Sys Rev Pharm 2020;11(9): 89-94
A multifaceted review journal in the field of pharmacy

CYP24A1 and *AHR* Gene Expression in Iraqi Colorectal Cancer Patients

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ABSTRACT
Previous studies indicated colorectal cancer (CRC) is the third leading cause of cancer in the world and the second in terms of mortality , CRC is spread between men and women , CRC spreads in different location of the colon and rectum, . Colorectal cancer affects in different age and the number of CRC patient has increased in the past two decades, especially within the younger age. Molecular studies indicated, many changes in genetic, epigenetic and gene expression in many genes contributed to the emergence and development of colorectal cancer. This study aimed to measure the gene expression for CYP24A1 and AHR genes in iraqi patients with CRC, and study age groups and locations of CRC within the colon and rectum. The results showed an overexpression of CYP24A1 gene (6.1) fold change and AHR expression had(1.8) fold change ,and males are more likely to develop colon rectal cancer than women's, and most patient colorectal cancer ranged between 45 to 68 years old .

Keywords: Colorectal cancer, *CYP24A1*, *AHR* gene.
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INTRODUCTION
colorectal cancer (CRC), is the most common malignancy of gastrointestinal system , Colorectal cancer (1.8 million cases, 10.2% of the total) is the third most commonly diagnosed cancer in the word and the second cause of death in the world (881 000 deaths, 9.2%) per year[1] . The risk for developing CRC increases with age and there are suggestions that the distribution of incident cancers in the colorectal is becoming more proximal [2]. the etiology of CRC is complicated and is not clearly identified; however, several risk factors such as family history, increased BMI, cigarette smoking and low physical activity have been associated with the disease [3]. Abnormal regulation of vitamin D metabolism pathways contributes to the pathogenesis of CRC [4]. The most active form of vitamin D metabolite (1α,25-dihydroxyvitamin D3, 1,25[OH]2D3) reduces epithelial cell proliferation and promotes the differentiation in human colon-derived cells by several mechanisms such as cell cycle arrest at G0/G1 phase [5] . Cytochrome P450 Family 24 Subfamily A Member 1(CYP24A1) is a mitochondrial enzyme that catalyzes the 1,25-2D3 (calcitriol) into 24-hydroxylated (1,24,25 Tri hydroxy vitamin3) product that also decreases the anti-proliferative actions of endogenously produced 1,25-2D3 [6] . It's altered expression has been observed in many different cancers, including CRC, and hence is considered to function as an oncogene [7]. In CRC CYP24A1 levels are increased when compared with matched normal tissues. The autocrine/ paracrine growth regulation of colonocytes by the active vitamin D seems to be functional only in the early stages of tumorigenesis, and it becomes impaired during tumor progression, owing to CYP24A1 overexpression. [8]. Numerous ligands for the AHR have been described. Xenobiotics, which are mostly aromatic hydrocarbons (dioxins), tobacco smoke contains a variety of polycyclic hydrocarbons long term environmental exposure results in more extensive toxic effects among which are immune toxicity and carcinogenic to humans which lead AHR gene over expression [9].

Material and method
Collection of the samples
Collected 98 biopsy samples from reviewers to colonoscopy unit at the Gastroenterology and Hepatology Hospital in Baghdad Medical City, in 2019. and the histological diagnosis ware classified biopsy in to three group: healthy, benign tumor and colorectal cancer, according to TNM staging system. Biopsy samples for RNA extraction collected in 1.5ml eppendorf tube containing 1ml TRIzol and freezing immediately under -20 C. Note: Weight of sample not more than 25mg.

RNA extraction and Amplification of Real-Time qRT-PCR
TRIzol reagent (GENEzol; Geneaid; korea,) was used to save tissue (biopsy) samples. RNA extract was made according to the manufacturer's instructions, use (Genomic RNA extraction kit (Z3100) promega, USA). Total RNA was then reverse transcribed using a (GoScript™ Reverse Transcriptase kit, promega, USA). According to the manufacturer's protocol with 3µl of total RNA, 2µl oligoprimer and 1µl Nuclease-Free Water to final volume 5 µl in a tube. Heated in a 70°C water bath for 5 minutes, then mix with 15 µl GoScript master mix and, Heated in a 25°C for 5 minutes, then 42°C for 60 min. final 70°C for 15 min and incubate at -20 °C. For RT-qPCR, the analysis of mRNA levels was performed using SYBR-Green Reagents (GoTaq® qPCR and RT-qPCR Systems, Promega, USA). Amplification conditions as shown in table (1).

