Original Article

Circulating lncRNA EGFR-AS1 as a diagnostic biomarker of colorectal cancer and an indicator of tumor burden

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Background: Colorectal cancer (CRC) is one of the most common malignancies. Although CRC treatment has been significantly improved, patient survival remains low because most patients already have advanced disease at diagnosis. Early screening and diagnosis of tumors is critical; however, the current tissue biopsy and radiological evaluation methods have very limited effectiveness. Therefore, establishing new convenient and non-invasive biomarkers is urgently needed for timely detection, therapeutic assessment, and prognostic prediction. At present, non-coding RNAs (ncRNAs) have attracted research attention owing to their potential oncological applications.

Methods: The long ncRNA epidermal growth factor receptor antisense RNA 1 (EGFR-AS1) is overexpressed in multiple malignancies including CRC. The present study examined the circulating EGFR-AS1 level in CRC, and the results showed that EGFR-AS1 could be considered an indicator of tumor burden.

Results: Elevated circulating EGFR-AS1 levels were detected in CRC cases (n=128) compared with control cases comprising endoscopy confirmed CRC-free individuals [n=64, median expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1.578 *vs.* 0.780, P<0.001]. Individuals with larger tumors (≥5 cm) had elevated circulating EGFR-AS1 levels compared to those with smaller tumors (<5 cm, 1.739 *vs.* 1.290, P<0.001). The expression of serum EGFR-AS1 in stage III/IV CRC was higher than that in stage I/II CRC (1.691 *vs.* 1.412, P<0.05). Plasma EGFR-AS1 levels were markedly reduced following surgical resection of colorectal lesions in a subset of patients [n=32, 1.192 (pre-surgery) *vs.* 0.692, P<0.001]. Furthermore, the expression of EGFR-AS1 in resected CRC tissues was significantly higher than that in paracancerous tissues (n=32, 1.336 *vs.* 0.487, P<0.001).

Conclusions: These results highlight the potential of EGFR-AS1 as a diagnostic biomarker in CRC.

Keywords: Biomarker; circulating; colorectal cancer (CRC); diagnostic; epidermal growth factor receptor antisense RNA 1 (EGFR-AS1)

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Introduction

Colorectal cancer (CRC), a common malignancy affecting the gastrointestinal tract, is characterized by a high recurrence rate and reduced survival and constitutes the second and third most prevalent malignancy in women and men, respectively, accounting for 10% of new cancer diagnoses and cancer-associated deaths worldwide (1,2). About 50–60% of CRC cases metastasize, with 80–90% exhibiting unresectable liver tumors (3,4). CRC treatment mostly includes surgical resection, chemotherapy, as well as radiation and biological therapies. Although these treatments have increased the survival of CRC patients, advanced CRC remains a leading cause of cancer-associated deaths, with only 6% of cases surviving for 5 years or more.

Advanced methods have been developed recently for treating metastatic colorectal cancer (mCRC) (5). Previously, 5-fluorouracil (5-FU) was the unique active compound, conferring an overall survival (OS) approximating 11–12 months. Currently, new treatments could lead to an OS of 3 years (6). Such improvements are mostly attributed to novel active compounds, including oxaliplatin and irinotecan, as well as monoclonal antibodies that suppress angiogenesis and cell division pathways by regulating vascular endothelial growth factor and the epidermal growth factor receptor (7-9). Recently, important insights into tumor immune checkpoints and their suppressors have been provided for the treatment of multiple cancers (10). Programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) suppressors were shown to play essential roles (11).

However, the heterogeneity of mCRCs has hampered efforts by oncologists to design targeted therapeutic approaches and optimize personalized treatment. Therefore, developing predictive tools that are clinically applicable to individual patients prior to treatment initiation is critical (12,13). Additionally, identifying circulating biomarkers is urgently required to improve patient management, since the current approaches, including tissue biopsy and radiological assessment, are very limited in terms of follow-up and prognosis. Thus, in the era of precision medicine, liquid biopsy constitutes a critical decision-making tool for designing therapeutic strategies (14,15).

