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ORIGINAL RESEARCH ARTICLE

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Circulating exosomal CPNE3 as a diagnostic and prognostic biomarker for colorectal cancer

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Exosomal proteins are emerging as relevant diagnostic and prognostic biomarkers for cancer. This study was aimed at illustrating the clinical significance of exosomal Copine III (CPNE3) purified from the plasma of colorectal cancer (CRC) patients. The CPNE3 expression levels in CRC tissues were analyzed by real-time PCR, western blot, and immunohistochemistry. Plasma exosomes were isolated to examine the CPNE3 level using ELISA. Pearson's correlation analysis was performed to investigate the CPNE3 levels between CRC tissues and matched plasma samples. Receiver operating characteristic curve analysis was developed to measure the diagnostic performance of exosomal CPNE3. The Kaplan–Meier method and Cox's proportional hazards model were utilized to determine statistical differences in survival times. CPNE3 showed increased expressions in the CRC tissues. A moderately significant correlation was found between CPNE3 expression in CRC tissues by immunohistochemistry and matched serum exosomal CPNE3 expression by ELISA ($r = 0.645$, ($r = 0.645$, $p < 0.001$). < 0.001). Exosomal CPNE3 yielded a sensitivity of 67.5% and a specificity of 84.4% in CRC at the cutoff value of 0.143 pg per 1ug1 ug exosome. Combined data from carcinoembryonic antigen and exosomal CPNE3 achieved 84.8% sensitivity and 81.2% specificity as a diagnostic tool. CRC patients with lower exosomal CPNE3 levels had substantially better disease-free survival (hazard ratio [HR], 2.9; 95% confidence interval [CI]: 1.3–6.4; $p = 0.009$) = 0.009) and overall survival (HR, 3.4; 95% CI: 1.2–9.9; $p = 0.026$) = 0.026) compared with those with higher exosomal CPNE3 levels. Exosomal CPNE3 show potential implications in CRC diagnosis and prognosis.

KEYWORDS
biomarker, colorectal cancer (CRC), Copine III (CPNE3), exosome

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent causes of cancer deaths worldwide (Siegel, Miller, & Jemal, 2018). Symptoms usually manifest in the late stages of disease progression, with the most common being rectal bleeding and changes in bowel habits and obstruction (Imperiale & Ransohoff, 2017). Early detection and diagnosis are essential to improve the prognosis of CRC patients. To

Abbreviations: CRC, colorectal cancer; CEA, carcinoembryonic antigen; DFS, disease-free survival; OS, overall survival; CPNE3, Copine III; NSCLC, non–small cell lung cancer; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis.

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date, colonoscopy examination coupled with pathologic biopsy remains the standard gold test. However, this test can be uncomfortable and is related to some risk of complications, including bleeding and colon perforation. Although carcinoembryonic antigen (CEA) is widely adopted as a diagnosis and monitoring biomarker, it is not sensitive enough for an early diagnosis (Garborg et al., 2013). The development of valid biomarkers for CRC diagnosis and prognosis evaluation remains an urgent, unmet medical need.

Exosomes are 40–150 nm membrane vesicles secreted into extracellular space by most cells (Kalluri, 2016). Exosomes are reported to enrich DNAs, RNAs, and proteins selectively from parent cells. Because they have cellular origins, exosome proteins can function as cancer predictive and prognostic biomarkers. The cell surface proteoglycan glypican-1 (GPC-1) has been found in serum exosomes from patients with pancreatic cancer and patients with breast cancer (Etayash, McGee, Kaur, & Thundat, 2016; Melo et al., 2015). The macrophage migration inhibitory factor was upregulated on exosomes harvested from the serum of patients with pancreatic ductal adenocarcinoma when compared with those from healthy control subjects (Costa-Silva et al., 2015). However, the clinical application of exosomal proteins in CRC has not been explored as thoroughly as it has been for other diseases.

Copine III (CPNE3) is a calcium-dependent membrane-binding protein (Creutz et al., 1998). It was reported to participate in several biological functions at the interface of the cell membrane and the cytoplasm. CPNE3 is increased in prostate, breast, and ovarian tumors (Heinrich et al., 2010). CPNE3 is located at focal adhesions and plays an essential role in ErbB2-dependent cell migration in breast cancer. CPNE3 may improve the invasion and metastasis of non-small-cell lung cancer (NSCLC), and it may act as an attractive therapeutic target for NSCLC metastasis (Lin et al., 2013). However, the potential of CPNE3 as a diagnostic and predictive biomarker in CRC has not been studied.

In this study, we found that CPNE3 mRNA and protein significantly elevated in CRC tissues as compared with corresponding noncancerous tissues. Plasma exosomal CPNE3 was further found upregulated in CRC patients in comparison with healthy controls by western blot. We thus detected the CPNE3 level in 124 plasma exosome samples from 92 CRC patients and 32 healthy controls by ELISA. The utility of exosomal CPNE3 as a diagnostic and prognostic biomarker was then analyzed.