Table 1. Amplification conditions for qRT-PCR.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	**95 °C**	**2 min**	**1**
Denaturation	**95 °C**	**15 sec**	**40**
Annealing	**60°C for *CYP24A1* , AHR And *GAPDH***	**1 min**	
Extension	**72°C**	**30 sec**	
and extension	**72°C**	**7 min**	**1**

Gene expression of the target gene *CYP24A1* gene and housekeeping gene *GAPDH* were analyzed using specific primers as shown in table (2).

Table 2, specific primers for qRT-PCR.

Primer	Sequence (5´-3´)	Reference	Annealing temperature (°C)	Product size (bp)
GAPDH	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA	(Nabokina *et al* ., 2014)	60°C	131 bp
AHR	F : ACATCACCTACGCCAGTCGC R : TCTATGCCGCTTGGAAGGAT	Nguyen *et al* .,2017)	60°C	101 bp
CYP24A1	F: CATCATGGCCATCAAAACAAT R: GCAGCTCGACTGGAGTGAC	(Schulz *et al*., 2017)	60°C	56 bp

In the current study were depended to choose $2^{-\Delta\Delta Ct}$ method to determine the differences in the gene expression because it is forceful and suitable [10], so the choice was for this purpose. Real-time PCR was performed in duplicate for each sample; then mean value of them were used at final calculation for mRNA expression level.

Results

Distribution of study samples

In this study, 98 samples for (40 adenocarcinoma, 23 Benign tumor, 35 Apparently healthy) gender and average age as shown in table 3

Table 3: Distribution of study samples.

Type of sample	Number of samples	age	gender	family history No. (%)
Negative control	35	(16- 80)	18 men 17 women	2(5%)
Benign tumor	23	(32- 64)	13 men 9 women	4(17%)
adenocarcinoma	40	38-70) (	27 men 13 women	14 (35%)

Age distribution of peoples in study:

Age distribution of peoples in study as shown in table (4).

Table 4. Age distributions for people in the research samples

Type of sample / Age	Negative control samples = 35	Benign tumor samples = 23	Adenocarcinoma samples = 40
10 -20	2(5.71%)	1 (4.36%)	
20 – 30	3(8.57%)	2 (8.6%)	
30 40	6 (17.14%)	2 (8.6%)	3 (7.5%)
40 – 50	7 (20%)	4 (17.3%)	12 (30%)
50 – 60	7(20%)	6 (26,0%)	13 (32.5%)
60 – 70	7(20%)	5 (21.7%)	12 (30%)
70 - 80	3 (8.57%)	3 (13%)	

Colorectal cancer Locations:

The locations of cancer were distributed as follows cecum 7(17.5%) , ascending colon 2(5%) , transverse colon 6(15%) , descending colon 2(5%) , sigmoid 17(42%) and rectum 6(15%) as shown in figure (1) .

