The Value Of Tissue Retinoblastoma Gene (RB1) Expression Levels In Colorectal Cancer And Inflammatory Bowel Disease

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ABSTRACT
Background: Colorectal cancer (crc) is one of the most important and prevalent digestive tract malignant tumors with poor prognosis. Cancer biomarkers play a crucial role in outlining the prognosis of a disease independently of any treatment (known as prognostic biomarkers) or in predicting how a cancer will respond to a specific treatment, which helps anticipate treatment outcomes (referred to as predictive biomarkers). Retinoblastoma (Rb1) represents a critical tumor suppressor gene in different tumor types, governing diverse cellular processes implicated in cancer biology Subjects and Methods: 49 paired colorectal tumors and non-tumor marginal tissues, as well as 21 tissue biopsies taken from patients with inflammatory bowel disease. Total RNA was extracted from the samples and cDNAs were synthesized. Their expression was quantified by qRT-PCR. Results: We observed significant upregulation of RB1 in the colorectal tumors compared with non-tumor as well as inflammatory tissues sample from IBD patients. RB1 expression was significantly elevated with higher tumor marker (CA19-9 and CA125). ROC curve analysis showed that RB1 expression levels could discriminate tumor from non-tumor and IBD. Conclusion: RB1 expression levels could discriminate colorectal tumors from non-tumor tissues, as well as from IBD, highlighting their potential as biomarkers for colorectal cancer development

Introduction

Colorectal cancer (CRC) is the third most common diagnosed cancers worldwide, of which males rank third and females rank second[1] with about 1.9 million new patients, and caused 900,000 deaths per year [2] . In Egypt, it is estimated to rank as the eighth most common cancer diagnosis in some reports. The incidence rate is estimated to be 9.8 per 100,000 cases[3].

In recent decades, CRC incidence rate is increasing due to lifestyle changes including dietary habit, obesity, smoking, inflammatory bowel disease, diabetes mellitus and lack of physical activity [4]. Distant metastasis and recurrence of disease are the cause of most CRC-related deaths [5]. Therefore, it is essential to identify novel biomarkers closely linked to the disease morbidity that could hold potential as diagnostic and/or prognostic biomarker for the disease [6].

IBD has a higher risk of developing gastrointestinal cancer, especially colorectal cancer (CRC) [7]. It is ranked as the third-highest risk factor for development of CRC [8]. The risk of CRC increases with the duration of IBD and the anatomic extent and severity of the disease [9].

Retinoblastoma (Rb1) represents a critical tumor suppressor gene in different tumor types, governing diverse cellular processes implicated in cancer biology [10]. Dysregulation or deletion in RB1 contributes to the development and progression of various cancers making it a prime target for therapeutic intervention. [11].

Aim of Work

This study aimed to assess the diagnostic value of tissue RB1 expression levels in patients with CRC in different stages of the disease.

Subjects and methods

Patients

After obtaining the approval from Institutional Research Board (IRB) Committee of Mansoura Faculty of Medicine, Mansoura University, a written informed consent will be obtained from all patients to be included in this study who present to Tropical Medicine Department at Mansoura University Hospital in collaboration with Surgical Department at Oncology Center, Mansoura University and clinical pathology Department. Study will be carried out for two years from 2021 to 2023 and will include 70 subjects divided into two groups.The first group contained 21 patients with IBD, and the second group contained 49 patients with colorectal cancer.

Inclusion criteria for the study were patients with pathologically confirmed colorectal cancer and cases with pathologically confirmed IBD.

Exclusion Criteria for this study were patients with extra colonic malignancy or lack of histological diagnosis for CRC and Patients on chemotherapy or radiotherapy.

all the patients were subjected to complete history taking , physical examination, laboratory investigations including complete blood picture , liver function tests (serum albumin, serum bilirubin total& direct, SGPT,SGOT, prothrombin time.), serum creatinine , tumor markers (CEA-CA19),radiological investigations as pelvi abdominal us and dynamic study (CT-MRI) ,colonscopy ,tissue biopsy and blood samples.

