



Different primers for diagnosing circulating cell-free DNA of colorectal cancer

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Background: Cell-free DNA (cfDNA) primers have been designed to screen for cancer diagnosis, but the results have been inconsistent. The study aimed to compare different primers for diagnosing cfDNA of colorectal cancer (CRC).

Methods: Peripheral blood specimens were collected from 71 patients with CRC and 20 patients with proliferative intestinal polyps. Quantitative polymerase chain reaction (q-PCR) was used to detect the concentration of cfDNA of these primers, including ALU elements (ALU), Beta-actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The receiver operator characteristic curve (ROC) was performed to analyze the diagnostic value.

Results: The concentration of cfDNA in plasma of the CRC group determined by primers ALU and GAPDH was significantly higher than that of the intestinal polyp group ($P < 0.05$). There was no significant difference in cfDNA level determined by primer ACTB between the CRC group and the intestinal polyp group ($P > 0.05$). The area under curves (AUCs) of ALU, GAPDH, and ACTB were 0.734, 0.800, and 0.503, respectively.

Conclusions: The GAPDH and ALU in plasma are expected to be sensitive indicators for the diagnosis of CRC.

Keywords: Colorectal cancer (CRC); cell-free DNA (cfDNA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); ALU; prognosis

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Introduction

Colorectal cancer (CRC) is currently the fourth most common cancer (1). Five-year survival for stages I and II is 90%, which drops to 14% for stage IV disease (2). Therefore, early detection and treatment are significant for the management of CRC. Many diagnostic methods have been used to detect cancer, such as protein tumor markers, imaging techniques, and tissue biopsy (3). Serum protein

biomarkers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) have limited sensitivity and specificity (4,5). Imaging techniques are challenged by small lesions and may be influenced by structures. Tissue biopsy, a gold standard for diagnosing cancer, has disadvantages from invasion and medical complications.

Liquid biopsy has advantages such as easy accessibility, minimal invasiveness, and repeatability (6). Cell-free DNA (cfDNA), one of the markers of liquid biopsies, has

been applied in all phases of cancer patient management, including predictive and early detection, monitoring disease progression, and survival recurrence and minimal residual disease (7). The cfDNA is released by viable and necrosis cancer cells and may reflect the entire tumor genome. Molecular characteristics of cfDNA have been extensively studied, including concentration, genomic alterations, copy number mutations, methylation changes, single-nucleotide mutations, cancer-derived viral sequences, and microsatellite alterations (8,9).

The concentration of cfDNA has been identified as a potential prognostic biomarkers in CRC. The concentration of cfDNA based on different primers has been found to be significantly higher in CRC than in healthy volunteers and intestinal polyp patients (10-13). However, different primers in CRC have not yet been compared. We presented a comparison study in plasma samples to monitor the variation and efficiency of cfDNA primers.

Methods

Sample collection

Seventy-one patients with CRC and 20 patients with proliferative intestinal polyps at Yongchuan Hospital of Chongqing Medical University from May 2018 to February 2019 were enrolled. The CRC group consisted of 45 non-metastatic colorectal cancer (non-mCRC) patients, 13 patients with metastatic colorectal cancer (mCRC), and 13 patients with recurrent colorectal cancer (rCRC). The diagnosis of all patients was confirmed by biopsy, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) scans. All peripheral venous blood samples were collected in EDTA-K₂-coated tubes and separated by centrifugation at 1,600 g for 10 min at 4 °C (Neofuge 15R, Heal Force). The supernatant was transferred to a new tube and centrifuged again at 16,000 g for 10 min at 4 °C (Neofuge 13R, Heal Force). Purified plasma was carefully removed without disturbing the lower residual layer and stored at -80 °C for further analyses (14). Plasma cfDNA was extracted from 400 µL blood plasma using the plasma cfDNA extraction kit (GO-MXCF-100, Changchun China, <http://www.geneonbio.com/>) according to the manufacturer's protocol. The total eluted cfDNA (~40 µL) was transferred into 0.2 mL Eppendorf tubes and then stored at -20 °C.