2 | METHODS

2.1 | Patients and clinical information

This study was restricted to patients diagnosed with CRC and treated at the Fudan University-Affiliated Huashan Hospital. Ninety-two patients with pathologically proven CRC and 32 healthy controls were enrolled between March 2012 and February 2017. All participants signed an informed consent. Pathological and radiological examination provided evidence for CRC diagnosis. Data on clinical variables including sex, age, family and personal history, tumor grade, tumor size, lymph node invasion, distant metastases, tumor budding, and selected laboratory results were gathered. All patients recruited were sporadic. Tumor budding is assessed according to the recommendations of the International Tumor Budding Consensus Conference (ITBCC) 2016 (Lugli et al., 2017). Briefly, tumor budding is defined as a single tumor cell or foci of ≤4 tumor cells at the invasive front of the tumor. Cases with ≥10 buds in a 25× field were classified as high-grade budding, those with 5–9 buds were classified as intermediate-grade budding, and those with 0–4 buds were classified as low-grade budding. Treatments that patients received after enrollment were reviewed, and the patients were followed-up until February 28, 2017. Disease-free survival (DFS) was determined as the period from the initial surgery to documented disease recurrence. Overall survival (OS) was ascertained as the interval between the initial surgery and death or last contact. Follow-up data were available for all included patients. This study was approved by the Ethics Committee of Fudan University-Affiliated Huashan Hospital.

2.2 | Sample collection and exosome isolation

Peripheral venous blood samples were collected into citrate-treated tubes from healthy subjects and CRC patients before their undergoing invasive tests and therapeutic measures. Samples were centrifuged at 2,000*g* for 15 min at 4°C for the preparation of plasma samples. The resultant plasma was filtered through a 0.22-μm pore mesh, aliquoted in 0.5-ml tubes and stored at −80°C for subsequent exosome isolation.

For exosome isolation, 0.5-ml plasma aliquots were thawed and processed through different centrifugation and filtration steps at 4°C. First, plasma was centrifuged at 10,000*g* for 30 min to remove small cell debris and then ultracentrifuged at 100,000*g* for 75 min to pellet the exosome. Finally, the exosome was washed with phosphate-buffered saline (PBS) and pelleted again by ultracentrifugation at 100,000*g* for 75 min. The pellet of exosome was resuspended in 50 μl of PBS for subsequent use.

2.3 | Exosome identification

Exosome morphology was determined by transmission electron microscopy (TEM). Briefly, isolated exosomes were loaded onto a copper grid and stained with 1% phosphotungstic acid for 2 min at room temperature and observed with TEM (Hitachi HT7700, Japan). The size distribution of plasma exosomes was measured by nanoparticle tracking analysis (NTA).

2.4 | Real-time PCR

Total RNA was isolated from CRC tissues and the corresponding noncancerous tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and cDNA was synthesized from 2 μg of total RNA using an Access RT System (Promega, Madison, WI). Real-time PCR was conducted using the 7500 Real-time

PCR System (Life Technologies, Carlsbad, CA). The primer sequences were: forward 5′-GTCAGACCCTTTATGTGTGTTGT-3′; reverse 5′-TGG AAAATTGGGGATTCAAGCAA-3′. Gene expression was normalized to GAPDH. The comparative quantification was carried out by the $2^{-\Delta\Delta Ct}$ method. Each sample was tested three times.

2.5 | Western blot

Exosomes were prepared with RIPA buffer supplemented with Complete Protease Inhibitor Mixture tablets (Roche Diagnostics, Mannheim, Germany). Protein concentration was estimated using the BCA Protein Assay Kit (Pierce, Waltham, MA). Protein prepared from exosomes and the exosome-depleted supernatant were loaded onto SDS–polyacrylamide gel (10%) and processed with electrophoresis. The protein was then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk for 1 hr and probed overnight at 4°C with different primary antibodies including an anti-CD9 antibody (Abcam, Cambridge, MA, ab92726, 1:2,000), an anti-CD81 antibody (Abcam, ab109201, 1:5,000) and an anti-CPNE3 antibody (Proteintech, Rosemont, IL, 11186-1-AP, 1:1,000), respectively. The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (Abcam, ab6721, 1:5,000). The ECL Western Blotting Reagent (Piece) was used for visualization.

2.6 | Immunohistochemistry

Immunohistochemistry was performed in 35 pairs of CRC tissues and adjacent noncancerous tissues. The clinical variables of the 35 CRC specimens are shown in Table 1. Tissue paraffin sections were deparaffinized, rehydrated, and pre-treated with 10 mM sodium citrate buffer at a sub-boiling temperature for 10 min to unmask the antigen. The sections were subsequently incubated with 3% H_2O_2 for 10 min at room temperature and dark conditions to block endogenous peroxidase activity, followed by incubation with a blocking solution for 1 hr to avoid unspecific binding of the primary antibody. The sections were then incubated overnight at 4°C with the anti-CPNE3 antibody (Proteintech, 11186-1-AP, 1:100) followed by incubation for 30 min at room temperature with a biotinylated ECL anti-rabbit IgG (GE Healthcare, Chalfont St. Giles, UK). The color was developed using the diaminobenzidine substrate (Roche Diagnostics), and the sections were counterstained with hematoxylin. Two pathologists examined the CPNE3 immunostaining. The CPNE3 immunostaining intensity was scaled as 0 (absent staining), 1 (low staining), 2 (mediate staining), and 3 (strong staining). The percentage of immunoreactive cells was designated as 1 (0%–25%), 2 (26%–50%), 3 (51%–75%) and 4 (76%–100%). The final score was then obtained by multiplying the intensity and reactivity extension values (range, 0–12).

