulocytes were upregulated in female patients with HCC, and showed significantly increased discriminatory power when differentiating female patients with early-stage HCC from controls or differentiating female patients with HCC from patients with CHB and cirrhosis, compared with alpha fetoprotein (AFP). Then, another lncRNA Jpx that was an activator of Xist was upregulated in exosomes, mononuclear cells and granulocytes of female patients with HCC. Furthermore, our results showed that Jpx could be delivered from HCC cells to blood cells *via* exosomes and activate Xist expression of blood cells by repressing the transregulatory effects of CCCTC-binding factor (CTCF).

Conclusions: This study revealed an exosome-mediated regulation of Xist expression in blood cells and suggested that Xist expressions of mononuclear cells and granulocytes would be promising biomarkers for diagnosis of female patients with HCC.

Keywords: exosomes, granulocytes, HCC, Jpx, mononuclear cells, Xist

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Introduction

Hepatocellular carcinoma (HCC) is one type of primary liver tumor with a high mortality, and ranks as one of the most common cancers in humans. Epidemiological studies have shown several key risk factors for the development of HCC, such as viral hepatitis infection and cirrhosis.[1,2] However, the majority of patients with HCC are diagnosed at a late stage, in which severe liver dysfunction has occurred and curative treatments are not feasible.[3] Therefore, it is necessary to search for a convenient and reliable strategy for early-stage HCC diagnosis. Currently, serum biomarker is one of the diagnostic tools for HCC and alpha fetoprotein (AFP) is the most common of the serum biomarkers used for HCC diagnosis.[4] However, it has been reported that the specificity of AFP detection for HCC diagnosis is poor in clinical application.[5] In view of this, the development of new noninvasive biomarkers, which could be used for large-scale clinical investigations with high specificity and sensitivity, is greatly needed in HCC diagnosis.

In the past few years, many studies have focused on the specific expressions of noncoding ribonucleic acids (RNAs), including long noncoding RNA (lncRNA) and microRNA (miRNA) as new diagnostic biomarkers in the plasma of patients with HCC. More and more evidence has shown that there are significant differences in plasma lncRNA and miRNA expression profiling between HCC patients and healthy donors. Tang *et al.* found that the upregulation of lncRNA RP11–160H22.5, XLOC_014172 and LOC149086 in plasma could serve as potential biomarkers in the diagnosis of HCC.[6] Another study indicated that miR-483-5p in plasma might serve as a novel noninvasive biomarker for diagnosis of HCC.[7] However, there is lack of suitable internal control for plasma RNA and difficulty in the extraction of plasma RNA because of the low concentration. More work is needed to achieve the clinical application of plasma noncoding RNAs.

Recently, the abnormal expressions of noncoding RNAs in peripheral blood cells of patients with cancers have attracted more and more attentions, and provided new strategy for cancer diagnosis. For instance, miR-129 was downregulated in peripheral blood mononuclear cells of prostate cancer patients and was a novel independent diagnostic biomarker for prostate cancer.[8] Another study showed that lncRNA MALAT1 was a blood-based biomarker for the diagnosis of lung cancer.[9] Therefore, lncRNA or miRNA in peripheral blood cells, which could be more convenient to detect and quantify, would be a more promising biomarker for the diagnosis of tumors. However, there were few reports focusing on the diagnostic application of lncRNA or miRNA in peripheral blood cells of HCC patients.

Our previous study showed that lncRNA X-inactive-specific transcript (Xist) could regulate the proliferation and metastasis of HCC cells.[10] By microarray analysis of lncRNA expression profile, we also found that Xist expression of peripheral blood was upregulated in HCC patients. Therefore, we supposed that circulating Xist in peripheral blood might be a promising biomarker for HCC diagnosis. In this study, we analyzed the diagnostic performance of Xist expressions in peripheral blood cells for differentiating female patients with HCC from controls. In addition, we also focused on investigating the regulatory mechanism of Xist expressions in peripheral blood cells by exosomal JPX transcript, XIST activator (Jpx). These findings provide new insights in understanding the roles of Xist from peripheral blood cells in the diagnosis of female patients with HCC.

Materials and methods

Patients and samples
Peripheral blood samples were collected from healthy volunteers (HVs) and patients with chronic hepatitis B (CHB), cirrhosis and HCC. HCC patients who underwent partial liver resection were selected at random from Department of Hepatobiliary and Pancreatic Surgery, The Affiliated Hospital of Qingdao University. Patients with CHB and cirrhosis were diagnosed and randomly selected at Department of Infectious Diseases, the Affiliated Hospital of Qingdao University. The characteristics of patients and HVs were listed in Supplementary Table S1. This study was approved by the Ethics Committee of Affiliated Hospital of Qingdao University (approval number: 20150617) and all participants provided their informed consent to participate in this study.

Density gradient separation
Whole blood with ethylenediaminetetraacetic acid (EDTA) anticoagulant was diluted in phosphate buffer saline and layered on the Ficoll–Paque solution (Solarbio, Shanghai, China) in a centrifuge tube. The tube was centrifuged at 2000 rpm for 20 min. Plasma, mononuclear cells, granulocytes and erythrocytes were separately isolated to different tubes. For the isolation of mononuclear cells and granulocytes, the remaining erythrocytes were lysed using Red Blood Cell Lysis Buffer (Roche, Mannheim, Germany).

Ribonucleic acid extraction and quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and quantified by spectrometry. Reverse transcription of RNA was performed using PrimeScript RT-PCR kit (Takara, Otsu, Japan). QRT-PCR was performed using SYBR Green Premix (Takara, Otsu, Japan). Xist and Jpx expression were normalized to the expression of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The data were analyzed by delta-delta Ct method. The primers were as follows: Xist: 5'-CGGGTCTCTTCAAGGACATTTAG

CC-3' and 5'-GCACC AATACAGAGGA ATGGAGGG-3'; Jpx: 5'-TGCAGTCAGAA GGG AGCAAT-3' and 5'-CACCGTCATCA GGCTGTCTT-3'; β-actin: 5'-CATCCTCA CCCTGA AGTACCC C-3' and 5'-AGCCTGG ATGCAACGTACATG-3'; GAPDH: 5'-GAA GATGGTGAT GGGATTTC-3' and 5'-GA AGGTGAAGGTCGGAGT-3'.

