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ORIGINAL ARTICLE

# Cancer stage-dependent alterations in cell-free DNA in patients with colorectal caner

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## Summary

***Purpose:*** *Cell-free DNA (cfDNA) in plasma is a useful resource for liquid biopsy. The concentration and integrity of cfDNA may be clinical informative for detecting and predicting cancer progression.*

***Methods:*** *Plasma from 40 healthy controls and 90 colorectal cancer patients was assessed. qPCR targeting the arithmetic-logic unit (Alu) repeats were performed using two different sets of primers amplifying the long and short segments. DNA integrity was calculated by the ratio of the long to the short fragments of amplified Alu repeats.*

***Results:*** *cfDNA concentration was significantly higher in the patients than that in healthy controls. Patients with stage III colorectal cancer showed no significant difference in their cfDNA levels as compared with the healthy controls. In colorectal cancer, cfDNA level of stage IV patients was higher than that of stage 0-III (p=0.049). The DNA integrity was significantly lower in patients with stage I and II cancer than that in normal controls (p=0.007, 0.029 respectively). The receiver operating characteristic (ROC) curve for discriminating patients with colorectal cancer from normal controls had an area under the curve of 0.672 (95%CI, 0.572 to 0.772) and cfDNA concentration increased within 21 days following surgery and dropped by 3 months after surgery.*

***Conclusion:*** *Concentration of cfDNA is a promising molecular marker for assessing colorectal cancer progression. Both the cfDNA concentration and its integrity are highly variable. Some cancer stage dependent changes were observed, which warrants further investigation with more patients included.*

***Key words:*** *cancer stage dependent change, cell-free DNA, colorectal cancer, plasma*

## Introduction

Colorectal cancer is the third and the second most common cancer in men (746,000 cases, 10.0% of the total) and in women (614,000 cases, 9.2% of the total) respectively [1]. The most significant prognostic indicator is TNM stage [2]. Diagnostic imaging methods such as endoscopic ultrasound or computed tomography are effective for the detection of tumor depth, lymph node (LN) status and distant metastasis when they are relatively large. However, there is no clinically established blood test with the predictive ability to determine colorectal cancer for clinical stages. Therefore, it is

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necessary to develop a blood test for assessment of clinical stage of colorectal cancer as well as for the evaluation of therapeutic effects.

Cell-free DNA in blood is a promising diagnostic and prognostic cancer biomarker [3]. The level of cfDNA elevates in patients with cancers and drops down after radical surgery. However, in patients with cancer recurrence or metastasis, the levels of cfDNA increased [4]. In metastatic colorectal cancer, high level of cfDNA could be used to predict short overall survival [5]. In addition to the amount, DNA fragments released into blood also vary in their lengths [6]. cfDNA integrity measured as the ratio of long to short DNA fragments has been widely studied in patients with different cancers [7-10]. Several studies have assessed the cfDNA integrity in colorectal cancer, but the results were inconsistent. Some reports found increased DNA integrity [8,9,11,12], and others reported a reduced integrity [13-15].

In this study, we measured the concentration of circulating DNA by quantitative real-time PCR of arithmetic-logic unit (Alu) repeats on 130 plasma samples from 90 colorectal cancer patients and 40 controls. The relationship between the circulating DNA results and pathological findings was analyzed to assess the diagnostic and prognostic values of these genetic markers of colorectal cancer.

## Methods

*Collection of plasma samples and clinical information*

Blood samples were collected from 90 patients with colorectal cancer and 40 healthy volunteers. Blood samples were drawn before antitumor therapy and at selected time points following operation. Postoperative histopathology findings were used for initial staging (stage 0 to III), and imaging diagnoses were used for final determination of stage IV (Table 1). Inclusion criteria: Patients who were treated from January 1st of 2013 to December 31st of 2014 at the second affiliated hospital of Soochow University; Patients who had histologically confirmed colorectal adenocarcinoma; Patients aged 18-78 years. Exclusion criteria: Patients with second malignancies, connective tissue diseases and pregnancy. Written informed consent was obtained from all participants prior to this non-interventional treatment. This study was approved by the human research ethics committee of the second affiliated hospital of Soochow University.