2.7 | Quantification of exosomal CPNE3

The protein concentration of lysed exosomes was estimated by the BCA Protein Assay Kit (Pierce). The CPNE3 level was quantified using a commercially available ELISA according to the manufacturer's instructions (YX-031614H, SinoBestBio, Shanghai, China). The absorbance at 540 nm was determined using a microplate reader. The assay was performed in triplicate. The resultant exosomal CPNE3 expression was defined as the CPNE3 protein content per ug exosome.

TABLE 1 Clinical characteristics

Total number of tissues	35
Age	
<65	19
≥65	16
Gender	
Male	26
Female	9
Location	
Right hemicolon	13
Left hemicolon	11
Rectum	11
Grade	
Well or moderate	27
Poor	8
Pathologic stage	
1–2	11
3–4	24
T stage	
1–2	7
3–4	28
N stage	
0	12
1–2	23
Distant metastasis	
0 (No)	25
1 (Yes)	10
Tumor budding	
Low or intermediate grade	23
High grade	12
CEA	
≤5	14
>5	21

Note. CEA: carcinoembryonic antigen.

2.8 | Statistical analysis

Statistical analyses were performed using the SPSS Statistics 22.0 (SPSS, Chicago, IL). Correlations of exosomal CPNE3 with clinicopathological characteristics were examined using the chi-square test. Pearson's correlation analysis was performed to compare the CPNE3 levels between CRC tissues and matched plasma samples. Receiver operating characteristics (ROC) curves were conducted to determine the performance of exosomal CPNE3 in a CRC screening test. DFS and OS were analyzed by using the Kaplan–Meier method and compared via the log-rank test. Cox's proportional hazards model was utilized to identify factors influencing DFS and OS. Factors significantly associated with DFS and OS on univariate analysis ($p < 0.05$) were subjected to the multivariate models. All statistical tests were two-sided. p-values less than 0.05 were considered significant.

3 | RESULTS

3.1 | CPNE3 increased in CRC tissues

Real-time PCR analysis demonstrated that CPNE3 was significantly elevated in cancer tissues compared with adjacent noncancerous tissues (Figure 1a). The relative abundance of CPNE3 mRNA was >two fold in all examined cancer samples as compared with matched noncancerous samples (Figure 1b). Western blot illustrated that CPNE3 protein was upregulated in eight examined cancer tissues compared with matched noncancerous tissues (Figure 1c). The CPNE3 expression was further detected by immunohistochemistry in 35 pairs of paraffin-embedded specimens of CRC and adjacent noncancerous tissues. CPNE3 protein was determined in 32 of 35 (91.4%) cases of cancer tissues (Figure 1d), whereas there was no or low signal intensity in adjacent noncancerous tissues in all samples examined.

3.2 | Characteristics of plasma exosomes

TEM, NTA, and western blot were utilized for the analysis of the characteristics of plasma exosomes. TEM analysis exhibited round-shaped structures that ranged in size from 30 nm to 150 nm (Figure 2a). NTA showed that the average size of plasma exosomes was around 100 nm (Figure 2b). In addition, the specific exosomal protein markers CD9 and CD81 were detected from the plasma exosome by western blot (Figure 2c). These results indicated the successful isolation of plasma exosomes.

3.3 | Exosomal CPNE3 levels in CRC patients versus healthy controls

We performed western blot to detect the plasma exosomal CPNE3 level from CRC patients and healthy controls. Exosomal CPNE3 was significantly elevated in CRC patients as compared with healthy controls ($n = 8$; Figure 3a,b). Furthermore, the CPNE3 expression in plasma exosomes was higher in Stage III-IV patients than that in Stage I-II patients ($n = 8$).

To better understand the expression pattern of CPNE3 in plasma exosome, ELISA was performed in 92 patients with CRC and 32 healthy controls. Spearman's correlation analysis was conducted to determine the correlation of CPNE3 expression