Technique

For RB1 gene, two samples were taken from each patient of CRC, one from the diseased tissue and the other from the adjacent nonmalignant tissue (control sample) and one sample taken from IBD patient. Each sample was evaluated for RB1 gene expression analysis.

The second portion will be transferred to RNA preservative media. For gene expression analyses, biopsies were immediately stored in RNA later and kept at 4°C overnight. The following day biopsies were frozen and stored at -80°C then Total tissue RNA extraction will be done using TRIZOL method and Total RNA will be reversed, using reverse transcriptase enzyme, into cDNA and finally, cDNA will be assessed using quantitative RT-PCR.

Also, for detection of the prognostic value of these genes in CRC, patients will be classified according to TNM staging by monitoring the correlation between levels of these genes with clinical prognosis, intra operative staging as regards extension of the tumor and metastasis.

RNA extraction, cDNA synthesisand expression analysis

RNX-plus Reagent (SinaClone, Iran) was used to isolate total RNA from tissues according to the manufacturer's instructions. The quality and quantity of isolated RNAs were measured using agarose gel electrophoresis and NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher). A total of 1000 ng RNA was reverse transcribed into cDNA in a volume of 10 µl using PrimeScript RT Reagent kit (TaKaRa) following the manufacturer's protocol. Primers were RNU6 Forward: 5′-CTCGCTTCGGCAGCACAT-3′ and Reverse: 5′-GGAACGCTTCACGAATTTGC-3′, RB1 Forward: 5′-GCGTGCGCTCTTGAGGTT-3′ and Reverse: 5′-AGCCATGCAAGGGATTCCA-3′. The genes expression levels were quantified by qRT-PCR using Master Mix Green (RealQ plus 2x, AMPLIQON) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems).

All reactions were performed in a total volume of 20 µL in duplicate format. The reaction conditions were: Initial denaturation at 95˚C for 10 min, followed by 40 repeats of a cycling stage consisting of 20 sec at 95˚C (denaturation), 30 sec at 58˚C (RB1), at 65 ˚C (RNU6) (primer annealing) and 20 sec at 72˚C (extension). Data were normalized to RNU6 and the relative expression of FER1L4 and RB1 were calculated by the 2-ΔCt method.

Data Analysis

IBM's SPSS statistics (Statistical Package for the Social Sciences) for windows (version 25, 2017) was used for statistical analysis of the data collected. Shapiro-Wilk test was used to check the normality of the data distribution. All tests were conducted with 95% confidence interval. P (probability) value < 0.05 was

considered statistically significant. Charts were generated using SPSS' chart builder and Microsoft Excel for windows 2019. A survival analysis was conducted using Kaplan-Meier graph to assess the median duration for preventing recurrence in the treated patients. Reading from both groups were compared using the log rank test.

Results

As illustrated in table (1), this study included 70 individuals with mean age (50.5 ± 9.9), 41.1% of them were males and the rest 58.9% were females. They classified into 2 groups; the 1st group (contained 21 patients diagnosed as IBD patients and 2nd group contained 49 patients diagnosed as CRC patients. Two groups were matched as regards gender, DM, HTN and smoking. As regards age control group was matched with both IBD and CRC group, while CRC patients were significantly older than IBD patients.

As illustrated in table (2), CRC group contained most frequent finding in radiological investigation was circumferential mural thickening in 42.9% followed by irregular wall thickening in 34.7% and mass in 18.4%. The most common affected site was rectal in 13 patients followed by descending colon on 9 patients and transverse colon in 8 patients. The pathological examination revealed that well differentiated adenocarcinoma in 5 patients, moderate differentiated adenocarcinoma in 28 patients, poorly differentiated adenocarcinoma in 10 patients, undifferentiated adenocarcinoma in 2 patients and invasive adenocarcinoma in 4 patients. AJCC staging revealed that stage I in 7 patients, stage II in 13 patients, stage III in 22 patients and stage IV in 7 patients.