Quantitative polymerase chain reaction (q-PCR)

Three primer sequences were used to compare the efficiency of different genes: ALU elements (ALU), Beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously published and used to quantify the level of cfDNA. Three fragments of 108 bp of the GAPDH gene, 115 bp of the ALU gene, and 100 bp of the ACTB gene were amplified with the following primers to quantify the yield of cfDNA. The ALU primer forward: 5'-CCTGAGGTCAGGAGTTCGAG-3' and reverse: 5'-CCCAGTAGCTGGGATTACA-3' (11); The ACTB primer forward: 5'-GCACCACACCTTCTACAATGA-3' and reverse: 5'-GTCATCTTCTCGCGGTTGGC-3' (15). The GAPDH primer forward: 5'-ATGTTTCGTCATGGG TGTGAA-3' and reverse: 5'-GGTGCTAAGCAGTTG GTGGT-3' (13). The total q-PCR reaction volume of 25 µL contained 0.4 µM of forward and reversed primers, 12.5 µL of TB Green[®] Premix Ex Taq[™] II (Tli RNaseH Plus) (Takara, Japan), 8.5 µL double-distilled water and 2 µL of cfDNA. The subsequent progress involved incubation at 95 °C for 30 s, 39 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and ended with a melt curve from 65 to 95 °C for 5 s in Real-time Quantitative PCR Detecting System (Bio-Rad CFX96, USA). The absolute equivalent amount of cfDNA in each sample was determined using a standard curve with serial dilutions (from 9.813 to 98,130 ng/mL) of human genomic DNA. Standard curves were created for ALU, ACTB, and GAPDH primer sets by PCR amplifying 10-fold serially diluted DNA samples. The concentration of cfDNA for different primers was represented by the concentration of ALU, ACTB, and GAPDH, respectively. The results were presented as the median and interquartile range.

CEA analysis

Venous blood (5 mL) was collected from the patients simultaneously. The concentrations of CEA were measured at the Clinic Pathology Laboratory of Yongchuan Hospital affiliated to Chongqing Medical University by Elecsys. COBAS (e601 Germany) was adopted to calculate their quantity.

Receiver-operating characteristic (ROC) curve analysis

The ROC curve was used to assess the diagnostic value

Table 1 The concentration of cfDNA in intestinal polyp and CRC

Groups	Intestinal polyp	CRC	Z	P
ALU			-2.983	0.003
n	18	65		
C (ng/mL)	13.03 (0.01, 120.43)	97.51 (20.48, 412.70)		
ACTB			-0.246	0.806
n	12	67		
C (ng/mL)	364.49 (143.97, 2,749.28)	484.33 (162.79, 2,390.97)		
GAPDH			-3.757	0.000
n	20	71		
C (ng/mL)	79.54 (27.83, 115.15)	168.63 (113.68, 270.76)		

cfDNA, cell-free DNA; CRC, colorectal cancer.

of ALU, ACTB, GAPDH, CEA and the combination diagnostic for non-mCRC.

Statistical analysis

The Mann-Whitney U-test and Kruskal-Wallis H-test were used to compare the ALU, ACTB, and GAPDH between the groups. Statistical analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant at $P < 0.05$. The corresponding figures were drawn in GraphPad Prism v 8.01 software (Graphpad Software Inc.).

Results

Patient characteristics

A total of 91 individuals were enrolled. The data obtained from plasma samples of 71 CRC patients (male/female: 35/36) were compared to 20 patients with intestinal polyps (male/female: 11/9) as controls. For controls and CRC patients, the median of age was 55.00 (range, 44.75–64.00) and 66.00 (range, 61.00–71.00) years, respectively. The patients did not differ statistically by age and gender, as verified by independent χ^2 and t -tests ($P > 0.05$ for both). The abnormal value was excluded in further analysis.