*Plasma preparation for Alu-qPCR*

Six ml of blood were collected into an EDTA-containing tube, stored at 4°C, and were processed within 6 h. The blood samples were centrifuged at 3000 rpm for 10 min at room temperature to remove the cellular components. Two ml of plasma were cryopreserved at -80°C. DNA was purified from 2 ml of plasma using QIAamp® circulating nucleic acid kit according to the manufacturer's instruction.

*Quantitative PCR of Alu repeats*

qPRT-CR was performed using two different primer sets for Alu repeats, amplifying 115bp short and 219bp long products. The primer set for the 115bp amplicon (Alu115) could amplify products from Alu fragments harboring the nucleotides of the Alu115 region. It could also amplify a few nucleotides shorter and all Alu fragments longer than the Alu115 region. However, the primer set for the 219bp amplicon (Alu219) only amplified Alu DNA fragments about or longer than 219 bps. The sequences of the Alu115 primers were: forward, 5'-CCTGAGGTCAGGAGTTCGAG-3' and reverse, 5'-CCCGAGTAGCTGGGATTACA-3'as described previously [8]; the sequences of Alu219 primers were: forward, 5'- CACGCCTGTAATCCCAGCACTTT-3' and reverse, 5'-CACGCCTGTAATCCCAGCACTTT-3'. The primers Alu219 were optimized from screening several primers including previously reported Alu247 primers [8]. DNA integrity index is calculated as the ratio of Alu219over Alu115 by qRT-PCR.

The reaction mixture for each Alu-qPCR consisted of 1µl DNA template, 0.5µl of forward and reverse primers, 12.5µl Quantifast sybr green PCR mix (Qiagen) and 10.5µl RNase-free water in a total 25µl volume. RT PCR amplification was performed with pre-cycling heat activation of DNA polymerase at 95°C for 5min, followed by 40 cycling of denaturation at 95°C for 20 s, annealing and extension at 65°C for 30 s using Bio-Rad CFX96 PCR. The absolute equivalent amount of DNA in each sample was calculated according a standard curve obtained with serial dilutions (37.5ng-0.375pg) of prepared genomic DNA from peripheral blood leukocytes of nor-

**Table 1.** Clinicopathologic characteristics of colorectal cancers

| *Variables* | *Patients n (%)* |
|---|---|
| AJCC primary tumor | |
| Tis | 2 (2.22) |
| T1 | 6 (6.67) |
| T2 | 28 (31.11) |
| T3 | 7 (7.78) |
| T4 | 47 (52.22) |
| AJCC regional lymph nodes | |
| N0 | 59 (65.56) |
| N1 | 19 (21.11) |
| N2 | 12 (13.33) |
| AJCC distant metastasis | |
| M0 | 83 (92.22) |
| M1 | 7 (7.78) |
| AJCC stage | |
| 0 | 5 (5.56 |
| I | 27 (30.00) |
| II | 26 (28.88) |
| III | 25 (27.78) |
| IV | 7 (7.78) |

mal control. A negative control (without template) was contained in each plate. All qPCR assays were analyzed without knowledge of specimen identity. Each assay was carried out in duplicate.

*Statistics*

The Mann-Whiney U-test was used to compare Alu115 and Alu219/Alu115 between groups of colorectal cancer and controls. Kruskal-Wallis H-test was used for multiple comparisons between the groups. ROC curves and area under the ROC curve were used to assess the diagnosis value of using Alu115 and Alu219/Alu115 for colorectal cancer. Statistical analyses were performed using SAS9.2 software and results were considered statistically significant when p<0.05 (two-0tailed).

## Results

*Clinical and pathologic characteristics of colorectal cancer*

The mean age was 49.68±11.74 (standard deviation) years in 40 normal controls and 65.08±10.97 years in 90 patients with colorectal cancer. The cancer group included 49 men and 41 women. Among the 90 patients with colorectal cancer, 4 had high grade carcinomas, 66 had middle grade carcinomas, and 11 had low grade carcinomas. Histopathologic grade in 9 patients was unknown. Sixteen patients with colorectal cancers had lymphovascular invasion and 74 patients had no lymphovascular invasion. Table 1 shows AJCC stages for patients whose plasma was preoperatively sampled.