Long noncoding ribonucleic acid microarray

Total RNAs from peripheral blood of eight HCC patients and eight HVs were respectively pooled and sent to Shanghai Biotechnology Corporation (Shanghai, China) for profiling lncRNAs using the SBC 4 × 180K human lncRNA microarray (Agilent Technologies, Santa Clara, CA, USA). Hybridization signals were detected with an Agilent DNA microarray scanner (Agilent Technologies). The lncRNA expression profiles were sorted using Cluster 3.0 software (University of Tokyo, Human Genome Center).

Cell lines and coculture system

In this study, cell lines QGY-7703, Hela and HL-60 were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 10% fetal-bovine-serum-containing Dulbecco's Modified Eagle's medium or serum-free medium (Gibco, Grand Island, NY, USA) at 37°C in humidified air with 5% CO_2.

QGY-7703 and HL-60 cells were cocultured using a transwell chamber culture system containing 0.4 μm pore polycarbonate membrane inserts (Corning, Corning, NY, USA). HL-60 cells were cultured in the upper chamber of the transwell culture system and QGY-7703 cells were cultured in the lower chamber. In this system, both cell lines shared the same culture media.

Exosome isolation

For exosome isolation from culture medium, cells were cultured in serum-free medium for 48 h and the culture supernatant was collected. Then the culture supernatant was centrifuged at 2000 g for 10 min and filtered on a 0.22 μm filter to remove cell debris. Exosomes from culture media were extracted by ExoQuick-TC Exosome Precipitation solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions.

For Exosome isolation from plasma, whole blood samples from HVs and patients were collected into EDTA-containing tubes and centrifuged at 3000 g for 15 min. Then 250 μl plasma was transferred to a sterile vessel for exosome isolation. Exosomes from plasma were extracted by ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions.

Transmission electron microscopy and particle size analysis

The exosomal fraction was suspended in phosphate buffer saline, spreaded onto formvar/carbon-coated copper grid (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with 3% aqueous phosphotungstic acid for 5 min at room temperature. Filter paper was used to blot off excess solution. The grids were viewed and imaged using transmission electron microscope (JEOL, Tokyo, Japan).

The sizes of isolated exosomes suspended in phosphate buffer saline were analyzed by a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) according to the manufacturer's instructions. Malvern Zetasizer software (version 7.11, Malvern Instruments Ltd., Worcestershire, UK) was used to analyze the data.

Acetylcholinesterase activity

Acetylcholinesterase activity was used to evaluate the quantity of exosomes. Briefly, exosome fraction isolated from 10 ml cell culture supernatant was suspended in 50 μl phosphate buffer saline and transferred to a 96-well plate. Then acetylthiocholine iodide (1.25 mmol) and 5, 5-dithio-bis (2-nitrobenzoic acid) (0.1 mmol) were added to each well of the plate in a final volume of 200 μl per well. The plates were incubated at 37°C for 20 min and the absorbance at 412 nm was measured.

Western blot analysis

Cells or exosomal pellets were lysed using RIPA lysis buffer (Boster, Wuhan, China). Protein concentrations were detected using bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Then protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to 0.45 μm polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with

primary antibodies. The primary antibodies used in this study were listed in Supplementary Table S2.

Transfection and Luciferase assay

Small interfering RNAs (siRNAs) targeting Jpx and siRNAs targeting non-specific control (NC) were obtained from GenePharma (Shanghai, China). Jpx and CTCF-expression plasmids were purchased from Biogot Technology (Nanjing, China). A 2kb fragment encoding Xist gene promoter was fused to PGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). SiRNAs were transfected by Hiperfect transfection reagent (Qiagen, Valencia, CA, USA) and the transfection of plasmids was conducted using Lipofectamine 3000 transfection reagent (Invitrogen). Luciferase activity was measured using the Dual Luciferase Assay System (Promega) and pRL-TK plasmid was used as an internal control.

Chromatin immunoprecipitation (ChIP)

ChIP assay was conducted using EZ ChIP kit (Millipore) and CTCF antibody (Cell Signaling Technology, Danvers, MA, USA) according to the instruction of the manufacturer. The primers specific to the Xist promoter were listed as follows: 5'-ATCACAAAGATGTCCGGCTTTCA ATCTTCTAGGC-3' and 5'-CTTCCAGCC CCGAGAGAGTAAGAAATA TGGCTG-3'. Nonspecific immunoprecipitations were carried out with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The statistical significance of differences between two groups was calculated by Student's *t* test. The statistical significance of differences between two areas under the curve (AUCs) was calculated and compared by using Delong's algorithm. Pearson's analysis was conducted in correlation analysis. A value of $p < 0.05$ was considered as statistically significant.

Results

X-inactive-specific transcript is upregulated in mononuclear cells and granulocytes of female patients with hepatocellular carcinoma

First, total RNAs of whole blood from eight HCC patients and eight HVs, including four males and four females, respectively, were separately pooled and analyzed by human lncRNA microarray. The results showed that Xist that was previously reported to be involved in HCC progression,[10] was more highly expressed in HCC patients than that in HVs (Figure 1A). Considering that Xist was hardly detected in the blood of males, we measured Xist levels of whole blood from 20 female patients with HCC and 20 female HVs by qRT-PCR. Consistent with microarray data, Xist levels of whole blood from female patients with HCC were significantly higher than that of HVs (Figure 1B).

Then, density gradient separation was used to isolate plasma, mononuclear cells, granulocytes and erythrocytes from whole blood of 206 female participants including HVs and patients with CHB, cirrhosis and HCC (Figure 1C). The monocyte marker CD14 and lymphocyte marker CD3 were used to verify mononuclear cells, while CD15 and CD235a were used as granulocyte and erythrocyte marker respectively (Figure 1D). QRT-PCR analysis showed that Xist expressions in mononuclear cells and granulocytes of female patients with HCC were significantly higher than that of any other group (Figure 1E–F), while no significant difference was observed in Xist levels of erythrocytes (Figure 1G) and plasma Xist was not detected. These results indicated that Xist was upregulated in mononuclear cells and granulocytes of female patients with HCC.

Interestingly, our results showed that Xist level of either mononuclear cells or granulocytes was significantly lower after surgery than that before surgery in 10 female patients with HCC (Figure 1H–I).