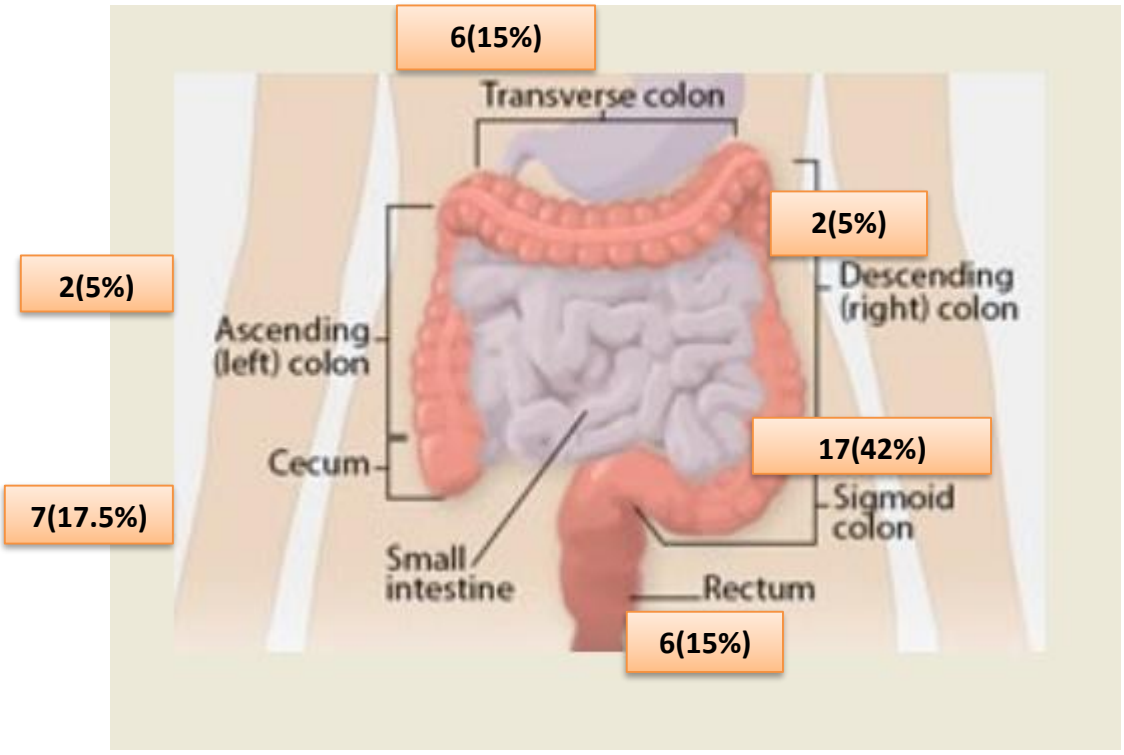

Figure 1. The distribution of cancer regions was shown in this study.

TNM classification

According to the TNM classification, the colorectal cancer samples distributed as follows. Stage IIIB was the most frequent stage in the study =15(40%),while Stage III-A was= 11(27.5%), Stage II-B= 10(25%) Stage II-A= 4(10%) as shown in figure (2).

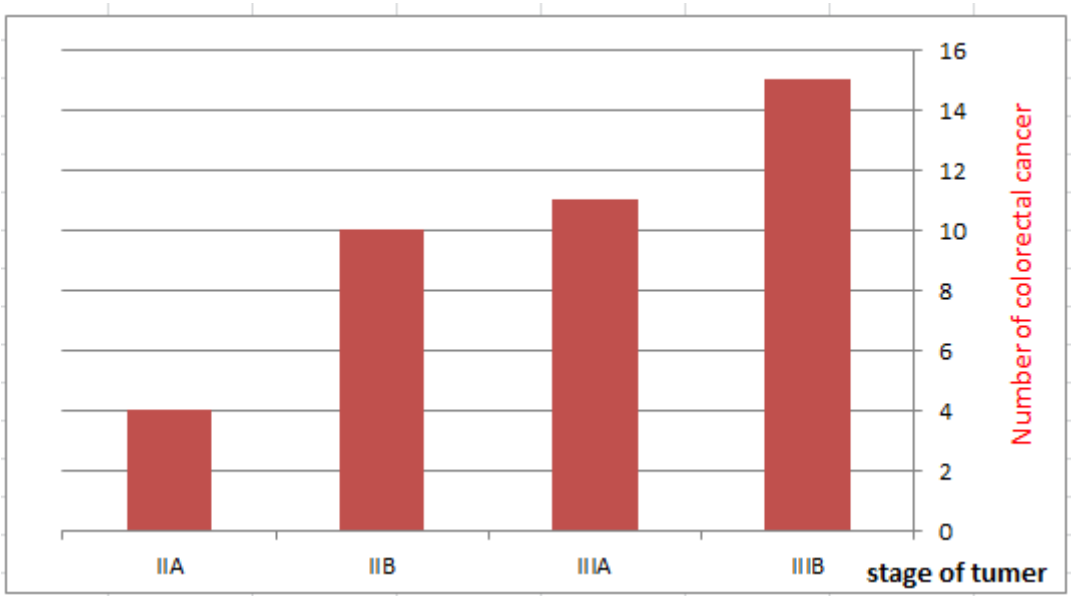

Figure 2. stages of tumor agents' colorectal cancer in this study.

From the above data, it indicates lack of health awareness, in addition colorectal cancer with outs clear symptoms are shown before the worsening of colorectal cancer, and thus leads to difficult treatment.