*Selecting and optimizing primers for measuring cfDNA concentration*

To evaluate the performance of Alu-qPCR for quantification of cfDNA, we tested the specificity of the primer by conventional PCR. The efficiencies and qualities of amplification were tested by RT-PCR of a 10-fold serial dilution of genomic DNA extracted from normal control leukocytes. The efficiencies of Alu-qPCRs with Alu115 primer, Alu247 primer and Alu219 primer were about 90%, 70%, and 90%, respectively. Agarose gel electrophoresis of PCR products obtained with Alu115 and Alu219 confirmed that the target DNAs could be specifically amplified without major aberrant bands (Figure 1).

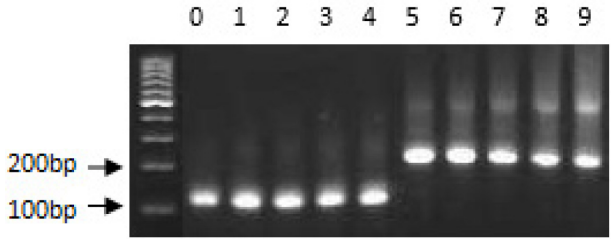


**Figure 1.** Representative results of agarose gel electrophoresis of PCR products obtained with Alu115 (0-4) and Alu219 (5-9) primer sets. Concentration of genomic DNA template from (0-1) and (4-9) is 0.00375, 0.0375, 0.375, 3.75 and 37.5ng $ml^{-1}$.

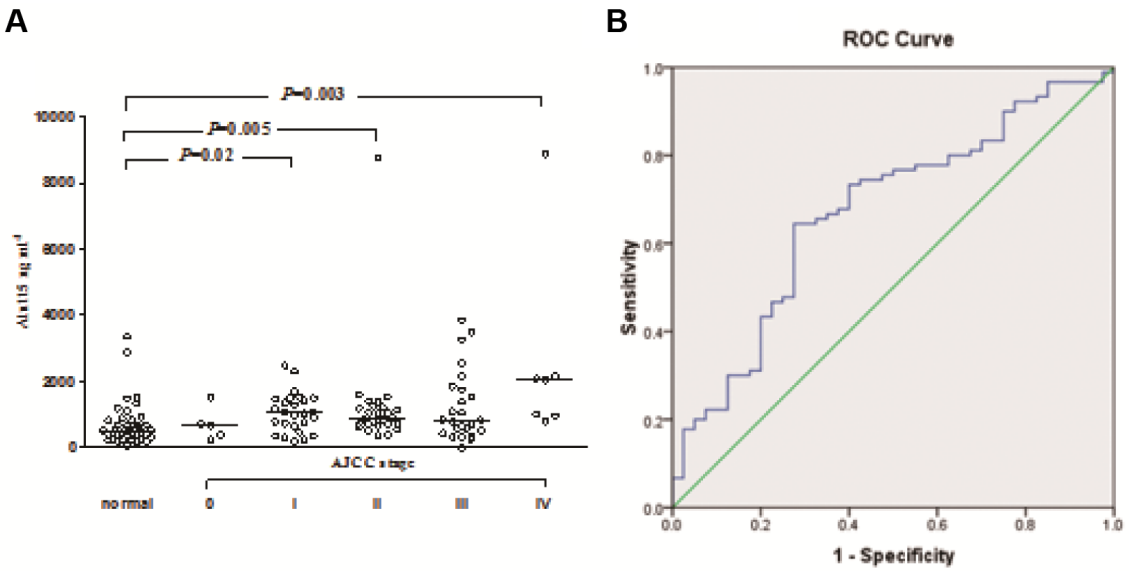


**Figure 2.** Comparison of Alu115-qPCR values in plasma from normal controls and patients with AJCC stage 0 to IV colorectal cancer. **A:** Alu115-qPCR values in plasma from normal controls (n=40) and patients with AJCC stage 0 to IV colorectal cancer (n=5, 2726,25, 7 respectively). Horizontal lines indicate the median for each group. The Alu115-qPCR values were significantly higher in patients with stage I, II and IV cancer than those in nornal controls. **B:** The ROC curve of Alu115-qPCR values for discriminating colorectal cancer from heath control had an area under the curve value of 0.672 (95%CI, 0.572 to 0.772).