Diagnostic efficacy of X-inactive-specific transcript levels from blood cells in differentiating female patients with hepatocellular carcinoma

The evaluation of diagnostic effects of mononuclear cell and granulocytic Xist in differentiating female patients with HCC from non-HCC patients and HVs was conducted by calculating the area under the receiver operating characteristic (ROC) curve (AUC). AUCs for AFP, mononuclear cell Xist and granulocytic Xist were 0.752, 0.815 and 0.827, respectively (Figure 2A–C and Table 1). This indicated that AFP and Xist in mononuclear cells or granulocytes had similar effects on differentiating female patients with HCC from non-HCC patients and HVs.
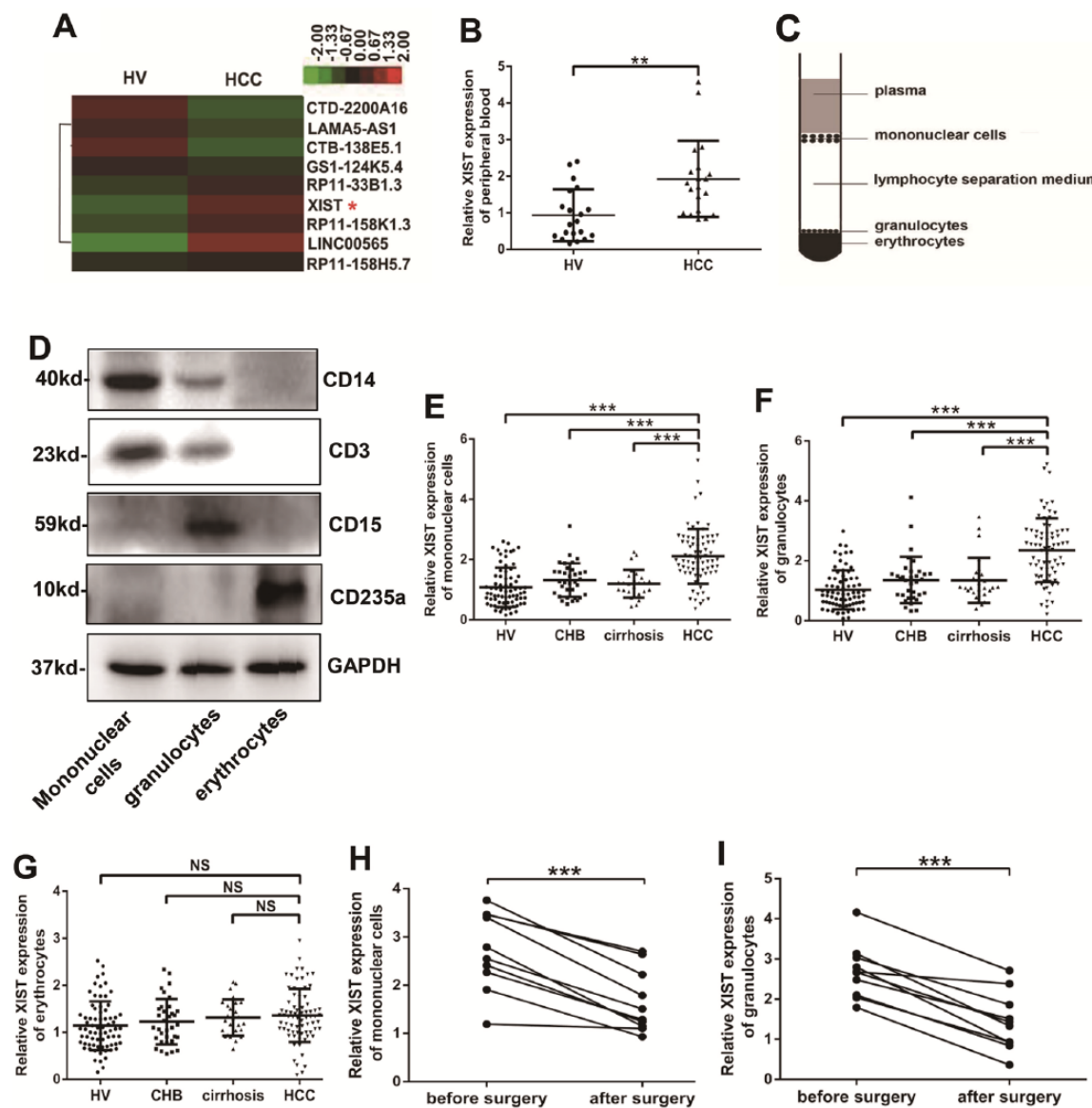
Figure 1. Xist expressions in peripheral blood cells of female patients with hepatocellular carcinoma.
(A) Partial long noncoding ribonucleic acid (lncRNA) expression profiles from peripheral blood of healthy volunteers (HVs) and hepatocellular carcinoma (HCC) patients. The red color in the heatmap indicates high expression and the green color indicates low expression according to the color bar in logarithmic scale shown above the heatmap. (B) Xist expressions in peripheral blood of female patients with HCC ($n = 20$) and female HVs ($n = 20$) were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to β-actin expression. (C) A schematic diagram for the separation of different peripheral blood cells based on centrifugation. (D) CD14, CD3, CD15 and CD235a expressions of mononuclear cells, granulocytes and erythrocytes were determined by western blot. (E–G) Xist expressions of mononuclear cells: (E) granulocytes (F) and erythrocytes (G) from female HVs ($n = 72$) and female patients with chronic hepatitis B (CHB) ($n = 34$), cirrhosis ($n = 26$) and HCC ($n = 74$). The results were determined by qRT-PCR and normalized to β-actin expression. (H–I) Xist levels of mononuclear cells: (H) and granulocytes (I) before and after curative resection in female patients with HCC ($n = 10$).
$^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant.

Meanwhile, the diagnostic effects of the combination of mononuclear cell or granulocytic Xist with AFP were also analyzed. The combination of mononuclear cell Xist with AFP exhibited significantly increased differentiation power compared with AFP alone, while the AUC for combination of granulocytic Xist with AFP reached 0.863 which was also significantly superior to that for AFP alone (Figure 2D–E and Table 1).