Also As regards RB1 expression, there was a significant elevation of RB1 in CRC group compared to IBD and control group. According to median value of RB1 expression CRC patients were classified into low and high expressor groups (Table 3).

positive tumor markers (CA19-9 and CA125) were more frequent in high RB1 expressor compared to low RB1 expressor (P=0.052 and 0.046 respectively) as shown in table (4).

RB1 expressions for prediction of IBD from non-tumor marginal tissue. ROC showed the RB1 best cut-off values for prediction of IBD were above 0.014. The area under the curve (AUC) was 0.533 (P=0.668) (figure 1).

Table (1): Demographic data among the groups studied:

			IBD N=21	**CRC N=49**	**P**
Age (years)*		Mean ± SD	42.42 ± 5.27	54.51 ± 9.81	P=≤0.001 P1=0.052 P2=0.062 P3=≤0.001
Gender	Male	N/ (%)	10 (47.6%)	17 (34.7%)	P=0.396
	Female	N/ (%)	11 (52.4%)	32 (65.3%)	
DM	Negative	N/ (%)	14 (66.7%)	38 (77.6%)	P=0.199
	Positive	N/ (%)	7 (33.3%)	11 (22.4%)	
HTN	Negative	N/ (%)	17 (81.0%)	33 (67.3%)	P=0.230
	Positive	N/ (%)	4 (19.0%)	16 (32.7%)	
Family history	Negative	N/ (%)	-----	33 (67.3%)	-----
	Positive	N/ (%)	-----	16 (32.7%)	
Smoking	Negative	N/ (%)	16 (76.2%)	38 (77.6%)	P=0.743
	Positive	N/ (%)	5 (23.8%)	11 (22.4%)	

Table (2): Staging, radiological and pathological data of CRC group at presentation:

Parameters		CRC patients (N=)	
		N	%
Radiology	Irregular wall thickening	17	34.7%
	Circumferential mural thickening	21	42.9%
	Irregular luminal narrowing	1	2.0%
	Polypoidal soft tissue	1	2.0%
	Mass	9	18.4%
Site of tumor	Caecal	5	10.2%
	Ascending colon	3	6.2%
	Transverse colon	8	16.3%
	Descending colon	9	18.4%
	Sigmoid colon	5	10.2%
	Rectosigmoid	6	12.2%
	Rectal	13	26.5%
Pathology	Well differentiated adenocarcinoma	5	10.2%
	Moderate differentiated adenocarcinoma	28	57.1%
	Poorly differentiated adenocarcinoma	10	20.4%
	Undifferentiated adenocarcinoma	2	4.1%
	Invasive adenocarcinoma	4	8.2%
T staging	T1	3	6.1%
	T2	6	12.2%
	T3	31	63.3%
	T4	9	18.4%
N staging	N0	19	38.8%
	N1	20	40.8%
	N2	8	16.3%
	Nx	2	4.1%
M staging	M0	20	40.%
	M1	8	16.3%
	Mx	21	42.9%
AJCC staging	Stage I	7	14.3%
	Stage II	13	26.5%
	Stage III	22	44.9%
	Stage IV	7	14.3%

Table (4): Comparison of RB1 expression among studied groups:

Parameter	Non tumor marginal tissue N=49	IBD tissue N=21	CRC tumor tissue N=49	P	P1	P2	P3
RB1 expression	0.015 (0.001-1.072)	0.014 (0.001-0.871)	0.329 (0.015-6.063)	≤0.001	0.690	≤0.001	≤0.001

Table (5): Clinical and laboratory characteristics of high and low RB1 expressing CRC patients.