Detecting blood biomarkers is a promising alternative diagnostic tool in CRC (16). Carcinoembryonic antigen (CEA) has been broadly utilized as a circulating molecular marker for detecting tumor recurrence (17,18). Other circulating biomarkers have also been considered for postoperative CRC surveillance, including cancer antigen 19-9 (CA19-9) and cancer antigen 125 (CA125). Despite their usefulness for patient monitoring, these markers are also associated with non-neoplastic conditions such as inflammatory bowel disease and endometriosis, as well as other types of cancer, including breast, gastric, pancreatic, bladder, and lung cancers (19,20). Presently, no circulating biomarker is available for early CRC detection.

An emerging field of biomarker research is the analysis of ncRNAs. Based on the lengths of their functional transcripts, human ncRNAs are largely grouped into two main classes, including small (<200 nt) and long (>200 nt) ncRNAs. MicroRNAs (miRNAs), as central members of the small ncRNA class, suppress gene expression (21). Diversity is considerably higher in the long ncRNA (lncRNA) class, which includes approximately 19,000 functionally distinct RNA molecules with multiple molecular mechanisms (22). Accumulating evidence suggests that the regulation of lncRNAs is impaired in cancer cells, implying that they might constitute potential biomolecular markers for cancer diagnosis and prognosis (23). Meanwhile, their regulatory effects on cell proliferation, invasion, and apoptosis were detected in CRC cells. Epidermal growth factor receptor (EGFR) antisense RNA1 (EGFR-AS1) is a lncRNA that is 2.8 kb in length. In the present study, circulating EGFR-AS1 levels were examined in 128 CRC patients and 64 healthy individuals by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). We comparatively investigated the circulating EGFR-AS1 levels in CRC patients versus control cases comprising endoscopy-confirmed CRC-free cases. In addition, pre- and post-surgical plasma levels of EGFR-AS1 were determined in a subset of these patients to examine its value as a biomarker of tumor load. Furthermore, the expression of EGFR-AS1 in the CRC tissues of these surgical patients was significantly higher than that in paracancerous tissues, which may help to further confirm the key role of EGFR-AS1 in the diagnosis and progression of CRC and can be used for early CRC diagnosis. We present the following article in accordance with the STARD reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-968/rc).

Methods

Study subjects

This trial enrolled individuals with resectable (no distant metastasis) CRC without prior surgery (n=128). The

Table 1 Associations between circulating EGFR-AS1 and clinical characteristics in CRC patients

Patient characteristics	No. of patients, n (%)	Relative plasma EGFR-AS1 level, median (range)	P value
Age, years			0.941
≥60	79 (61.7)	1.576 (0.968–2.155)	
<60	49 (38.3)	1.583 (1.762–1.275)	
Gender			0.461
Male	66 (51.6)	1.611 (0.860–2.516)	
Female	62 (48.4)	1.543 (0.897–2.579)	
Tumor size, cm			0.0001**
≥5	82 (64.1)	1.739 (0.892–3.579)	
<5	46 (35.9)	1.290 (0.860–2.101)	
TNM stage			0.003*
I/II	52 (40.5)	1.412 (0.860–2.275)	
III/IV	76 (59.5)	1.691 (0.897–3.579)	
Lymphatic metastasis			0.718
No	23 (18.0)	1.417 (0.860–2.275)	
Yes	105 (82.0)	1.614 (0.932–3.034)	

*, P<0.05; **, P<0.001. EGFR-AS1, epidermal growth factor receptor antisense RNA 1; CRC, colorectal cancer; TNM, tumor-node-metastasis.

control group comprised age and sex-matched CRC-free individuals (n=64). The absence of CRC in control individuals was verified by endoscopic investigation, and by biopsy in certain cases (if necessary).

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethical Review Board of Second Affiliated Hospital of Navy Medical University (No. 2017SL627) and informed consent was taken from all the patients. Sample collection was conducted at the surgery unit of Second Affiliated Hospital of Navy Medical University from March 2018 to December 2020. Most patients had locally advanced disease. The detailed clinical characteristics of all patients in the CRC group are listed in *Table 1*. Blood was collected from all CRC patients before partial colorectal surgery and post-surgically from a subset of patients (n=32), in whom post-surgical blood samples were available (taken within 7–12 days after surgery). Blood specimens (5 mL) were collected in Ethylene Diamine Tetraacetic Acid (EDTA) tubes, kept at 4 ℃ for 2 h, and centrifuged at 1,500 ×g for 10 min at 4 ℃ within 4 h of withdrawal. Following immediate second centrifugation (12,000 ×g for 10 min at 4 ℃), the plasma samples were collected and kept in aliquots at –80 ℃. In total, 32 pairs of CRC tissue and paracancerous tissue specimens were surgically removed in Second Affiliated Hospital of Navy Medical University and stored at –80 ℃.

