J Surg Med. 2025;9(7):104-108.
https://doi.org/10.28982/josam.8069

JOSAM

Journal of Surgery and Medicine

e-ISSN: 2602-2079

Research article
https://jsurgmed.com/

CDC42 and EZH2 are overexpressed in colorectal cancer: Are they minimal invasive diagnostic markers?

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Ethics Committee Approval
The study was approved by the Ethics Committee of SANKO University (2019/15-01). All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

Financial Disclosure
This research was funded by TÜSEB with project grant no. TA-4614.

Published
2025 July 11

Abstract

Background/Aim: Bioinformatics methods have been used to show that cell division cycle 42 (CDC42) and enhancer of zeste homolog 2 (EZH2) have potential oncogenic effects in colorectal cancer (CRC). In this study, we performed experimental validation of these genes.
Methods: We considered the possible role of CDC42 and EZH2 genes in SW480 and SW620 cells. Furthermore, blood samples were gathered from CRC patients and healthy controls to compare CDC42 and EZH2 levels, and relative mRNA and protein levels were measured.
Results: CDC42 and EZH2 expression levels were significantly increased in the SW480 and SW620 cell lines when compared with normal CRL-1790. In addition, when we examined CDC42 and EZH2 expression levels in blood samples of 20 CRC patients and 20 healthy controls by RT-qPCR, the levels of CDC42 and EZH2 were significantly upregulated in patients with CRC compared with healthy control subjects. Similar results were obtained in terms of the protein expression levels of CDC42 and EZH2.
Conclusion: These data reveal that CDC42 and EZH2 are significantly overexpressed in CRC. Considering that high gene and protein expression levels of CDC42 and EZH2 were found in the serum of patients suffering from CRC, these two genes may be developed as minimally invasive diagnostic markers for CRC detection.

Keywords: colorectal cancer, CDC42, EZH2, noninvasive biomarker

How to cite: Denkçeken T, Onur E, Şahin M, Milli R. CDC42 and EZH2 are overexpressed in colorectal cancer: Are they minimal invasive diagnostic markers? J Surg Med. 2025;9(7):104-108.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world and one of the leading causes of cancer-related deaths for both males and females [1]. Nearly 60% of patients with CRC develop metastases, which is a major cause of mortality [2]. The identification of cancer-related genes to define critical events in the development and progression of CRC is therefore extremely valuable.

Recent advances in bioinformatics analyses open up exciting new opportunities for understanding cancer genetics. Via the use of high-throughput sequencing and other technologies, scientists are now able to analyze vast amounts of genetic data, uncovering patterns, biological processes, signaling pathways, molecular functions, and relationships that were previously unknown [3,4]. In our previous study, expression profiling was downloaded from Gene Expression Omnibus (GEO) databases to determine potential biomarkers that may play an important role in CRC. Significant genes were determined via computational and bioinformatics analysis, and hub genes were verified using The Cancer Genome Atlas (TCGA) [4]. In the present study, we examined the expression and protein levels of cell division cycle 42 (CDC42) and enhancer of zeste homolog 2 (EZH2), which are among the hub genes determined to play a role in CRC.

CDC42, a member of the Rho GTPases family, is involved in the regulation of critical cellular functions. Some of those functions are cell cycle control and metastasis, rearrangement of the actin cytoskeleton, intracellular trafficking, cell fate determination, cell cycle control, cell polarity, and gene transcription [5,6]. Recently, accumulating evidence has suggested that CDC42 is highly expressed in approximately 60% of incidences in human CRCs and plays important roles in cancer development and progression [7,8]. This situation suggests a potential role of this gene in tumor development.

EZH2 is a core component of the polycomb repressor complex 2 and mediates gene silencing. EZH2 has been found to be over-expressed in many malignancies [9-12]. EZH2 downregulation can reduce growth of invasive breast carcinoma [13], tumor angiogenesis [14], and in vitro cell migration/invasion of CRC cell lines [15]. Various studies have elucidated the complex role of EZH2 in biological processes and cancer-related events.