Gene expression for *CYP24A1* and *AHR* genes

Quantitative Real time PCR assay were validated and performed. The gene expression of 98 tissues was quantified for samples from the three groups: control, benign tumor and malignant of both *CYP24A1* and *AHR* genes. These results showed that gene expression levels for (*GAPDH*, *CYP24A1* and *AHR*). The control samples (apparently healthy) the mean of *GAPDH* gene expression higher than other genes 15.31 (±0.2), where *AHR* gene mean level 22.87 (±0.6), but the CYP*24A1* gene the lowers 30.08 (±2.3). The mean gene expression for colorectal cancer samples defer from control samples (apparently healthy) samples in *CYP24A1* gene expression by 6.1-fold change, while AHR gene had 1.8-fold change as shown in table (5).

Table 5. mean fold change for CYP24A1 and AHR genes with housekeeping gene GAPDH, between control samples (apparently healthy) and colorectal cancer patients

Type of genes	Colorectal cancer samples			Calibrator (apparent healthy)			ΔΔCt	Fold change
	Ct of target gene	Ct of GAPDH	ΔCt	Ct of target gene	Ct of GAPDH	ΔCt		
CYP24A1	**27.17 ±2.83)(**	**15.01 (±0.5)**	**12.16**	**30.08 (±2.3)**	**15.31 (±0.2)**	**14.77**	**2.61-**	**6.10**
AHR	**21.72 (±0.41)**	**15.01 (±0.5)**	**6,71**	**22.87 (±0.6)**	**15.31 (±0.17)**	**7.56**	**-0.85**	**1.80**

The *GAPDH* gene defer 0.3 ΔCt which that head the gene expression have 1.23 fold change as shown in table (6) for this result of *GAPDH* over 1 fold of gene expression, that mean *GAPDH* is not the beast housekeeping gene and we need found other gene as housekeeping gene more stable , or use two or more housekeeping gene for colorectal cancer gene expression . Xu *et al* 2019 referd , given the proliferative drive of malignant cells, many reference genes such as beta-actin (ACTB) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) which play critical roles in cell membrane organization and glycolysis, may be dysregulated in tumors versus their corresponding normal controls [10] .

Table 6: Comparison of *GAPDH* Fold expression between study groups

Group	Means Ct of *GAPDH*	2^{-Ct}	experimental group/ Control group	Fold of gene expression
apparent healthy (control)	15.31	0.246E-4	0.246E-4/0.246E-4	1
Colorectal cancer	15.01	0.303E-4	0.303E-4/0.246E-4	1.23

The result of benign tumor when comber with control found 2.63-fold change for CYP24A1 gene and found 1.58 fold for AHR gene, as shown in table (7).

Table 7. mean fold change for CYP24A1 and AHR genes with housekeeping gene GAPDH, between control samples (apparently healthy) and Benign tumor patients.

Type of genes	**Benign tumor samples**			**Calibrator (apparent healthy)**			**ΔΔCt**	**Fold change**
	Ct of target gene	**Ct of GAPDH**	**ΔCt**	**Ct of target gene**	**Ct of GAPDH**	**ΔCt**		
CYP24A1	**28.45 ±2.64)(**	**15.09 (±0.3)**	**13.36**	**30.08 (±2.3)**	**15.31 (±0.2)**	**14.77**	**1.41-**	**2.63**
AHR	**21.99 (±0.41)**	**15.09 (±0.3)**	**6.90**	**22.87 (±0.6)**	**15.31 (±0.17)**	**7.56**	**-0.66**	**1.58**

Conclusions

The results show highly significant relationship between CYP24A1 gene expression and may be incidence colorectal cancer. this action reduce the protection effect of vitamin D3 against cell proliferation, and may be in the other hand may be play a role in the imbalance of calcium inside the cell (increase) and this activates CAMKII gene which activates HDAC gene, and leads to the lack of supercoiling of the DNA which lead appears many oncogenes for transcription and induce colorectal cancer accrue . AHR gene expression may be increasing a little as response to stress within a colorectal cancer cell, but not a cause for accrue the colorectal cancer. We need a more stable housekeeping gene than *GAPDH* gene for gene expression in colorectal cancer research or use two housekeeping gene.

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