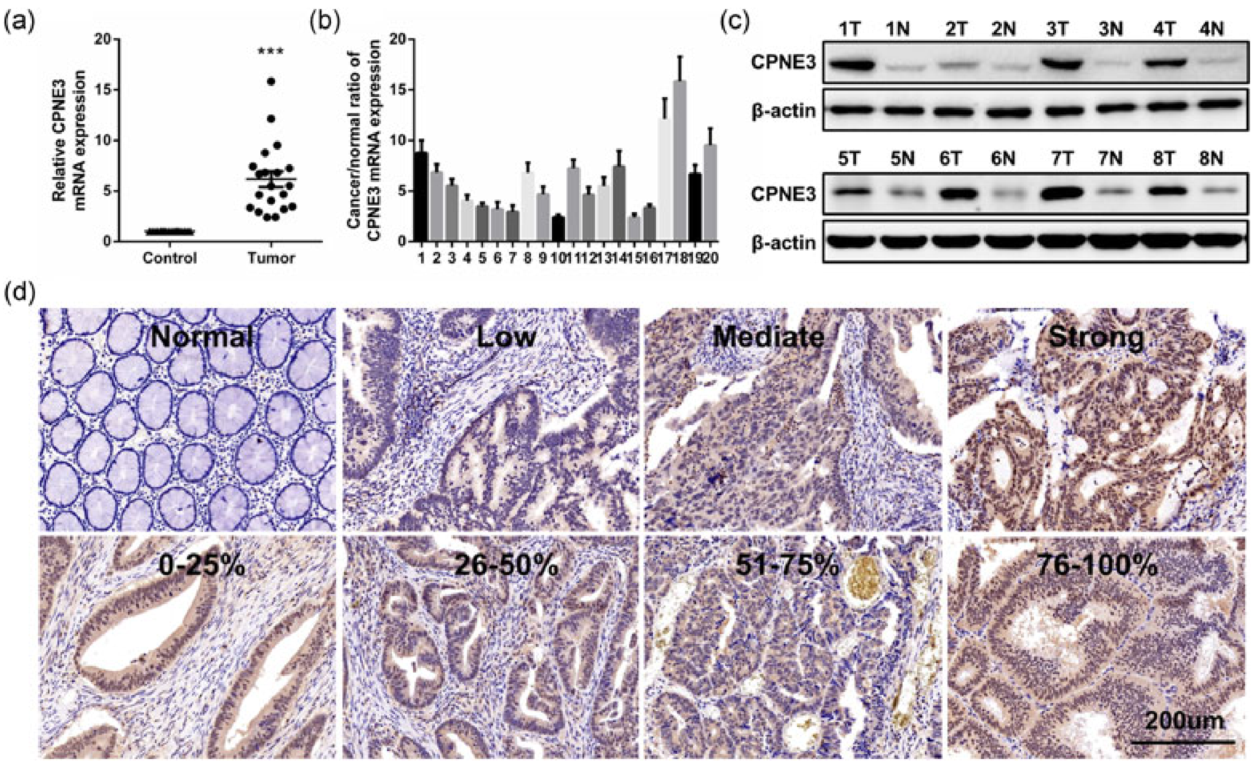

FIGURE 1 CPNE3 expression increased in CRC. (a,b) CPNE3 mRNA level markedly increased in cancer tissues compared with that in paired non-cancerous tissues by real time-PCR. (c) Western blot showed that the CPNE3 protein level increased dramatically in eight examined CRC tissues compared with non-cancerous tissues; β-actin was the loading control. (d) Examples of CRC tissues and para-carcinoma tissues immunostained for CPNE3. Various percentages of CPNE3-positive cells and different CPNE3 staining intensities were exemplified. CPNE3: Copine III; CRC: colorectal cancer [Color figure can be viewed at wileyonlinelibrary.com]

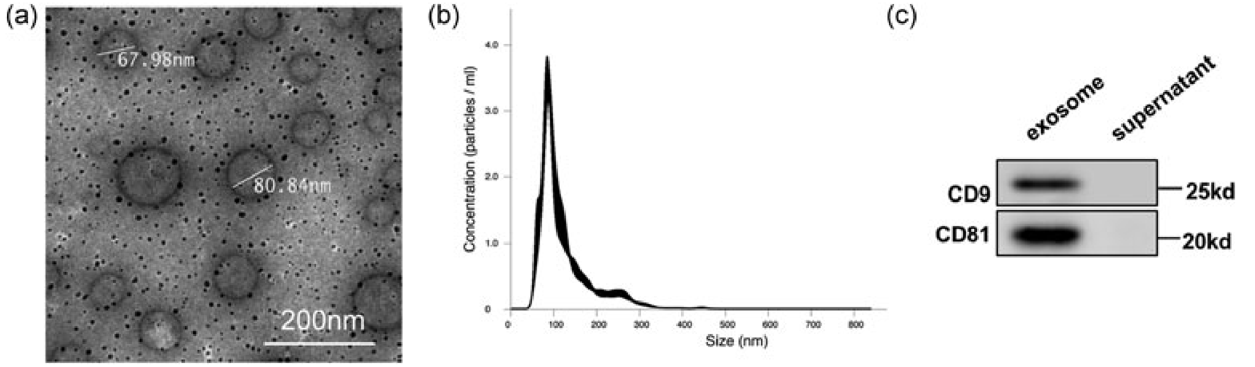

FIGURE 2 Characteristics of plasma exosomes. (a) TEM of exosomes showed round-shaped structures with diameters between 30 and 150 nm. (b) The average size of exosomes measured by NTA was estimated to be around 100 nm. (C) Western blot analysis on the plasma exosomal biomarkers CD9 and CD81. NTA: nanoparticle tracking analysis; TEM: transmission electron microscopy

level between CRC tissue samples by immunohistochemistry and matched plasma exosomal CPNE3 expression by ELISA. As shown in Figure 3c, a moderatey significant correlation was found between CPNE3 expression in CRC tissues and matched plasma exosome ($r = 0.645$, $p < 0.001$). These results demonstrated that plasma exosomal CPNE3 reflected the CPNE3 expression level in CRC tissues.