The cfDNA concentration in intestinal polyp and CRC patients

The concentration of ALU and GAPDH in the CRC group

was significantly higher than in the intestinal polyp group ($P = 0.003$ and $P = 0.000$, respectively). However, there was no difference in ACTB ($P = 0.806$) (Table 1; Figure 1A,B,C). Compared with the control group, the concentration of ALU and GAPDH in non-mCRC patients and mCRC patients was significantly higher ($P < 0.05$), while there was no significant difference in the concentration of ACTB. The rCRC group also had higher concentration than the control group, except for ACTB. Compared with the non-mCRC and mCRC groups, the concentration of the rCRC group was decreased, although there was no statistical difference (Table 2; Figure 1D,E,F).

ROC curve analysis between the non-mCRC and intestinal polyp groups

The area under curves (AUCs) of ALU, GAPDH, and ACTB were 0.734, 0.800, and 0.503, respectively. The AUC of ALU, GAPDH, and CEA combined was 0.974 (Table 3; Figure 2A,B).

Clinical variables of CRC patients

The analysis showed that the concentration of ALU was correlated with size ($P = 0.004$), histological differentiation ($P = 0.001$), and lymphatic metastasis ($P = 0.004$). The concentration of ACTB was correlated with histological differentiation ($P = 0.018$), local invasion ($P = 0.003$), and lymphatic metastasis ($P = 0.011$). The concentration of GAPDH was correlated with histological differentiation ($P = 0.017$) (Table 4).