In the next stage, we selected tumor-node-metastasis (TNM) stage I as the early stage of HCC ($n = 59$), and the diagnostic values of Xist in mononuclear cells and granulocytes for
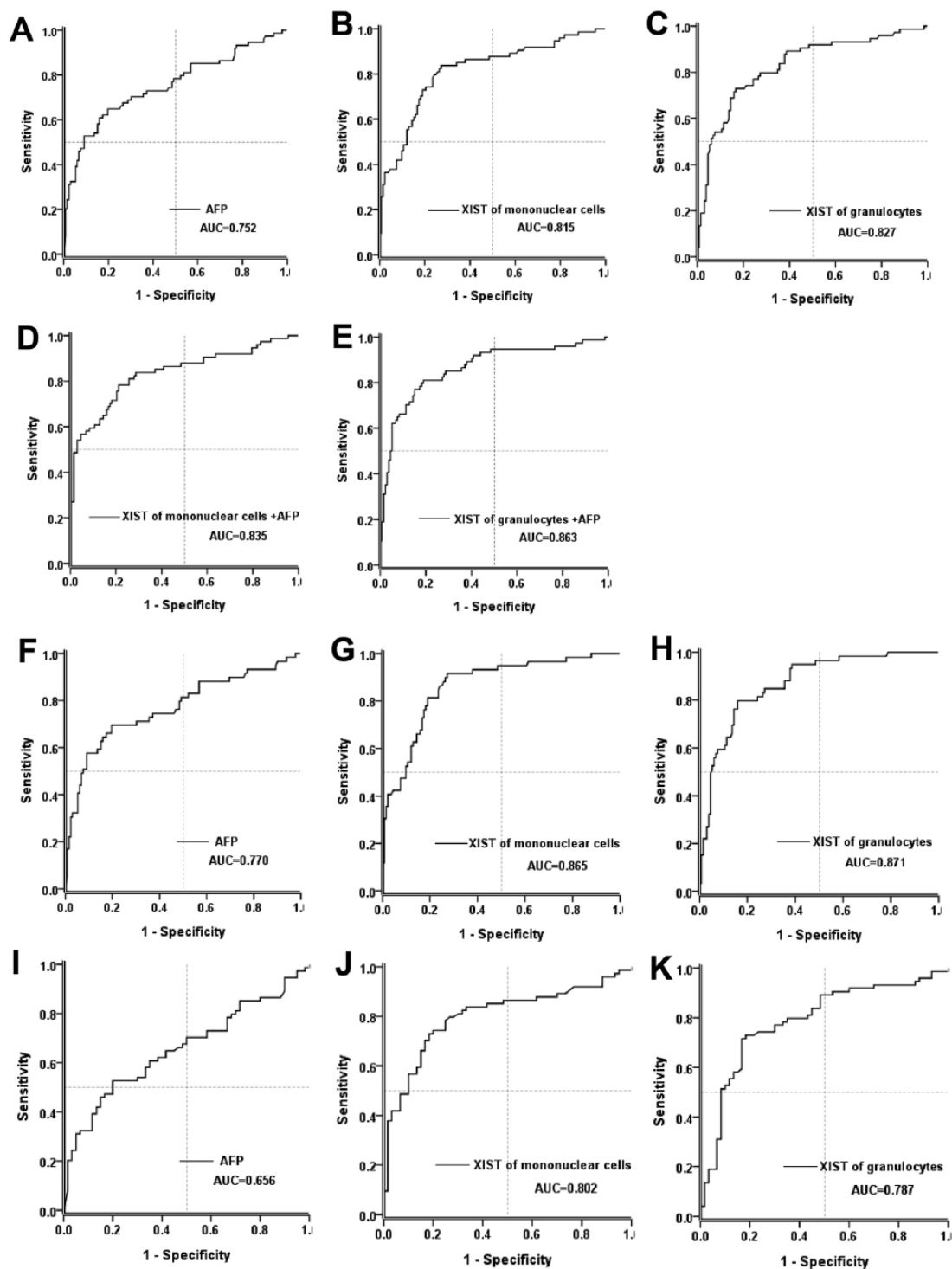
Figure 2. Diagnostic power of alpha fetoprotein (AFP) and X-inactive-specific transcript (Xist) for female patients with hepatocellular carcinoma (HCC).
(A–C) receiver operating characteristic (ROC) analysis of AFP: (A) mononuclear cell Xist (B) and granulocytic Xist (C) in differentiating female patients with HCC (n = 74) from controls (n = 132). (D–E) Power of the combination of AFP with mononuclear cell Xist (D) or granulocytic Xist (E) in differentiating female patients with HCC from controls. (F–H) ROC analysis of AFP: (F) mononuclear cell Xist (G) and granulocytic Xist (H) in differentiating female patients with early-stage HCC (n = 59) from controls (n = 132). (I–K) ROC analysis of AFP: (I) mononuclear cell Xist (J) and granulocytic Xist (K) in differentiating female patients with HCC (n = 74) from female patients with chronic hepatitis B (CHB) and cirrhosis (n = 60).

Table 1. Differentiating power of alpha fetoprotein and X-inactive-specific transcript of mononuclear cells and granulocytes for female patients with hepatocellular carcinoma against controls.

	AUC	95% CI	Sensitivity (%)	Specificity (%)	Youden index	p value (*versus* AFP)
HCC patients *versus* controls						
AFP	0.752	0.678–0.826	64.9	80.3	0.452	
Xist of mononuclear cells	0.815	0.751–0.878	83.8	72.7	0.565	0.130
Xist of granulocytes	0.827	0.766–0.888	73.0	83.3	0.563	0.097
Xist of mononuclear cells +AFP	0.835	0.772–0.897	78.4	78.8	0.572	**0.024**
Xist of granulocytes +AFP	0.863	0.807–0.919	81.1	81.1	0.622	**0.004**

Bold numbers indicate significant differences ($p < 0.05$).
AUC, area under the curve; CI, confidence interval; AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; Xist, X-inactive-specific transcript.

Table 2. Differentiating power of alpha fetoprotein and X-inactive-specific transcript of mononuclear cells and granulocytes for female patients with early-stage hepatocellular carcinoma against controls.