We further analyzed the exosomal CPNE3 expression level in different groups. Higher exosomal CPNE3 levels were observed in patients with CRC compared with healthy controls ($p < 0.001$; Figure 3d). Besides, more significantly elevated levels of plasma exosomal CPNE3 were found in patients with CRC in advanced stages ($p < 0.001$; Figure 3e). Moreover, there were much higher levels of exosomal CPNE3 in plasma samples from patients with CRC with distant metastasis than those without metastasis ($p < 0.001$; Figure 3f). Taken together, these results indicate that the exosomal CPNE3 levels were increased in patients with CRC, particularly those at later TNM stages or with distant metastasis.

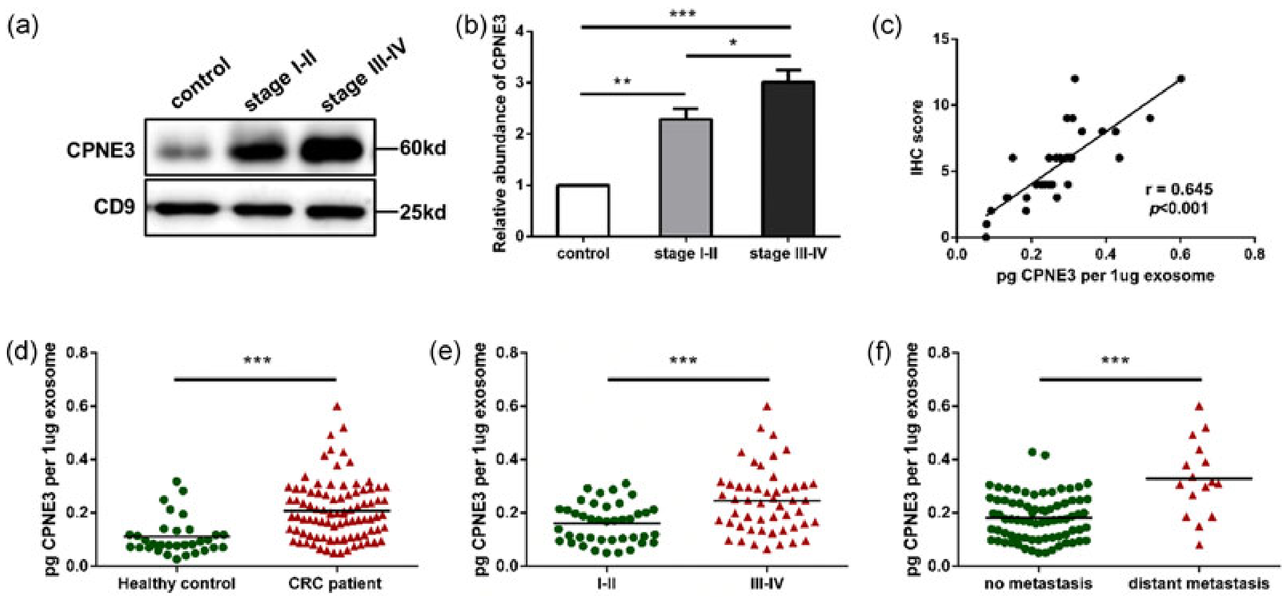

FIGURE 3 Plasma exosomal CPNE3 levels in patients with CRC versus healthy controls. (a,b) CPNE3 levels in plasma exosomes isolated from healthy controls (control), Stage I-II CRC patients (Stage I-II), and Stage III-IV CRC patients as determined by western blot; CD63 served as the internal control of exosomes ($n = 8$). The data are presented as mean ± standard error of the mean. (c) Correlation between a histological score of the CPNE3 expression in CRC tissue samples and the matched plasma exosomal CPNE3 expression level detected by ELISA. (d) The exosomal CPNE3 levels were significantly higher in CRC patients than those in healthy controls. (e) Exosomal CPNE3 levels were significantly higher in Stage III-IV patients compared with those in Stage I-II patients. (f) Exosomal CPNE3 levels were significantly higher in patients with distant metastasis than those in patients without metastasis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by the Student t test. CPNE3: Copine III; CRC: colorectal cancer [Color figure can be viewed at wileyonlinelibrary.com]