Parameter		Low expression (N=24)	High expression (N=25)	P
Age*	Mean ± SD	54.7 ± 10.60	54.2 ± 9.20	0.869
Gender	Male	11 (45.8%)	6 (24.0%)	0.108
	Female	13 (54.2%)	19 (76.0%)	
CEA	Negative	12 (50.0%)	9 (36.0%)	0.322
	Positive	12 (50.0%)	16 (64.0%)	
CA19-9	Negative	18 (75.0%)	12 (48.0%)	0.052
	Positive	6 (25.0%)	13 (52.0%)	
CA125	Negative	19 (79.2%)	13 (52.0%)	0.046
	Positive	5 (20.8%)	12 (48.0%)	
Site of tumor	Colon	15 (62.5%)	15 (60.0%)	0.858
	Rectal	9 (37.5%)	10 (40.0%)	
Pathology	Well	3 (12.5%)	2 (8.0%)	0.838
	Moderate	14 (58.3%)	14 (56.0%)	
	Poorly	5 (20.8%)	5 (20.0%)	
	Undifferentiated/invasive	2 (8.4%)	4 (16.0%)	
T staging	T1/T2	2 (8.3%)	7 (28.0%)	0.138
	T3/T4	22 (91.7%)	18 (72.0%)	
N staging	N0	8 (36.4%)	11 (44.0%)	0.867
	N1	10 (45.4%)	10 (40.0%)	
	N2	4 (18.2%)	4 (16.0%)	
AJCC	Stage I/II	9 (37.5%)	11 (44.0%)	0.644
	Stage III/IV	15 (62.5%)	14 (56.0%)	

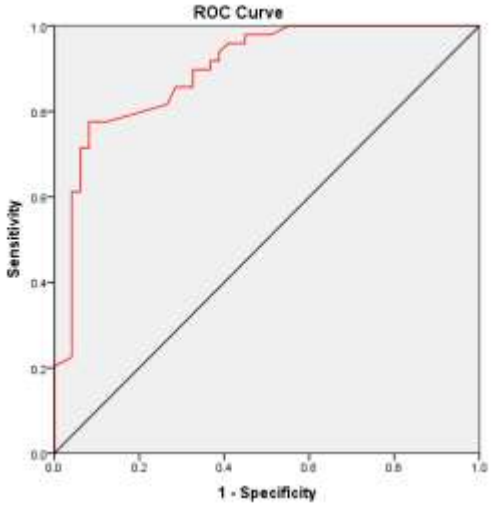

Figure (1) ROC analysis identifies the RB1 expressions for prediction of CRC from non-tumor marginal tissu.

Discussion

Colorectal cancer (CRC) is one of the most important and prevalent diseases with poor prognosis. It ranks second in cancer-related deaths overall and is the leading cause in men younger than 50 years. In addition, a large proportion of CRC incidence and mortality is preventable through the receipt of regular screening, surveillance, and high-quality treatment[11].

So, identification of the pathogenic molecular pathways and determination of therapeutic targets are needed.

RB1 is one of the first described tumor suppressor genes in a wide variety of human cancers. It represents a critical tumor suppressor gene, governing diverse cellular processes implicated in cancer biology [12].

We observed in this study significant upregulation of RB1 in the colorectal tumor compared with their matched marginal non-tumor tissues with the sensitivity and specificity were 81.6% and 73.5%, respectively. Also, we found that RB1 was significantly elevated with higher tumor markers CA19-9 and CA125 but not significantly change with tumor depth, lymph node invasion nor distant metastasis. But in IBD patients we found no significant difference between levels of RB1 in comparison to those with healthy controls but significant elevation in patients with colorectal cancer in comparison to patients with IBD with the sensitivity and specificity were 81.6% and 81%, respectively.

Our study in addition to Ostovarpour et al., 2021 and Collard et al., 2012 had found that RB1 expression is important for CRC development; while works from Ivkovic et al., 2

Ostovarpour et al., 2021 supported our results and for that we can say that the lncRNA FER1L4 and RB1 cooperate in a competing endogenous RNAs (ceRNAs) network involving in cancer biology, where the expression of FER1L4 positively correlates with the RB1 expression.

The discrepancies in findings of the above-mentioned studies can be explained by a number of factors including differences in genetic background and gene environment interactions, the etiology of cancer colon, dissimilar populations, selection of patients and limited sample size.

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