	AUC	95% CI	Sensitivity (%)	Specificity (%)	Youden index	p value (*versus* AFP)
Early-stage HCC patients *versus* controls						
AFP	0.770	0.691–0.849	69.5	80.3	0.498	
Xist of mononuclear cells	0.865	0.809–0.921	91.5	72.7	0.642	**0.035**
Xist of granulocytes	0.871	0.818–0.923	79.7	84.1	0.638	**0.036**

Bold numbers indicate significant differences ($p < 0.05$).
AUC, area under the curve; CI, confidence interval; AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; Xist, X-inactive-specific transcript.

distinguishing female patients with early-stage HCC from non-HCC patients and HVs were also analyzed. The values of AUCs for Xist of mononuclear cells and granulocytes were respectively 0.865 and 0.871, both of which were higher than that for AFP (AUC = 0.770, Figure 2F–H and Table 2). This indicated that Xist in either mononuclear cells or granulocytes could serve as more useful biomarkers for female patients with early-stage HCC compared with AFP.

Previous reports have indicated that AFP levels often increased in chronic hepatitis or cirrhosis patients, and had poor sensitivity in the diagnosis of HCC.[11] Accordingly, we combined the female patients with CHB and cirrhosis, and analyzed the differentiation power of Xist between the female patients with HCC and the combined patients with CHB and cirrhosis. The results showed that AUCs for Xist of both mononuclear cells and granulocytes were significantly superior to that for AFP (Figure 2I–K and Table 3). These results indicated that Xist levels of mononuclear cells and granulocytes would be better diagnostic tools than AFP in differentiating female patients with HCC from CHB and cirrhosis.

Jpx is upregulated in exosomes, mononuclear cells and granulocytes of female patients with hepatocellular carcinoma

What was the mechanism underlying the upregulation of Xist in mononuclear cells and granulocytes of female patients with HCC? Previous reports indicated that exosomes could deliver RNAs to peripheral blood cells and lncRNA Jpx could activate Xist expression.[12,13] Therefore, we isolated exosomes from plasma of female HVs and patients. Morphology and size of exosomes were verified by the transmission electron microscopic and nano-particle size analysis (Figure 3A-B). Exosomal markers such as CD9 and CD63 were also enriched in exosomes compared with whole blood cell lysates (Figure 3C).

Table 3. Differentiating power of alpha fetoprotein and X-inactive-specific transcript of mononuclear cells and granulocytes for female hepatocellular carcinoma against chronic hepatitis B and cirrhosis.

	AUC	95% CI	Sensitivity (%)	Specificity (%)	Youden index	*p* value (*versus* AFP)
HCC *versus* CHB + cirrhosis						
AFP	0.656	0.564–0.748	52.7	80.0	0.327	
Xist of mononuclear cells	0.802	0.725–0.878	73.0	81.7	0.547	**0.006**
Xist of granulocytes	0.787	0.707–0.867	71.6	83.3	0.549	**0.027**

Bold numbers indicate significant differences ($p < 0.05$).
AUC, area under the curve; CI, confidence interval; AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; Xist, X-inactive-specific transcript; CHB, chronic hepatitis B.

Fortunately, Jpx, but not Xist, was detected in exosomes of female HVs and patients. QRT-PCR analysis showed that Jpx levels were higher in exosomes, mononuclear cells and granulocytes, but not erythrocytes, of female patients with HCC than that of HVs and patients with CHB and cirrhosis (Figure 3D–G), which exhibited similar expression patterns of Xist in mononuclear cells and granulocytes (Figure 1E–F). Interestingly, correlation analysis showed that Jpx levels of exosomes had significant and positive relationship with that of mononuclear cells or granulocytes in female patients with HCC (Figure 3H–I), which suggested that Jpx of blood cells might be derived from exosomes.

Jpx is delivered from hepatocellular carcinoma cells to hematologic cells by exosomes and activates X-inactive-specific transcript expression

To explore the transport mechanism of Jpx between HCC and blood cells, we generated a coculture model by use of a transwell system. The basal levels of both Xist and Jpx were much higher in HCC cell line QGY-7703 than that in myelocytic cell line HL-60 (Figure 4A), and knockdown of Jpx in QGY-7703 cells could significantly decrease the expression of Xist (Figure 4B). A fixed number of QGY-7703 cells were transfected with Jpx expression plasmid or empty vector, and cultured in the lower well, sharing the same cultured medium of HL-60 cells cultured in the upper chamber (Figure 4C). After coculture, accompanied by the increased Jpx levels in exosomes extracted from cultured medium and the increased Jpx and Xist levels in QGY-7703 cells (Figure 4D), Jpx and Xist levels in cocultured HL-60 cells were also upregulated (Figure 4E). Then we treated QGY-7703 cells with GW4896 which was an inhibitor of exosome release, and found that both Jpx and Xist levels in cocultured HL-60 cells were downregulated accompanied by a reduced exosome release and an increased Jpx level in QGY-7703 cells (Figure 4F–H). These data suggested that Jpx could be delivered to blood cells by exosomes released from HCC cells and promoted Xist expression.

Exosomal Jpx inhibits the transregulatory activity of CTCF on X-inactive-specific transcript promoter

A previous study showed that Jpx could activate transcription of Xist by evicting CTCF protein.[13] In order to explore the regulatory mechanism of exosomal Jpx in promoting Xist expression, we extracted the exosomes from the medium of cultured QGY-7703 cells transfected with Jpx expression plasmid or empty vector, and added the purified exosomes into the cultured medium of Hela cells (Figure 5A). We found that exosomal Jpx could obviously upregulate Jpx and Xist levels of Hela cells (Figure 5B). Next, we constructed a reporter plasmid containing Xist promoter with CTCF binding sites[14] (Figure 5C). Hela cells were cotransfected with reporter genes and CTCF expression plasmid, and incubated with the corresponding exosomes. Luciferase reporter assay showed that CTCF repressed Xist promoter activity and exosomal Jpx antagonized the effects of CTCF (Figure 5D). Furthermore, quantitative ChIP assays also showed that the binding of CTCF protein to Xist promoter was significantly reduced when exosomes with Jpx overexpression were added (Figure 5E). These results indicated that exosomal Jpx could promote Xist transcription by evicting CTCF.