*Changes in cfDNA in plasma of patients with colorectal cancer*

Circulating cell-free DNA in patients before antitumor therapy was assessed. The median (IQR25-75) Alu115-qPCR values in the normal controls and patients with stage 0, I, II, III and IV colorectal cancer were 511.28 (342.28-865.30), 669.55 (239.5-705.05), 922.85 (466.1-1396.8), 784.73 (674.55-1142.6), 706.75 (391.15-1269) and 1383 (795.1-2047.5) ng $ml^{-1}$, respectively. The Alu115-qPCR values were significantly higher in patients with stage I, II and IV colorectal cancer than those in normal controls (p=0.02, 0.005, and 0.003 respectively) (Figure 2A). A trend of elevation in stage III cancer was observed but without statistical significance.

The Alu115-qPCR values showed a trend that for increase with AJCC stage. The ROC curve of plasma DNA concentration for discriminating colorectal cancer patients from healthy controls had an area under the curve value of 0.672 (95%CI, 0.572 to 0.772; Figure 2B).

The median (IQR25-75) Alu115-qPCR values in patients with stage 0-III were 782.65 (466.1-1280.5) ng $ml^{-1}$. The Alu115-qPCR values were significantly higher in patients with stage IV than those with stage 0-III (p=0.049; Figure 3A). The ROC curve of plasma DNA concentration for discriminating patients with stage IV disease had an area under the curve value of 0.725 (95%CI, 0.547 to 0.902; Figure 3B).

Alu219-qPCR value represents the circulating DNA concentration in longer sizes of plasma DNA. The median (IQR25-75) Alu219-qPCR values in the controls and patients with stage 0, I, II, III and IV colorectal cancer were 237.48 (140.88-345.78), 255.5 (85.23-361.75), 274.45 (158.7-329.2), 253.03 (182-429.75), 291.5 (197.7-534.2) and 463.15 (198.55-681.6) ng $ml^{-1}$, respectively. The Alu219-qPCR values were significantly higher in patients with stage IV disease than those in normal controls (p=0.027).

The plasma DNA integrity was calculated using the ratio of Alu219-qPCR value/Alu115-qPCR value of each sample. The median (IQR25-75) plasma integrities in the normal controls and patients with stage 0, I, II, III and IV colorectal cancer were 0.48 (0.31-0.68), 0.38 (0.36-0.45), 0.34 (0.27-0.43), 0.36 (0.27-0.46), 0.41 (0.35-0.49), and 0.33 (0.27-0.45), respectively. The DNA integrity was significantly lower in patients with stage I and II cancer than those in nornal controls (p=0.007, 0.029 respectively).

In 90 patients with colorectal cancer, median plasma DNA concentration was not correlated neither with depth of tumor invasion (p=0.129), nor lymph node metastasis (p=0.377).

*Cell-free DNA dynamics in patients with colorectal cancer undergoing surgery*

Postoperative cfDNA was assessed in the patients who agreed to be followed up in their cfDNA