RNA purification and reverse transcription

Total RNA was extracted from sera or tissues using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instructions. A One Step Real Time RT-PCR Kit (Takara, Dalian, China) was used for reverse transcription.

Analysis of EGFR-AS1 gene expression

EGFR-AS1 levels were determined from the sera or tissues of study participants. The primers were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3'. EGFR-AS1, forward 5'-CCATCACGTAGGCTTCCTGG-3' and reverse 5'-GCATTCATGCGTCTTCACCTG-3'. Quantitative real-time PCR was performed on a 7900HT (ABI, Arlington, USA) with SYBR Premix Ex Taq^{TM} (Takara, Dalian, China). PCR amplification was performed at 95 ℃ (10 min), followed by 40 cycles of 95 ℃ (30 s), 95 ℃ (10 s), and 60 ℃ (34 s). The relative levels of EGFR-AS1 in circulation were determined by the $2^{-\Delta\Delta Cq}$ method (22). Amplification of the appropriate product was confirmed by melting curve analysis.

Statistical analysis

To determine whether the plasma EGFR-AS1 levels were normally distributed, the Shapiro-Wilk test was performed, which indicated non-normal distribution. Thus, the EGFR-AS1 levels were presented as the median and range with the first and third quartiles. The Mann-Whitney U test was employed to compare the EGFR-AS1 levels between the study groups and determine associations with clinical features. Receiver operating characteristic (ROC) curve analysis was carried out to examine the diagnostic potential of plasma EGFR-AS1. Pre- and post-operative levels of EGFR-AS1 were compared in a subset of patients with

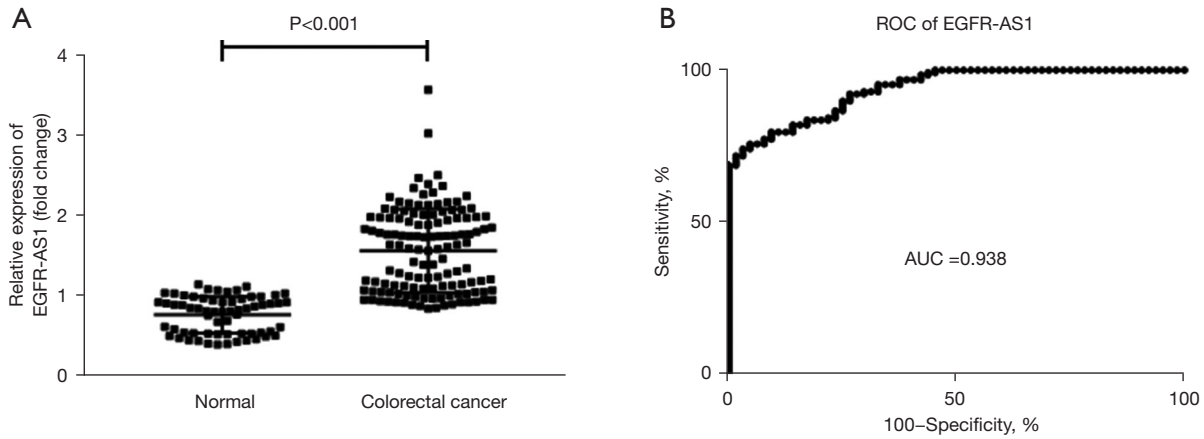

Figure 1 Plasma EGFR-AS1 levels in colorectal cancer and control cases. (A) Plasma EGFR-AS1 levels in case and control groups. The box plots depict the medians and ranges (with the first and third quartiles). The Y-axis represents EGFR-AS1 levels (log scale) relative to GAPDH. (B) ROC curve analysis of the diagnostic value of circulating EGFR-AS1 in CRC. EGFR-AS1, epidermal growth factor receptor antisense RNA 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROC, receiver operating characteristic; AUC, under the curve; CRC, colorectal cancer.

available data (n=32) using the Wilcoxon test. SPSS v.25 (SPSS, USA) was utilized for data analysis, with P<0.05 indicating statistical significance.