Discussion

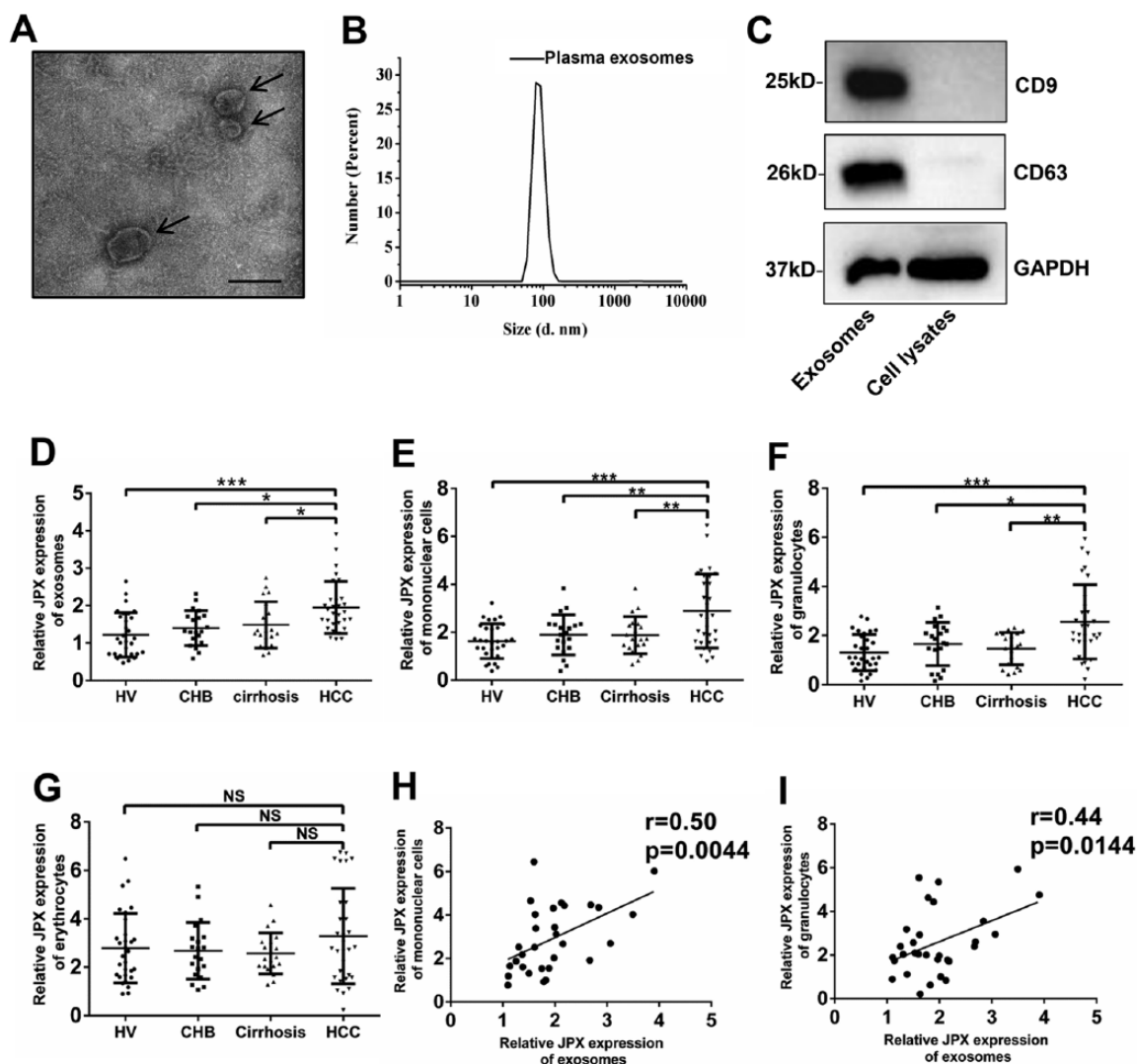Abnormal expressions of lncRNAs in HCC tissues and plasma were gradually revealed and laid

Figure 3. Jpx expression in exosomes and peripheral blood cells of female patients with hepatocellular carcinoma(HCC).

(A–B) Transmission electron microscopic (A) and particle size (B) analysis for exosomes isolated from plasma of HCC patients. The arrows indicate exosomes and the scale bar represents 100 nm. (C) Western blot analysis of CD9 and CD63 in exosomes and blood cell lysates. (D–G) Jpx expressions of exosomes (D) mononuclear cells (E) granulocytes (F) and erythrocytes (G) from female healthy volunteers (HVs) ($n = 30$) and patients with chronic hepatitis B (CHB) ($n = 20$), cirrhosis ($n = 20$) and HCC ($n = 30$). The results were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to GAPDH expression. (H) Correlation of Jpx expressions between exosomes and mononuclear cells of female patients with HCC. (I) Correlation of Jpx expressions between exosomes and granulocytes of female patients with HCC.
$^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant.

the foundation for analyzing circulating lncRNAs of blood cells in HCC diagnosis. Circulating lncRNAs from blood cells have more potential as biomarkers with invasiveness and reliability. Xist is the master regulator of X chromosome inactivation in mammalian cells[15] and many studies have revealed the relationship between Xist expression and tumor progression.[16,17] However, there are few reports considering Xist as a biomarker for tumor occurrence. Considering that