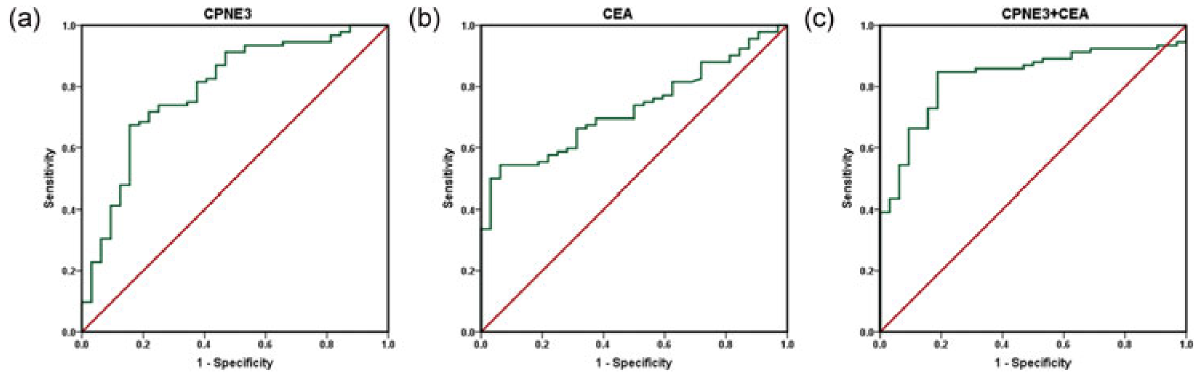

FIGURE 4 Diagnostic power of exosomal CPNE3, CEA, and exosomal CPNE3 plus CEA. (a) The area under the ROC curve was up to 0.791 (95% CI = 0.698–0.885, $p < 0.001$) for exosomal CPNE3. The optimal cutoff value was 0.143 pg CPNE3 per 1 ug exosome, yielding a sensitivity of 67.4% and specificity of 84.4%. (b) The area under the ROC curve was up to 0.728 (95% CI = 0.640–0.816, $p < 0.001$) for CEA. The optimal cutoff value was 5.0 ng/ml, yielding a sensitivity of 54.3% and a specificity of 93.7%. (c) The area under the ROC curve was up to 0.833 (95% CI = 0.758–0.907, $p < 0.001$) for exosomal CPNE3 plus CEA. CEA: carcinoembryonic antigen; CPNE3: Copine III; ROC: receiver operating characteristics [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Exosomal CPNE3 enhances the diagnostic power of CEA

We further built ROC curves to evaluate the utility of exosomal CPNE3 level as a diagnostic tool for CRC (Figure 4a). The area under the ROC curve reached 0.791 (95% confidence interval [CI] = 0.698–0.885, $p < 0.001$). Youden's index was then utilized to calculate the most appropriate cutoff value with maximum sensitivity and specificity; this value was 0.143 pg/μg plasma exosome, providing a sensitivity of 67.4% and specificity of 84.4%. A conventional tumor marker, CEA, was detected and compared with exosomal CPNE3 for diagnostic power. The AUC for CEA assay was 0.728 (95% CI = 0.640–0.816, $p < 0.001$), and the most appropriate cut-off value was 5.0 ng/ml, yielding a sensitivity of 54.3% and a specificity of 93.7% (Figure 4b). The results indicated that exosomal CPNE3 was better than CEA in distinguishing CRC from healthy controls.

We further combined CEA and exosomal CPNE3 with binary logistic regression to improve the diagnostic accuracy for CRC. ROC analysis illustrated that the AUC for the combined detection reached 0.833 (95% CI = 0.758–0.907, $p < 0.001$), which was superior to exosomal CPNE3 or CEA alone (Figure 4c).

3.5 | Exosomal CPNE3 levels and tumor characteristics

We analyzed the relationships between exosomal the CPNE3 levels and the clinicopathologic parameters (Table 1). Exosomal levels were associated with higher tumor size ($p = 0.002$), lymph node invasion ($p = 0.018$), distant metastasis ($p = 0.013$), advanced TNM stage ($p = 0.012$) and high-grade budding ($p = 0.031$). These results illustrated that higher exosomal CPNE3 levels are correlated with greater tumor burden. In addition, exosomal CPNE3 levels were associated with elevated CEA levels ($p = 0.007$).

3.6 | Factors associated with DFS and OS

The relationships between plasma exosomal CPNE3 and DFS are shown in Figure 5a. The median DFS rates were 29.4 and 21.8 months in patients with <0.143 pg CPNE3 per 1 ug exosome and ≥0.143 pg CPNE3 per 1 ug exosome, respectively ($p = 0.044$). The higher exosomal CPNE3 level was associated with poorer DFS on univariate analysis (HR: 2.9, 95% CI: 1.3–6.4, $p = 0.009$; Figure 5a and Table 2). Furthermore, higher T staging, lymph node invasion,

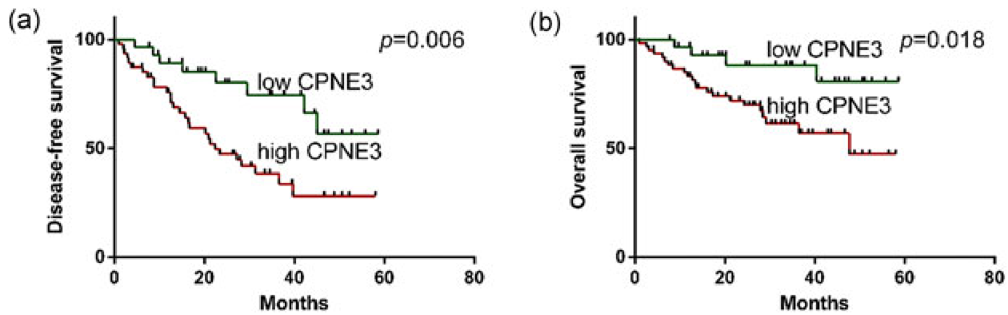

FIGURE 5 (a) Kaplan–Meier estimates of disease-free survival according to exosomal CPNE3 in patients with CRC. (b) Kaplan–Meier estimates of overall survival according to exosomal CPNE3 in patients CRC. CPNE3: Copine III; CRC: colorectal cancer [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Clinical characteristics