Results

Diagnostic value of EGFR-AS1 in CRC

Plasma EGFR-AS1 levels were measured in all study participants. As shown in *Figure 1A*, circulating EGFR-AS1 levels were higher in CRC cases compared to CRC-free control patients (median levels relative to GAPDH, 1.578 *vs.* 0.780, P<0.001). The ROC curve in *Figure 1B* illustrates the diagnostic power of circulating EGFR-AS1, with an area under the curve (AUC) of 93.8% (P<0.001).

Associations between EGFR-AS1 levels and clinicopathological parameters

The associations between plasma EGFR-AS1 levels and clinicopathological parameters in CRC patients were evaluated (*Table 1*). The results revealed that individuals with larger tumors (≥5 cm) exhibited significantly higher plasma EGFR-AS1 levels compared to those with smaller tumors (<5 cm, relative median levels 1.739 *vs.* 1.290, P<0.001, *Figure 2A*). Accordingly, the expression of serum EGFR-AS1 in stage III/IV CRC was higher than that in stage I/II CRC (relative median levels, 1.691 *vs.* 1.412, P=0.003, *Figure 2B*). Similarly, patients with no lymphatic metastasis (N0) exhibited lower plasma EGFR-AS1 levels compared to those with lymphatic metastasis (N1–3), although statistical significance was not reached (median relative levels 1.417 *vs.* 1.614, P=0.718). No associations were identified between plasma EGFR-AS1 and other parameters, including patient age and gender.

Circulating EGFR-AS1 may be a marker of tumor load

Pre-and post-operative levels of circulating EGFR-AS1 were compared in a subset of CRC patients (n=32). The results showed that EGFR-AS1 levels declined markedly following surgical tumor removal, from 0.544–2.077 to 0.493–0.995 [relative median levels, 1.192 *vs.* 0.692 (pre-surgery), P<0.001, *Figure 3A*]. In the vast majority of individuals, plasma EGFR-AS1 levels were decreased following surgery. These data indicated that circulating EGFR-AS1 may be an indicator of tumor burden (*Figure 3B*).

Differential expression of EGFR-AS1 in CRC tissues and paracancerous tissues

The expression of EGFR-AS1 in the CRC tissues of 32 surgical patients was significantly higher than that in paracancerous tissues (n=32, median levels relative to GAPDH, 1.336 *vs.* 0.487, P<0.001) (*Figure 4*). This result confirmed that EGFR-AS1 may play a key role in the early diagnosis of CRC.