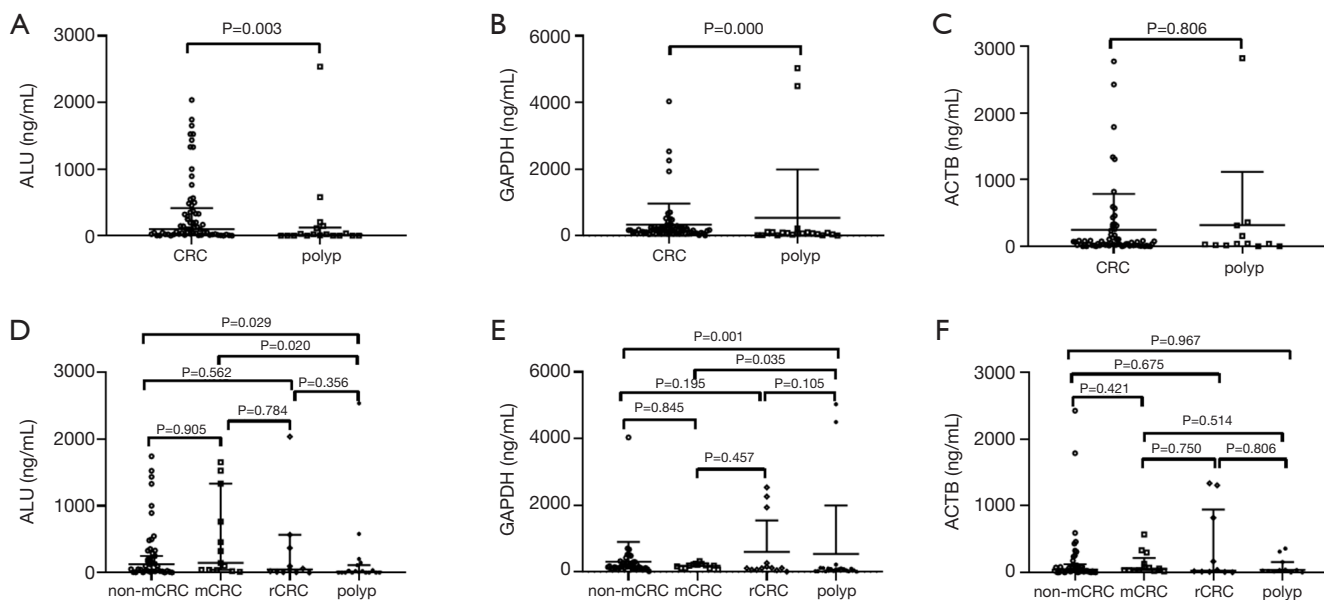


Figure 1 Scatter diagrams for *ALU*, *GAPDH*, and *ACTB* genes in intestinal polyp and colorectal cancer (CRC) patients. (A,B,C) The comparison in intestinal polyp and CRC patients; (D,E,F) the comparison in non-mCRC, mCRC, rCRC, and intestinal polyp. mCRC, metastatic colorectal cancer; rCRC, recurrent colorectal cancer.

Table 2 The concentration of cfDNA in non-mCRC, mCRC, rCRC, and intestinal polyp

Groups	Non-mCRC	mCRC	rCRC	Intestinal polyp	F	P
ALU					11.084	0.011
n	41	13	11	18		
C (ng/mL)	124.57 (21.92, 341.42) [#]	143.84 (39.04, 1,048.16) [#]	47.23 (6.42, 369.34)	13.03 (0.01, 120.43)		
ACTB					0.769	0.857
n	43	13	11	12		
C (ng/mL)	486.34 (134.86, 1,761.76)	593.29 (331.68, 2,152.72)	351.97 (112.49, 13,097.78)	364.49 (143.97, 2,749.28)		
GAPDH					15.832	0.001
n	45	13	13	20		
C (ng/mL)	171.44 (123.34, 287.50) [#]	194.78 (113.43, 235.29) [#]	117.68 (72.74, 1,102.51)	79.54 (27.83, 115.15)		

[#], compared with intestinal polyp, there were statistical differences (P<0.05). cfDNA, cell-free DNA; CRC, colorectal cancer; mCRC, metastatic colorectal cancer; rCRC, recurrent colorectal cancer.

Discussion

CfDNA consists of three constituents: circulating tumor DNA (ctDNA), non-malignant tumor cell-derived DNA, and normal cell germline DNA (16). The DNA half-life is short in the circulation, ranging from 15 min to several hours, which can present the real-time status of a tumor (17,18). It has been estimated that from a tumor weighing

100 g, corresponding to 3×10^{10} tumor cells, up to 3.3% of tumor DNA may enter the blood every day (19). CtDNA holds a lot of information, but the information is highly fragmented, of a low amount, and is costly to extract, which limits its usage (20,21). However, quantification of cfDNA by qPCR is simple, inexpensive, and reproducible (22). The concentration of cfDNA in plasma has been widely studied

Table 3 The diagnostic value of primers, CEA, and the combination diagnostic

Primers	AUC	Cut-off	Sensitivity (%)	Specificity (%)	95% CI	P value	PPV (%)	NPV (%)
ALU	0.734	0.17	100	44	0.579–0.889	0.004	80.39	100
GAPDH	0.800	122.09	77.8	85	0.660–0.939	<0.001	92.1	62.96
ACTB	0.503	451.79	51.2	66.7	0.315–0.692	0.967	84.61	27.58
CEA	0.843	3.19	73.3	86.7	0.727–0.959	<0.001	94.28	52
CEA + GAPDH	0.834	0.69	71.1	86.7	0.718–0.951	<0.001	94.12	50
CEA + ACTB	0.848	0.72	74.4	90.9	0.711–0.985	<0.001	96.96	47.62
CEA + ALU	0.882	0.76	73.2	92.3	0.789–0.974	<0.001	96.77	52.17
ALU + GAPDH + CEA	0.974	0.63	92.7	100	0.973–1.000	<0.001	100	81.25

CEA, carcinoembryonic antigen.