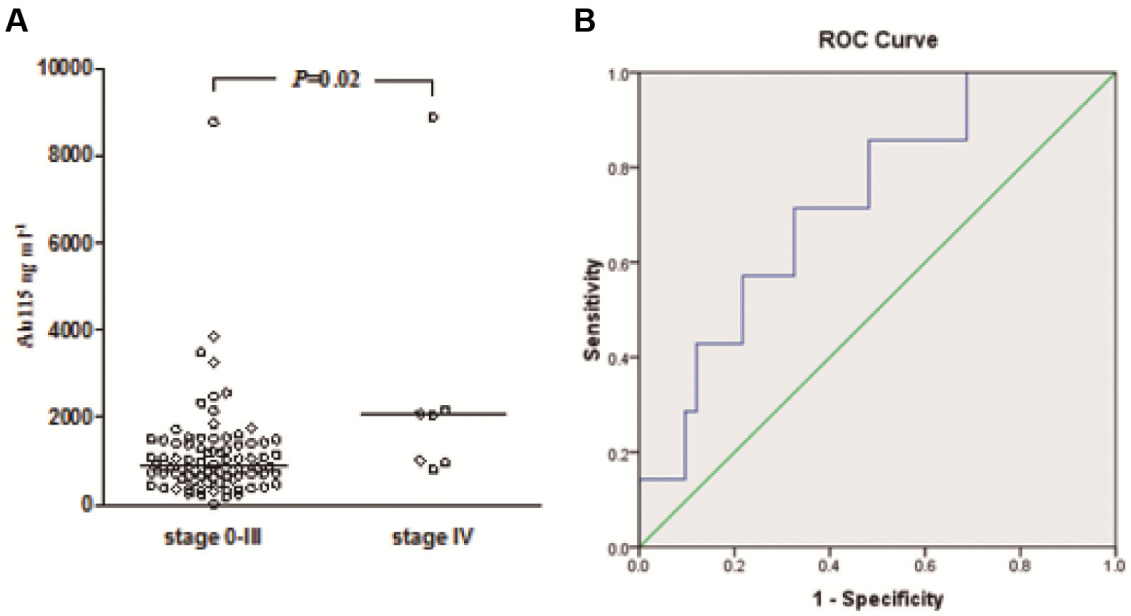


**Figure 3.** Comparison of Alu115-qPCR values in patients with stage IV and stage 0-III. **A:** The Alu115-qPCR values were significantly higher in patients with stage IV than in those with stage 0-III (p=0.049). **B:** The ROC curve of plasma DNA concentration for discriminating patients with stage IV colorectal cancer from all colorectal cancer had an area under the curve value of 0.725 (95%CI, 0.547 to 0.902).

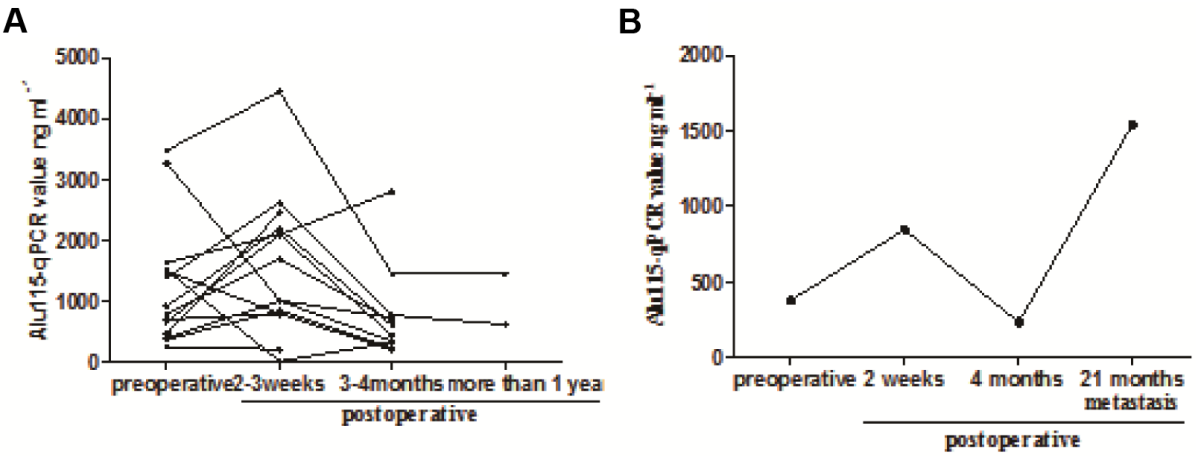


**Figure 4.** Cell-free DNA dynamics in patients with colorectal cancers undergoing surgery. **A:** Pre- and postoperative plasma Alu115-qPCR values in patients with colorectal cancer undergoing radical surgery (n=12) and palliative surgery (n=2). The Figures show that Alu115-qPCR values of most of the patients dropped compared with those before surgery. **B:** Alu115-qPCR values in one patient who had radical surgery and developed bone and celiac lymph node metastasis 21 months postoperatively.

(n=14). Of the 14 patients, 12 with postoperative AJCC stage I to III (n=4, 4, and 4, respectively) underwent radical colectomy, 1 patient with liver metastasis had palliative colectomy, and 1 patient with peritoneal carcinomatosis underwent diverting ostomy.

Alu115-qPCR values in plasma sampled preoperatively from the 14 patients varied from 257.25 to 3481.00 ng $ml^{-1}$(median=858.98 ng $ml^{-1}$).

Within three weeks after surgery, Alu115-qRT-PCR values elevated from the median by 119.0% (IQR 28.75%-169.3%) compared with those before surgery in 10 of the 14 patients. Alu115-qPCR values dropped in 2 patients with stage I and 2 patients with stage III cancer.