In our study, we used RT-qPCR and enzyme-linked immunosorbent assay (ELISA) to examine the possible role of CDC42 and EZH2 gene and protein expressions in SW480 and SW620 according to the CRL-1790 cell lines. We detected that CDC42 and EZH2 were strongly upregulated in cancer cell lines relative to the control. In addition, we investigated CDC42 and EZH2 expressions in blood samples of 20 CRC patients and 20 controls, finding that they were also upregulated in circulation. These findings suggest that dysregulation of CDC42 and EZH2 expression stimulates the oncogenic potential of CRC and that they can be developed as potential diagnostic biomarkers. Based on these results, we conclude that CDC42 in blood may serve as a viable and available biomarker for CRC diagnosis and prognosis.

Materials and methods

Cell culture

CRC cell lines SW620 (metastatic), SW480 (pre-metastatic tumor), and CRL-1790 (normal colon) were purchased from the American Type Culture Collection for in vitro analysis. They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100μg/mL streptomycin at 37°C in a 5% humidified CO_2 atmosphere. RT-qPCR and ELISA were used to determine the expression and protein level, respectively.

Clinical samples

Human CRC blood samples (n=20) and age- and sex-matched healthy controls (n=20) were collected from SANKO University, Faculty of Medicine, Department of Gastroenterology. G-power 3.1.9.4 was utilized for sample size calculation and power analysis. Based on the difference in means between the two groups, with a significance level (α) of 0.05 and a test power (1-β) of 0.8, it was determined that the power of the test was 80% and the minimum required sample size was 7 in each group, which indicated that the number of individuals in the present study was largely sufficient to detect differential expression of genes. The patient group consisted of individuals who did not receive antitumor therapy and who had undergone colonoscopy and histopathological confirmation, while controls were those who presented to primary care outpatient clinics without gastrointestinal symptoms. This study was reviewed and approved by the Ethics Committee of SANKO University (2019/15-01). The patients and controls provided their written informed consent to participate in this study. The research was conducted according to the ethical standards of our institution and the 1964 Helsinki Declaration and its later amendments.

Real-time PCR assays

Total RNA was isolated from cells and blood samples using the Quick-RNA Miniprep Plus Kit (Zymo Research). RNA isolates concentration was checked, and 5–10 ng/μL of RNA was utilized for cDNA synthesis using specific primers. For expression analysis of CDC42 and EZH2 markers by RT-qPCR, 5 μg of the RNA samples was reverse transcribed to utilize reverse transcriptase OneScript® Plus cDNA Synthesis kit (ABM), and SYBR green-based RT-qPCR was conducted with specific primers. Relative expressions of genes were normalized to GAPDH references, and fold changes between groups were calculated using the $2^{-\Delta\Delta Ct}$ method and represented as log2 fold change [16].

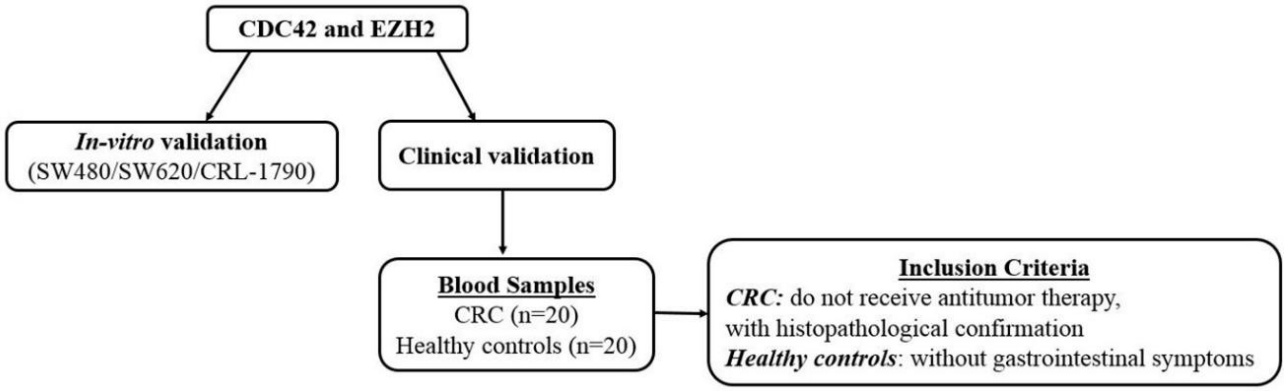
ELISA

Expression of CDC42 and EZH2 marker proteins in serum obtained from CRC patients was determined by ELISA kits (Cloud-Clone). Protein concentrations in serum samples were also measured with ELISA kits.