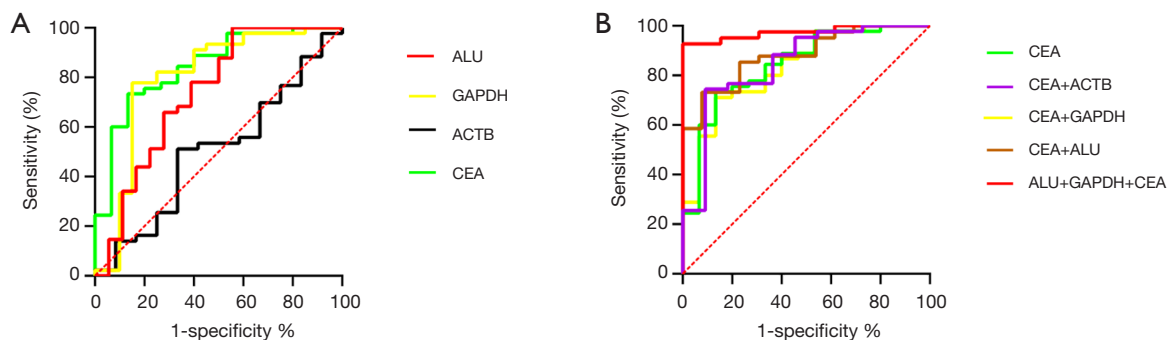


Figure 2 The ROC of *ALU*, *GAPDH*, *ACTB* genes, and CEA. (A) The diagnostic value of genes and CEA alone; (B) the diagnostic value of genes combined with CEA. ROC, receiver-operating characteristic; CEA, carcinoembryonic antigen.

in patients with various neoplasms, including hepatocellular carcinoma (HCC), breast cancer, renal cell carcinoma (RCC), gastric cancer, and CRC (10-13,23-27). The concentration of ALU and GAPDH in CRC was found to be significantly higher than in standard control groups (10-13). A higher concentration of ACTB has been observed in HCC and RCC than in healthy groups (23,26). To date, many genes have been reported for diagnosing cfDNA but are still technically challenging.

In our study, three genes served as the models to facilitate the comparison of the yields of cfDNA by q-PCR. The cfDNA concentration of ALU and GAPDH in the CRC group was higher than in the intestinal polyp group, and there was no significant difference in ACTB. Meanwhile, the yields of cfDNA with ALU and GAPDH were higher in malignancy. The results implied that ALU and GAPDH would be more suitable for detecting cfDNA.

The diagnostic value of the concentration of cfDNA

in ALU, ACTB, and GAPDH was compared between the non-mCRC and intestinal polyp groups. The GAPDH gene showed the best diagnostic value (AUC =0.800) compared with ALU (AUC =0.734) and ACTB (AUC =0.503). The AUC for distinguishing non-mCRC from normal controls by ALU has been reported as 0.85 (11). In comparing HCC and chronic hepatitis B virus (HBV)-infected groups, the AUC value of ACTB was found to be 0.56 (23). Consequently, the concentration of cfDNA in GAPDH and ALU is superior to ACTB in distinguishing between non-mCRC and intestinal polyp groups. The diagnostic value of ALU and GAPDH could be improved significantly by combining them with CEA. The concentration of cfDNA was correlated with clinical variables, as reported (11,13). The results showed that the yield of cfDNA was strictly related to tumor size, histologic differentiation, local invasion, and lymphatic metastasis.