Characteristics	Exosomal CPNE3 Low	High	p
Age			
<65	16	30	
≥65	14	32	0.656
Gender			
Male	19	42	
Female	11	20	0.675
Grade			
Well or moderate	26	49	
Poor	4	13	0.376
Pathologic stage			
1–2	19	22	
3–4	11	40	0.012
T stage			
1–2	13	9	
3–4	17	53	0.002
N stage			
0	19	23	
1–2	11	39	0.018
Distant metastasis			
0 (No)	29	47	
1 (Yes)	1	15	0.013
CEA			
≤5	40	31	
>5	17	36	0.007

Note. CEA: carcinoembryonic antigen; CPNE3: Copine III.

higher TNM staging, and high-grade budding predicted a significantly poorer DFS rate. All variables that showed a significant correlation on univariate analysis were subjected to Cox regression analysis. Independent predictors of DFS were lymph node invasion (HR: 2.1, 95% CI: 1.1–4.4, $p = 0.036$) and higher exosomal CPNE3 level (HR: 2.5, 95% CI: 1.1–5.5, $p = 0.029$).

The relationships between plasma exosomal CPNE3 and OS are shown in Figure 5b.The median OS rates were 32.0 and 24.7 months in patients with <0.143 pg CPNE3 per 1 ug exosome and ≥0.143 pg CPNE3 per 1 ug exosome, respectively ($p = 0.033$). The higher exosomal CPNE3 level was associated with poorer OS on univariate analysis (HR: 3.4, 95% CI: 1.2–9.9, $p = 0.026$; Figure 5b and Table 3). Demographic features, including age, sex, and tumor differentiation, were not associated with poorer OS. Tumor-related factors including higher T staging, lymph node invasion, distant metastasis, higher TNM staging, high-grade budding, and CEA > 5 ng/ml were significantly associated with reduced OS. On multivariate analysis, the independent predictors of OS were distant metastasis (HR: 2.6, 95% CI: 1.1–6.5, $p = 0.034$) and higher exosomal CPNE3 level (HR: 3.0, 95% CI: 1.0–8.9, $p = 0.047$) (Table 4).

4 | DISCUSSION

In this study, CPNE3 expression was found significantly elevated in CRC tissues as compared with the corresponding normal tissues. Plasma exosomal CPNE3 was more enriched in CRC patients as compared with healthy controls. ROC curve demonstrated its potential value in CRC diagnosis. Furthermore, exosomal CPNE3 might enhance the diagnostic power of CEA for CRC. Moreover, exosomal CPNE3 may be a possible prognostic factor for CRC patients regarding both DFS and OS.

Exosomes carry a variety of DNA, RNA, and proteins, reflecting the genetic and signaling changes in maternal cells. Due to their stability and specificity in most bodily fluids, exosomal proteins provide a high potential to serve as a liquid biopsy tool for some cancers (Thery, 2015). Proteomic analyses of exosomal proteins had been applied to identify potential biomarkers for many cancer types, including cancers of colon, pancreas, breast, prostate and ovarian. Ge et al. performed proteomic analyses to clarify the protein content in serum-purified exosomes from CRC patients and identified some proteins as potential biomarkers (Chen et al., 2017). Banales et al. studied proteome

TABLE 3 Factors affecting DFS in CRC patients

Variable	Univariate analysis HR (95% CI)	p value	Multivariate analysis HR (95%CI)	p value
Age (≥65)	1.3 (0.7–2.6)	0.385	—	—
Gender (male)	1.8 (0.8–3.7)	0.137	—	—
Poor differentiation	2.0 (0.9–4.3)	0.068	—	—
T staging	2.8 (1.2–6.4)	0.02	—	—
Lymph node invasion	2.6 (1.3–5.3)	0.008	2.1 (1.1–4.4)	0.036
Higher TNM staging	2.6 (1.3–5.3)	0.008	—	—
Higher CEA level	1.9 (1.0–3.6)	0.067	—	—
Higher exosomal CPNE3	2.9 (1.3–6.4)	0.009	2.5 (1.1–5.5)	0.029

Note. CEA: carcinoembryonic antigen; CI: confidence interval; CPNE3: Copine III; CRC: colorectal cancer; DFS: disease-free survival; HR: hazard ratio.

TABLE 4 Factors affecting OS in CRC patients

Variable	Univariate analysis HR (95% CI)	*p* value	Multivariate analysis HR (95% CI)	*p* value
Age (≥65)	1.7 (0.8–3.7)	0.206	—	—
Gender (male)	2.1 (0.8–5.2)	0.117	—	—
Poor differentiation	2.2 (1.0–5.2)	0.058	—	—
T staging	1.7 (0.7–4.3)	0.274	—	—
Lymph node invasion	2.7 (1.2–6.4)	0.022	—	—
Distant metastasis	3.2 (1.3–7.7)	0.011	2.6 (1.1–6.5)	0.034
Higher TNM staging	2.6 (1.1–6.2)	0.028	—	—
Higher CEA level	2.4 (1.0–5.5)	0.042	—	—
Higher exosomal CPNE3	3.4 (1.2–9.9)	0.026	3.0 (1.0–8.9)	0.047