Statistical analysis

Statistical analysis was done using RStudio. All data are presented as the means and standard deviations of three independent experiments. The t-test was used in paired group comparisons for normally distributed samples, one-way ANOVA was used in three-group comparisons, and the Mann-Whitney U test was utilized for non-normally distributed samples.

Figure 1: Flow diagram of study and patients



Results

The study and patient flow diagram is shown in Figure 1. CDC42 and EZH2 expression was significantly increased in the SW480 and SW620 cell lines when compared with normal CRL-1790 cells. CDC42 was expressed significantly higher in the SW620 (P=0.0011) and SW480 (P<0.0001) cell cultures (Figure 2A). EZH2 was also expressed significantly higher in the SW620 (P=0.0079) and SW480 (P=0.0073) cell cultures (Figure 2B). In addition, the CDC42 and EZH2 expression levels in the blood samples of 20 CRC patients and 20 healthy controls were also examined by RT-qPCR. The levels of CDC42 and EZH2 were significantly upregulated in patients with CRC compared with healthy control subjects. It was determined that CDC42 mRNA expression significantly increased in the CRC blood samples (P=0.0355) (Figure 3A), as did EZH2 mRNA expression (P=0.0422) (Figure 3B). Similar results were obtained in terms of the protein expression levels of CDC42 and EZH2. ELISA indicated that, compared with the healthy control, the serum CDC42 (P=0.005) and EZH2 (P=0.0004) were significantly increased in the CRC blood samples (Figure 4A–4B). These data revealed that CDC42 and EZH2 are significantly overexpressed in CRC.

Figure 2: (A) Relative CDC42 expression in CRC cell lines to CRL-1790 (three replicates per group), (B) Relative EZH2 expression in CRC cell lines to CRL-1790 (three replicates per group) by RT-qPCR (*P<0.05, ** P<0.01, ****P<0.0001).

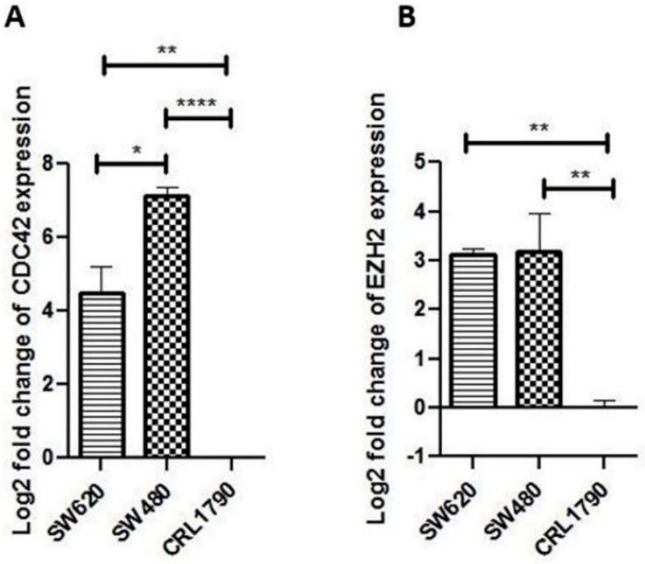


Figure 3: (A) Relative CDC42 expression in blood samples by RT-qPCR, (B) Relative EZH2 expression in blood samples by RT-qPCR (*P<0.05).

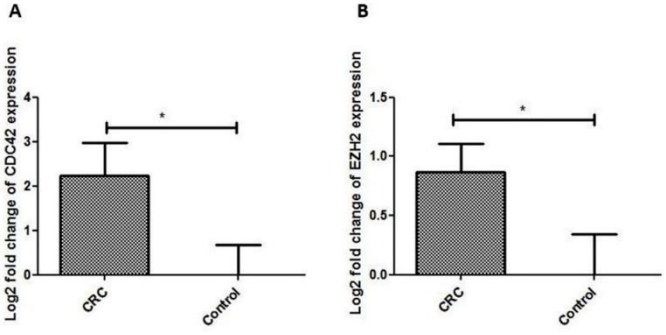
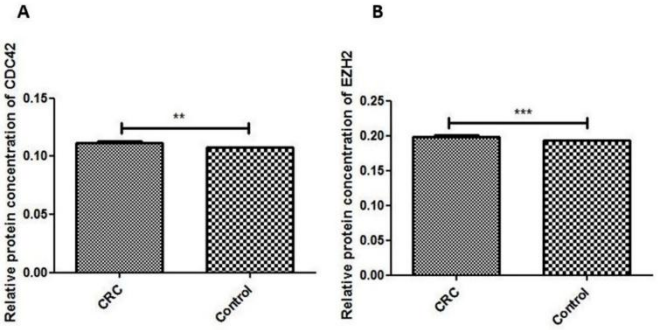