Table 4 The correlation analysis of ALU, ACTB, GAPDH with clinical variables

Clinical variables	ALU			ACTB			GAPDH		
	n	C (ng/mL)	P	n	C (ng/mL)	P	n	C (ng/mL)	P
Age (years)			0.334			0.647			0.215
≥60	50	111.50 (22.57, 463.21)		55	593.29 (149.98, 2,573.03)		56	175.62 (115.12, 276.54)	
<60	15	50.78 (10.90, 287.92)		12	355.24 (170.89, 845.99)		15	147.97 (89.14, 222.16)	
Gender			0.401			0.164			0.872
Male	33	143.84 (19.40, 532.62)		32	790.05 (214.93, 3,277.28)		35	165.56 (113.71, 270.76)	
Female	32	54.75 (21.27, 312.84)		35	364.77 (134.86, 860.94)		36	170.03 (112.60, 262.89)	
Location			0.955			0.682			0.296
Rectum	44	97.11 (21.27, 347.12)		44	422.55 (153.19, 2,233.67)		47	160.23 (113.19, 235.87)	
Colon	21	98.44 (19.28, 514.86)		23	677.44 (195.18, 3,105.92)		24	194.58 (115.12, 447.09)	
Tumor sizes (cm)			0.004			0.455			0.643
≥4	37	166.64 (49.07, 522.05)		38	744.93 (184.56, 2,675.31)		40	182.30 (122.94, 275.15)	
<4	28	30.59 (10.27, 235.21)		29	304.84 (156.39, 2,020.54)		31	151.21 (113.19, 268.83)	
Histologic differentiation			0.001			0.018			0.017
Low	17	456.06 (113.49, 1,113.15)		19	1,058.66 (769.23, 4,606.17)		20	209.79 (172.21, 447.09)	
Middle	37	97.50 (22.88, 341.42)		38	354.88 (121.19, 1,678.02)		40	154.44 (113.85, 276.54)	
High	11	20.33 (6.27, 41.64)		10	238.36 (140.61, 360.27)		11	113.19 (87.00, 160.23)	
Local invasion			0.749			0.003			0.711
Muscularis propria	10	48.85 (14.07, 401.77)		10	235.17 (8.20, 1,880.84)		11	268.83 (108.37, 328.22)	
Subserosa	14	121.57 (22.57, 796.18)		13	805.77 (218.45, 1,542.53)		14	165.83 (106.99, 272.22)	
Visceral peritoneum	41	98.44 (22.89, 392.74)		44	441.76 (172.38, 3,240.72)		46	167.09 (113.56, 238.90)	
Lymphatic metastasis			0.004			0.011			0.486
0	33	29.05 (10.36, 341.42)		32	331.81 (156.86, 1,066.39)		35	157.67 (108.37, 276.32)	
≤3	24	134.20 (41.89, 327.35)		27	486.33 (162.79, 3,334.40)		28	175.62 (113.69, 265.18)	
≥4	8	949.39 (146.90, 1,621.20)		8	1,893.96 (284.70, 7,585.63)		8	218.80 (144.06, 306.58)	
Distant metastasis			0.738			0.364			0.559
Yes	39	97.50 (20.62, 352.81)		40	441.76 (138.64, 1,587.98)		42	170.03 (123.61, 276.39)	
No	26	97.12 (19.66, 483.58)		27	593.29 (188.60, 3,334.40)		29	165.56 (108.27, 261.53)	
Tumor stage (TNM)			0.971			0.524			0.807
I	7	50.86 (18.47, 499.07)		8	235.17 (41.06, 2,096.51)		8	216.99 (112.51, 321.76)	
II	17	47.36 (19.43, 514.86)		17	774.32 (249.34, 2,076.36)		18	170.03 (120.67, 282.06)	
III	15	130.27 (25.44, 201.93)		15	357.79 (121.50, 1,058.66)		16	161.25 (124.70, 213.93)	
IV	26	97.12 (19.66, 483.58)		27	593.29 (188.60, 3,334.40)		29	165.56 (108.27, 261.53)	

Italic P values are statistic value in clinical variable (P<0.05).

Conclusions

In conclusion, the yields of cfDNA from the GAPDH and ALU genes are expected to be sensitive indicators for the diagnosis of CRC. The GAPDH and ALU genes are superior to ACTB. The diagnostic value of ALU and GAPDH could be significantly improved by combining them with CEA.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This research was approved by the Human Research Ethics Committee of Yongchuan Hospital Affiliated to Chongqing Medical University (approval ID: 20180401).

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