At 3-4 months after surgical intervention, Alu115-qPCR values dropped by 41.50% (IQR 29.25-72.75) compared with those before surgery in 10 of the 14 patients. One patient with diverting ostomy did not receive other anticancer therapy and Alu115-qPCR values increased (Figure 4A).

One year after surgery, Alu115-qRT-PCR values were still lower than those before surgery in 2 patients with stage II and III who were free of cancer recurrence. One patient who had radical surgery developed bone and celiac lymph node metastasis after postoperative 21 months, and Alu-qPCR values elevated again (Figure 4B).

## Discussion

As a biomarker for malignant tumor, cfDNA contains the DNA released from tumor cells. It is highly reliable and easy to analyze [16,17]. It was reported that the concentration of circulating DNA elevates in cancers, including lung cancer, breast cancer, gastric cancer, colorectal cancer, urologic tumor, head and neck tumor, etc [18,19]. Comparing with the concentration of total circulating DNA, the concentration of circulating tumor DNA (ctDNA) can more accurately reflect tumor burden [18]. However, there are several requirements to be fulfilled before detection of tumor-specific gene mutation. There must be tumor-specific gene mutation genetically identified in primary malignant tumor. The tumor-specific gene mutations are not known in a large portion of patients with colorectal cancer before operation. On the other hand, the low sensitivity and complexity of assays make them less attractive for routine clinical use, although they are highly informative in research using next generation sequencing [21].

In this study, we assessed the concentration of circulating DNA by Alu-qPCR methods. We found that the level of circulating DNA of colorectal cancer is higher than that of the healthy controls, especially in the stages I, II and IV. These data suggest the diagnostic potential of Alu parameters in combining with other tumor markers. Among patients with colorectal cancer, the level of cfDNA in stage IV is significantly higher than that of other earlier stages, indicating that cfDNA levels might be an indicator for cancer progression.

In addition to the changes in the cfDNA level represented by Alu amount, the present study identified that cfDNA integrity is lower in colorectal cancer patients than in healthy controls. However, the cfDNA integrity was not correlated with TNM stages in patients included in this study. These findings are in accordance with some pub-

lished studies [13-15]. However, there are some other studies showing a higher cfDNA integrity in patients with colorectal cancer [8,9,11,12]. These discrepancies in various studies may be related to many factors.

First, there may be qualitative differences in the measurement of the absolute amount and integrity of DNA using serum directly rather than DNA extracted from serum, particularly for the integrity. Different techniques of DNA purification have various efficiencies and may result in different DNA amount and DNA fragment size. Second, the size of the longer amplicon can be a variable substantially affecting the cfDNA integrity [15]. Third, cfDNA integrity has diversity in population and results from small sample studies may be not reliable. Fourth, the cfDNA level and the integrity are also different depending on whether the samples are serum or plasma. The relationship between cfDNA integrity and colorectal cancer needs to be confirmed by clinical trials including more colorectal cancer patients and uniform experimental method.

The high individual differences of the Alu parameters cannot overshadow their clinical values in longitudinal follow up for cancer patients. In most of patients, cfDNA rises in 21 days after surgery, and decreased to levels lower than those before operation until 3 months after surgery. Cell-free DNA increased continually in one patient who had stage IV cancer and did not receive anticancer therapy. In another patient who developed metastasis disease at 21 months after radical surgery, the concentration of circulating DNA was elevated again. These results demonstrate that detection of change in the circulating DNA may be useful for monitoring of the burden of colorectal cancer.

In conclusion, the present study reported a new pair of primers for Alu219 amplicon analysis. With Alu assay, we observed that cfDNA levels were higher in plasma from patients with colorectal cancer than in normal controls. In colorectal cancer, cfDNA level of stage IV patients was higher than in those of stage 0-III. The therapeutic effect of surgical removal of cancer was well reflected by the decrease of of free-DNA levels after operation. Although the cfDNA integrity is not consistently decreased in the colorectal cancer patients, significant decrease of the cfDNA integrity in patients with early stage disease may be useful for cancer diagnosis. Circulating cfDNA may be valuable in predicting and evaluating the therapeutic effects and the progression of colorectal cancer.

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## Conflict of interests

The authors declare no conflict of interests.

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