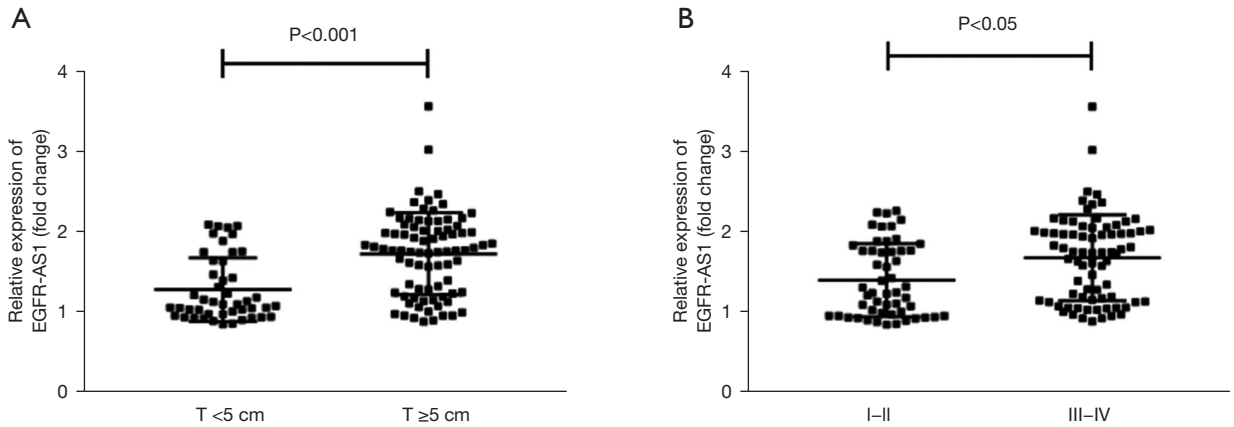

Figure 2 Associations between EGFR-AS1 levels and clinicopathological parameters. (A) Patients with larger tumors (≥5 cm) exhibited significantly higher plasma levels of EGFR-AS1 compared to those with smaller lesions (<5 cm). (B) The expression of serum EGFR-AS1 in stage III/IV CRC was higher than that in stage I/II CRC. EGFR-AS1, epidermal growth factor receptor antisense RNA 1; CRC, colorectal cancer.

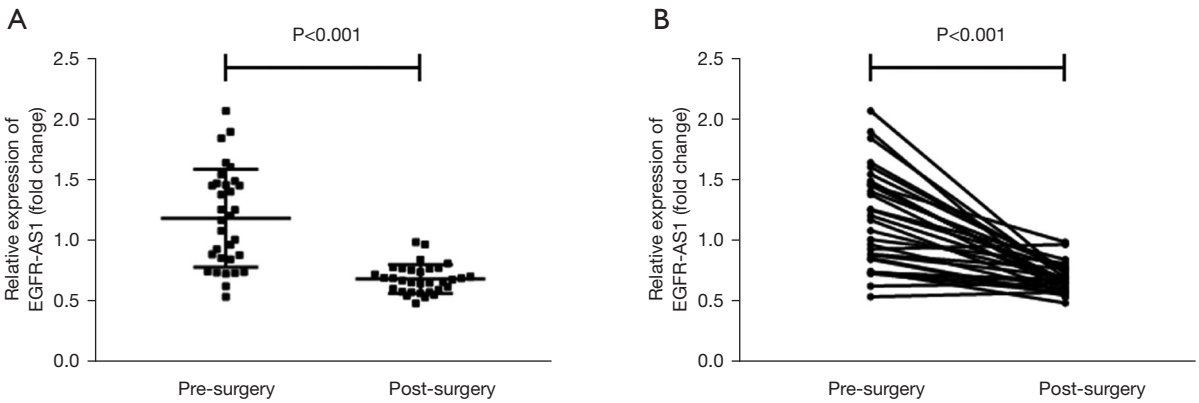

Figure 3 Circulating EGFR-AS1 were compared in pre-and post-operative CRC patients. (A) Pre- and post-operative plasma EGFR-AS1 levels in CRC in a subset of cases (n=32). (B) EGFR-AS1 level changes after surgery in individual cases. EGFR-AS1, epidermal growth factor receptor antisense RNA 1; CRC, colorectal cancer.

Discussion

Cancer biomarkers represent objectively quantifiable compounds in cells, tissues, and/or body fluids, and reflect the occurrence or prognosis of cancer. Since plasma constitutes the commonest specimen type for clinical diagnosis, multiple reports have focused on identifying efficient circulating biomarkers, with good availability, no requirement for invasive techniques, and low cost (24). Recently, circulating miRNAs were proposed as stably quantifiable and as reliable molecular markers of several cancers (25). In addition, typical circulating amounts of lncRNAs in cancer patients are currently considered a hot topic in molecular target research (22). Timely diagnosis and treatment of cancer, as well as early detection of precancerous lesions, including benign and adenomatous polyps, may significantly prolong the survival of cancer patients (26). The above results showed that EGFR-AS1 could not only discriminate CRC cases from healthy individuals but also help to diagnose CRC at both early and advanced stages, indicating its remarkable diagnostic value in differentiating various disease stages.