we could hardly detect Xist expressions in tumor tissues and blood cells of male HCC patients, we focused on its expression of blood cells as a biomarker for the diagnosis of female patients with HCC. Our results showed that Xist levels of both mononuclear cells and granulocytes were higher in female patients with HCC than that in controls. The difference of Xist levels between HCC patients and controls was not found in erythrocytes, possibly because of the deficiency of
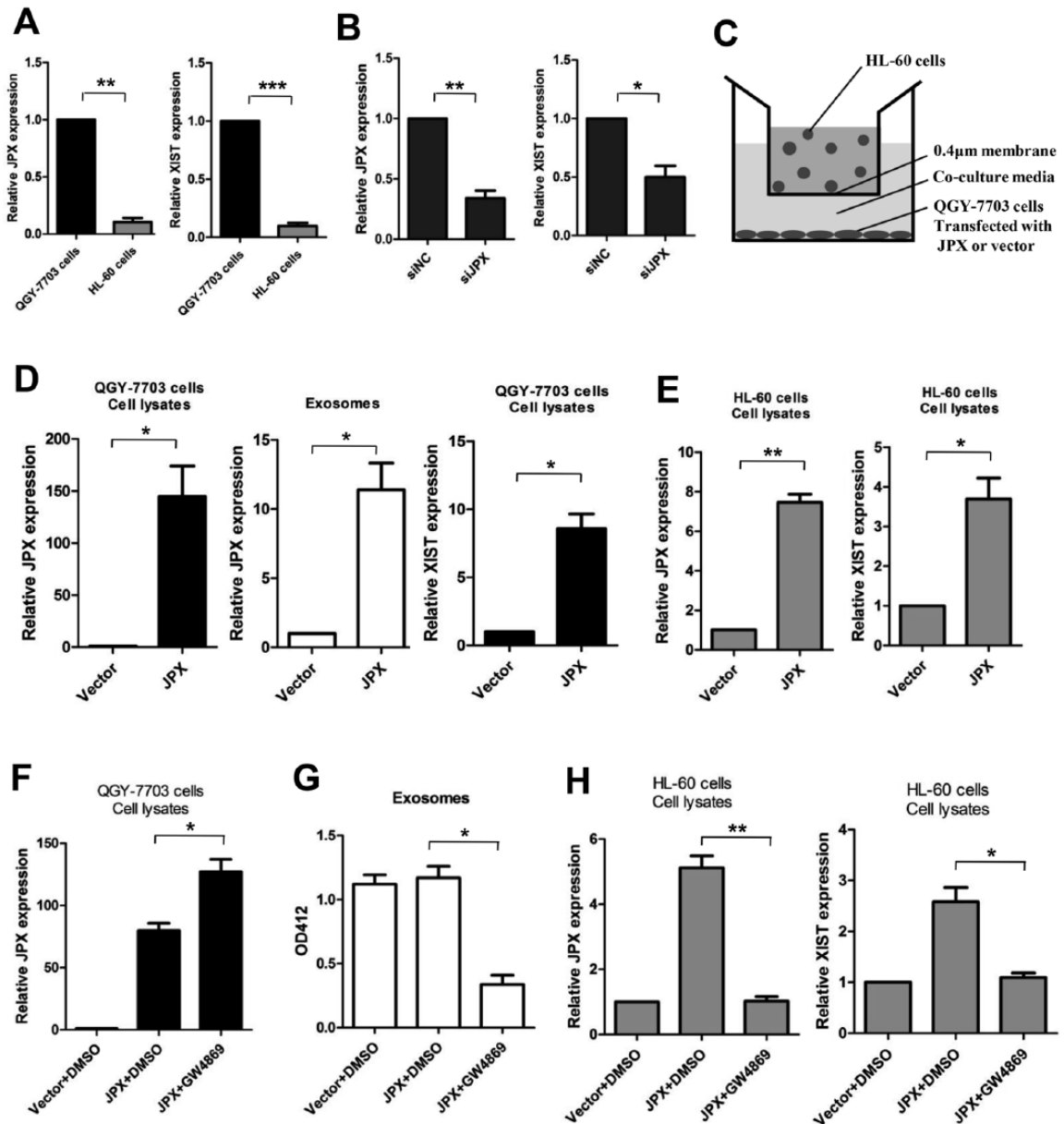
Figure 4. Jpx and X-inactive-specific transcript (Xist) expression of QGY-7703 and HL-60 cells in a coculture system.
(A) Relative Jpx and Xist expressions in QGY-7703 and HL-60 cells were determined by quantitative real-time polymerase chain reaction (qRT-PCR). (B) Relative Jpx and Xist expressions in QGY-7703 cells transfected with Jpx siRNA and NC siRNA. (C) Diagram of coculture system between HL-60 and QGY-7703 cells transfected with Jpx expression plasmid or empty vector. (D) Relative Jpx and Xist expressions in exosomes and cell lysates of co-cultured QGY-7703 cells transfected with Jpx expression plasmid or empty vector. (E) Relative Jpx and Xist expressions in cell lysates of cocultured HL-60 cells. (F) Relative Jpx expression in cell lysates of cocultured QGY-7703 cells with 10 μM GW4869 or dimethyl sulfoxide (DMSO) treatment. (G) Acetylcholinesterase activity of cocultured medium with 10 μM GW4869 or DMSO treatment. (H) Relative Jpx and Xist expressions in cocultured HL-60 cells with 10 μM GW4869 or DMSO treatment.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
siNC: NC siRNA; siJpx: Jpx siRNA.