Figure 4: (A) Relative protein concentration of CDC42 in blood samples by ELISA, (B) Relative protein concentration of EZH2 in blood samples by ELISA (**P<0.01, ***P<0.001).



Discussion

CRC is the third most common cancer worldwide, representing the second most common cancer in women and the third most common cancer in men. There were nearly 2 million new cases of CRC in 2020 [17]. With the improvement of high-throughput sequencing and screening technologies, CRC treatment through gene-targeted therapy, which requires cancer-associated biomarker identification, has become a novel potential approach. In this study, the gene and protein expression levels of the CDC42 and EZH2 genes, which we previously determined to play a role in CRC using bioinformatics methods [4], were determined in CRC cell lines and blood samples.

CDC42 is a small GTPase involved in multiple cellular processes such as cell cycle control, gene expression, cell migration, invasion, and metastasis whose aberrant expression and activity have been shown previously in different cancer types [18,19]. Since EZH2 regulates cell cycle progression, dysregulation in EZH2 accelerates cell proliferation and prolongs cell survival. This situation can indirectly lead to carcinogenesis and cancer development. EZH2 overexpression has been identified in many cancer types [20,21]. Therapies targeting

EZH2, which is associated with multiple diseases and plays a role in several biological processes, are an important strategy in the treatment of various types of cancer and diseases.

In this study, we used two CRC cell lines and one control cell which were isolated from normal human colon tissue to investigate the expression of CDC42 and EZH2 in CRC. We found that the CDC42 and EZH2 expression levels were significantly increased in the SW480 and SW620 cell lines when compared with normal CRL-1790 cells. These results are consistent with the literature [22-24]. Interestingly, when compared to the normal cell line for both genes, much higher expression was observed in the pre-metastatic tumor cell line than in the metastatic one. The literature describes a similar situation in studies conducted with these two cell lines, where it was determined that the expression of miR-26a, miR-26b, p27, and ZNF561-AS1 in pre-metastatic cells was higher than in metastatic cells [25-27]. However, there has been no discussion about this trend. These findings suggest that these genes serve as a promoter in pre-metastatic cells and that when the cell becomes metastatic, it may transfer its function to another gene with a decrease in its expression. This also raises the possibility of CRC being diagnosed with these genes while it is still in the pre-metastatic stage. This interesting phenomenon will be evaluated in more detail in future studies.

The present study also examined CDC42 and EZH2 expression levels in blood samples of CRC patients and healthy controls. CDC42 and EZH2 were significantly overexpressed in CRC patients compared with healthy controls. Similar results were obtained in terms of the protein expression levels of CDC42 and EZH2. These data suggest that CDC42 and EZH2 act as predictive markers in CRC. In the literature, validation studies of the mentioned genes have been mostly carried out on frozen tissues or fresh tissues. The results obtained in previous studies conducted with CRC patients' tissues are compatible with the results of our study, and the expression levels of both genes have been found to be high [7,28,29]. Our literature review only identified one validation study of the CDC42 gene in blood samples [30]. The results of that study are also compatible with our results. However, no study was found investigating the EZH2 gene in the circulation of CRC patients. Considering that the present study found high expression levels of CDC42 and EZH2 in the serum of patients suffering from CRC, these two genes may be developed as minimal invasive diagnostic markers for CRC detection. To fully understand the role of CDC42 and EZH2 in CRC, a longitudinal study design with a large sample set is needed, as well as studies in mouse and cell line models. This will be a challenging but promising task.

Limitations

The main limitation of our study is that it included a single ethnicity and a small number of participants. Our findings thus need to be supported by larger studies. Despite these limitations, this study is still an important preliminary step and provides the basis for future research.

Conclusion

In conclusion, CDC42 and EZH2 were found to be upregulated in CRC. Our findings also suggest that these genes may play a diagnostic role as novel biomarkers in CRC.

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