Note. CEA: carcinoembryonic antigen; CI: confidence interval; CPNE3: Copine III; CRC: colorectal cancer; OS, overall survival.

profiles based on mass spectrometry to reveal multiple differentially expressed proteins among cholangiocarcinoma and control (Arbelaiz et al., 2017). Proteomic signatures found in serum extracellular vesicles showed potential usefulness as diagnostic tools for cholangiocarcinoma. Urinary exosomes are easily accessible and have great potential to be used as biomarkers (Merchant, Rood, Deegens, & Klein, 2017). Quantitative proteomics of urinary exosomes led to the identification of promising biomarkers for prostate and bladder cancer (Overbye et al., 2015; Welton et al., 2010). Beak et al. demonstrated that developmental endothelial locus-1 protein (Del-1) was a potential biomarker for breast cancer by using the ELISA method (Moon et al., 2016). Khan et al. (2014), (2012) showed that exosomal Survivin was elevated in the plasma of prostate cancer patients and breast cancer patients by ELISA and western blot analysis. Exosomes isolated by ultracentrifugation from ovarian cancer patients' plasma carried TGF-β1 and MAGE 3/6, but not in exosomes from patients with benign tumors (Szajnik et al., 2013). In our study, western blot and ELISA were conducted to evaluate the exosomal CPNE3 amount from plasma. Exosomal CPNE3 was significantly elevated in CRC plasma, indicating that exosomal CPNE3 has potential as a diagnostic marker for CRC. To enhance the diagnosis quality of exosomal CPNE3, we combined exosomal CPNE3 with CEA for CRC diagnosis and found an improved diagnostic accuracy with AUC = 0.833.

The prognostic significance of exosomal proteins has been widely investigated in tumors. Bromberg et al. identified melanoma exosome proteins and found TYRP2, VLA-4, HSP70 and an HSP90 isoform as exosome-specific biomarkers with prognostic and therapeutic potential (Peinado et al., 2012). Wang et al. evaluated small RNAs circulating in the blood and confirmed that plasma exosomal miR-1290 and miR-375 had potential prognostic value for castration-resistant prostate cancer (Huang et al., 2015). Ozaki et al. identified 236 serum exosomal miRNAs through global miRNA profiling and ascertained circulating miR-25-3p as a valid biomarker for cancer surveillance and prognosis judgment in osteosarcoma patients (Fujiwara et al., 2017). Lahav et al. found that human telomerase reverse transcriptase (hTERT) mRNA levels in serum exosome were correlated with tumor burden and may serve as follow-up markers in different malignancies (Goldvaser et al., 2017). Mimori et al. purified miRNAs from serum exosome obtained from CRC patients with and without recurrence and validated exosomal miRNAs by quantitative real-time RT-PCR. Serum-based circulating miR-19a may serve as a non-invasive blood-based biomarker for CRC recurrence in patients (Matsumura et al., 2015). In our study, we showed that exosomal CPNE3 was associated with tumor burden. It has good discriminatory power for predicting the prognoses of CRC patients and thus may represent an ideal target for anticancer therapy.

There are several limitations to our study. We isolated plasma exosomes using differential ultracentrifugation in this study. Although it is a widely adopted method for exosome isolation, density gradient ultracentrifugation has a host of problems, including time-consuming and low recovery yield.

Consistent and dependable methods are desperately needed to isolate pure exosome population for exosome diagnostics. The current research is a retrospective analysis of patients with CRC. A prospective study will help in future to assess the clinical value of exosomal CPNE3. What's more, this study did not recruit participants with precancerous lesions and polyps. A further study is needed to investigate the role of exosomal CPNE3 in early stages of colorectal tumorigenesis and its potential utility as a cancer risk marker. As we did not draw blood from patients after treatment, we did not determine the impact of surgery and chemotherapy on exosomal CPNE3 expression levels in plasma. Although the diagnostic and predictive potential of plasma exosomal CPNE3 is well documented, further research is needed to clarify the particular role of CPNE3 in exosome and its potential therapeutic regulatory value. Furthermore, future efforts that combine exosomal RNA and DNA and immunoaffinity capture will aid in the promotion of exosome utilization for cancer diagnosis and prognosis.

5 | CONCLUSIONS

In summary, we report for the first time the diagnostic and prognostic relevance of exosomal CPNE3 in plasma as a protein marker for CRC. Exosomal CPNE3 levels are associated with the tumor extent and may serve as a diagnostic biomarker in CRC patients. Combining measurement of exosomal CPNE3 in plasma with CEA may further improve the diagnostic accuracy. The high exosomal CPNE3 level was a reliable indicator for worse DFS and OS. Further investigated is warranted on the role of exosomal CPNE3 in identifying therapeutic targets and determining follow-up treatments.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted with the support of the Ethics Committee of Fudan University-Affiliated Huashan Hospital.

CONSENT FOR PUBLICATION

A written informed consent was obtained from all subjects.

AVAILABILITY OF DATA AND MATERIAL

The datasets analyzed during the current study are available from the corresponding author on request.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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