organelles including the nucleus, mitochondria and endoplasmic reticulum in erythrocytes. In recent years, AFP has been one of the most common serum biomarkers for HCC diagnosis. Our results also showed that the diagnostic performance of Xist from either mononuclear cells or granulocytes in discriminating female patients with HCC from those with CHB and cirrhosis, or
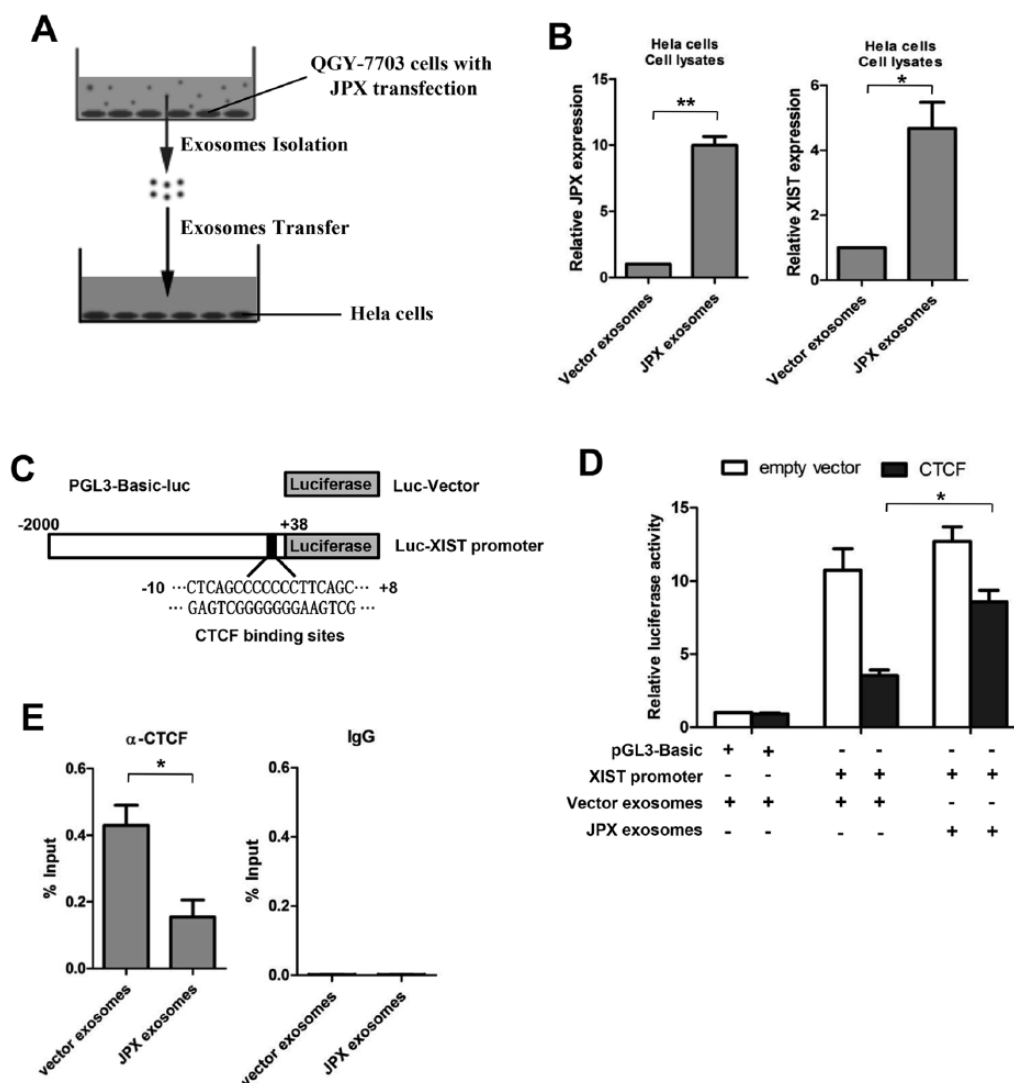
Figure 5. Effects of exosomal Jpx on X-inactive-specific transcript expression by evicting CTCF.
(A) Diagram of coculture between Hela cells and exosomes from QGY-7703 cells transfected with Jpx expression plasmid or empty vector. (B) Relative Jpx and X-inactive-specific transcript (Xist) expressions in Hela cells treated with exosomes from QGY-7703 cells transfected with Jpx expression plasmid or empty vector. (C) Luciferase reporter plasmids containing Xist promoter with CTCF binding sites were constructed. (D) Relative luciferase activities were analyzed after reporter plasmid and CTCF expression plasmid were transfected into Hela cells treated with the corresponding exosomes. (E) Chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction (qPCR) analysis was used to evaluate the effects of exosomal Jpx on CTCF binding at the Xist promoter. CTCF antibody (left) and normal rabbit immunoglobulin G (IgG) (right) were used. The data were normalized to the value of qPCR for Xist promoter amplification of Input DNA from the same cells.
$^*p < 0.05$; $^{**}p < 0.01$.

distinguishing female patients with early-stage HCC from controls, was better than that of AFP. All the results suggested that Xist levels in mononuclear cells and granulocytes might be useful biomarkers for diagnosis of female patients with HCC. At the same time, we should keep in mind that most of female patients with HCC were hepatitis B virus (HBV) infected and cirrhotic patients in the present study (Table S1). In our future study, we would collect the samples from HCC patients without HBV infection or cirrhosis, and investigate if the diagnostic efficacy of Xist in peripheral blood cells is associated with HBV or cirrhosis. On the other hand, HCC was commonly identified as a highly invasive and aggressive tumor, and recurrence remained one of the most prevalent causes leading to poor long-term survival of HCC patients after treatment.[18] In future, we would also conduct the long-term follow up of patients with HCC and explore whether

the Xist levels in peripheral blood cells would be a prognostic biomarker for female patients with HCC.

Combining the results that Xist expression of blood cells was downregulated after tumor resection for HCC patients (Figure 1H–I), we speculated that upregulation of Xist in blood cells might be related to HCC occurrence. Exosomes with a diameter of 30–150 nm were released from cells to the extracellular space and contained some molecules including protein, RNA and DNA, which could be transferred to and affect recipient cells.[19] Exosomes could play important roles in biological information exchange between cells. Considering the fact that exosomes could be detected in blood, urine and cerebrospinal fluid, cancer-related RNAs or proteins in exosomes might be novel biomarkers for cancer diagnosis.[20] In this study, we did not detect Xist expression in exosomes of female HVs and patients, which might partly be because of a high molecular weight of Xist (19 kilobases). Fortunately, we found that lncRNA Jpx, which was an activator of Xist, could be detected in exosomes of female HVs and patients. Moreover, our results also showed that exosomal Jpx levels in HCC patients was much higher than that in HVs and non-HCC patients, which exhibited a similar expression pattern of Xist in mononuclear cells and granulocytes. To investigate if the upregulation of Xist from peripheral blood cells was related to the increased expression of exosomal Jpx in female patients with HCC, we constructed a coculture system and found that exosomal Jpx from HCC cell line QGY-7703 could significantly increase the expression of Xist in myelocytic cell line HL-60. Then, the inhibitor of exosome release could abolish exosomal Jpx-induced upregulation of Xist in blood cells. These revealed a new exosome-mediated regulation of Xist expression in blood cells. In the future study, primary peripheral blood cells would be introduced into the coculture system and an animal model would be constructed to verify the conclusions.

The Jpx gene lies about 10 kilobases upstream of the Xist gene and is relatively conserved in its 5' region. A previous study showed that deleting the Jpx gene did not affect male cells but caused severe abnormalities in female cells,[21] which suggested that Jpx was also a sex-specific gene, like Xist. Jpx could interact with and inhibit the transregulatory function of CTCF, which was a transcription factor inhibiting Xist transcription. In the present study, mechanistic investigation showed that exosomal Jpx from HCC cells could promote Xist expression of blood cells by inhibiting the function of CTCF.

In conclusion, we have revealed for the first time that Xist levels of mononuclear cells and granulocytes were promising biomarkers for female patients with HCC, especially with early-stage HCC. Mechanism investigation indicated that exosomal Jpx from HCC cells could promote Xist expression of blood cells by evicting transcription factor CTCF. By lncRNA microarray screening, we should explore more sensitive and specific biomarkers in lncRNAs of blood cells for HCC diagnosis.

Conflict of interest statement
The authors declare no conflict of interest.

Supplemental material
Supplementary material is available online.

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