The lncRNA EGFR-AS1, found at chromosome 7p11.2,

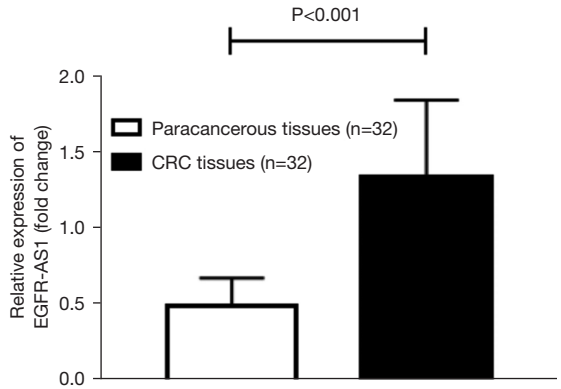

Figure 4 Differential expression of EGFR-AS1 in CRC and paracancerous tissues. EGFR-AS1, epidermal growth factor receptor antisense RNA 1; CRC, colorectal cancer.

constitutes an antisense transcript of EGFR. EGFR-AS1 and EGFR share a complementary sequence, which provides a basis for EGFR modulation. EGFR plays an essential role in the progression of multiple malignancies, including pulmonary, renal, gastric, and CRCs. In lung cancer, the specific EGFR suppressor gefitinib improves patient survival. Meanwhile, elevated EGFR amounts detected in renal cancer are closely associated with tumor development and metastasis (27). High EGFR levels induce several downstream pathways in cancer, e.g., the MAPK, PLCγ, STAT, and PI3K/AKT pathways (28). Also, EGFR-AS1 may exert a potential function in tumor development and survival via miRNA-133b sponging and EGFR/STAT3 axis modulation in CRC (29). However, the research about those of EGFR-AS1 in CRC remains largely unclear. Here, we have carried some studies, loss-of-function investigations disclosed that EGFR-AS1 silencing impaired CRC cell proliferation, migration and invasion and ascended cell apoptosis, which exhibited the oncogenic property of EGFR-AS1 in CRC.

In the present study, we examined the diagnostic and prognostic value of circulating EGFR-AS1 in CRC. Elevated circulating EGFR-AS1 levels were detected in CRC cases compared with endoscopy-verified CRC-free individuals. In addition, individuals with larger tumors (≥5 cm) exhibited higher plasma EGFR-AS1 levels compared to those with smaller lesions (<5 cm). Finally, plasma EGFR-AS1 levels were markedly reduced after surgical resection of colorectal tumors, as shown in a patient subset.

The current study had some limitations that should be noted. Firstly, only 128 CRC cases were examined, indicating low statistical power. Secondly, the mechanism by which EGFR-AS1 affects CRC was not explored. Thirdly, as a single-center trial, selection bias is unavoidable.

Indeed, many circulating lncRNAs remains have poor performance when taken individually. Several lncRNAs reportedly have either poor sensitivity or poor specificity towards a specific cancer type, affecting their potentials as diagnosis biomarkers. Overall, while the research of circulating lncRNAs is still at an early stage, the emergence of new technologies will improve their detection, specificity, and potential in clinical applications which will allow the early and accurate detection of cancer.

In conclusion, the present study confirmed previous data suggesting increased circulating EGFR-AS1 levels in CRC patients. However, this study also provided novel insights. Firstly, the establishment of a defined control group is considered to be important in determining the exact role of EGFR-AS1 in CRC. Indeed, establishing appropriate control groups is a universal challenge in case-control studies (29). Therefore, confirming the absence of malignancy in control individuals in the current study indicated that the obtained expression levels were accurate. Secondly, EGFR-AS1 expression was analyzed with respect to tumor load; following surgery, plasma EGFR-AS1 levels were decreased substantially in most patients, indicating that a significant proportion of plasma EGFR-AS1 originates from the tumor tissue. Furthermore, we examined the expression of EGFR-AS1 in 32 cases of paracancerous and CRC tissues and found that its expression in cancer was significantly higher than that in paracancerous tissues, further confirming that EGFR-AS1 in cancer tissues can be released into the plasma. The changing trend of EGFR-AS1 in cancer tissue and plasma is consistent, confirming that EGFR-AS1 can be used as a practical non-invasive biomarker. Despite certain limitations, including the small sample size and imbalanced case numbers in the subgroups, the present study revealed EGFR-AS1 as a marker with potential diagnostic usefulness in CRC. It may also be relevant in the assessment of therapeutic efficiency, which requires further investigation.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-968/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-968/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-968/coif). The authors have no conflicts of interest to declare.

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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethical Review Board of Second Affiliated Hospital of Navy Medical University (No. 2017SL627) and informed consent was taken from all the patients.

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