

# Realtime qRT-PCR Expression of P53, KRAS, and human Telomerase genes in circulating tumor cells, as potential biomarkers for early detection of sporadic colorectal cancer

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## Abstract:

**Objectives:** The objectives of present study were to investigate the potential use of mRNA of P53, KRAS, and human telomerase genes in circulating tumor cells as biomarkers for early detection of sporadic cases of colorectal cancer.

**Background:** In spite of colorectal cancer is a curable disease if detected early; it is still the third leading cause of death from cancer. There is an ultimate need for implementing an accurate and noninvasive screening method. The recent advances in molecular genetics have showed that the genes of P53, KRAS and human telomerase play major roles in development of colorectal cancer. Therefore the study of their expression in blood samples might be used as a mean for early detection of sporadic colorectal carcinoma cases.

**Methods:** A case control pilot study in which 20 patients with non-metastatic colorectal carcinoma in Duke's stages (A – C, TNM from 0 – III) and 20 healthy controls were enrolled. Expression of studied genes and the housekeeping gene Glyceraldehyde-3 phosphate dehydrogenase were determined using quantitative reverse transcriptase polymerase chain reaction. The study was performed in duplicate taking 1 µg of total RNA from each sample.

**Results:** The results showed significant overexpression of mRNAs of studied genes in samples taken from cases of proved colorectal carcinoma compared to that of controls group with a statistically significant P- value of <0.001.

**Conclusion:** From the above results, the expression of P53, KRAS, and human telomerase genes may be used as biomarkers in the early detection of sporadic non-metastatic colorectal cancer.

**Keywords:** P53, KRAS, human telomerase genes, Biomarkers, Colorectal cancer

## Introduction:

Colorectal cancer (CRC) remains the third leading cause of cancer related death in men and women in developed countries, despite of increased general awareness [1, 2]. It is a well-known malignancy, and its risk factors are almost well defined [3]. This cancer is a slow progressive with pre-neoplastic lesions (polyps) that can be detected and treated by colonoscopy [3, 4]. This cancer is staged by modified Duke's into stage-A (limited to mucosa), B (limited to the wall), C (with lymphnodes metastasis), and D (with distant metastasis) [5]. While TNM methods staged CRC into four stages I – IV [6]. Early detection and treatment is the best option of management, because of 5-year

survival rates for early cancer Dukes A, B, and C are 95%, 80%, and 60% respectively, while Duke's stage D it is only 10% [5, 6]. The search for presence of circulating tumor cells (CTCs) in blood samples of patients with malignant tumors, including CRC is one the great success in recent advances in molecular genetic studies of cancer [7, 8]. Detection of CTCs is achieved by quantitative reverse transcriptase realtime polymerase chain reaction (qRT-PCR) method by finding of molecular changes in genes that are the cause or the result of malignancy [9, 10]. Depending on new and recent advances in molecular genetic studies many markers has been used in the detection of circulating tumor cells including oncogenes, tumor suppressor genes, and signals transducing genes [8]. In the present study using qRT-PCR, the expression of messenger RNAs (mRNAs) of P53, KRAS, and human telomerase genes of circulating tumor cells (CTC) were chosen for detection of presence of CTCs and indirectly for presence of colorectal cancer

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(CRC), because their mutations or deletions are one of the causes or the results of CRC formation [8]. Therefore, genes of P53, KRAS, and human telomerase (hTE) are reliable target genes for detection of CRC [8]. They can be detected in peripheral blood of patients with CRC by means of qRT-PCR [9, 10]. Gene of p53 is well known tumor suppressor it prevents cells with genetic damage from completion of cell division, induce DNA repair enzymes, and switches cells with irreparable DNA damage into apoptosis [11, 12]. Expression of p53 gene has been seen in most of carcinoma of body organs [9], that makes p53 gene is a good target in the detection of circulating tumor cells and early detection of carcinoma [13, 14]. Kirsten viral oncogene homolog (KRAS) encodes for signals transducing protein. Function of KRAS oncogene is to inhibit apoptosis and induces cell proliferation in parenchymal cells which may result in carcinoma formation [15, 16]. Mutations in KRAS genes are commonly seen in cases of carcinoma of solid organs especially in colorectal cancer [17, 18]. Therefore the KRAS is a good marker for detection of CTCs and indirectly for presence of internal carcinomas including CRC [19]. Telomeres repeated sequences of nucleotides present at the ends of chromosomes protecting them and prevent their fusion [20]. With each cell division the telomere is shortened until reach a state in which the cell division will not possible [20]. Human telomerase gene is a complex lipoprotein composed of two units a catalytic and RNA unit [20]. The RNA unit acts as a template for synthesis of telomere [21]. Activation of human telomerase gene (hTE) has reported by many studies during the evolution of colorectal adenoma from adenoma of low grade dysplasia to adenoma of high grade dysplasia to carcinoma [22]. The finding of highly expressed hTE is a good index for presence of CTCs and internal carcinomas including CRC [21, 22]. Combination genes of P53, KRAS, and hTE may increase the specificity and sensitivity of the detection test [22, 23]. Glyceraldehyde-3 Phosphate Dehydrogenase (GAPD) gene was used as a housekeeping gene for normalization because it is expressed equally in all tissues and its expression remains the same in malignant benign conditions [24, 25]. The first goal of the present study was to detect expression of studied genes by means of qRT-PCR method in blood samples taken from patients with non-metastatic colorectal cancer and controls group. The second aim was to find significant differences in the expression of studied genes in samples taken from cases with CRC relative to that obtained from controls group. The third aim was to evaluate the benefits of using combination of markers in increasing sensitivity and specificity over one marker.

## Subjects and Methods:

The study was a prospective case-control study in which 20 patients and 20 controls were participated. Patients and controls were recruited at the Specialized Hospital of Gastroenterology and Liver Diseases / Baghdad Medical City during the period from June 2014 to March 2015. Pa-

tients group were 20 selected patients with colorectal cancer CRC without distant metastasis (modified Duke's stages (A, B & C) or TNM (I – III), proved by histopathology. They were 12 males and 8 females. The controls group was 20 selected cases without CRC proved by colonoscopy and histopathology and they were 7 males and 13 females. Patients included in the study were presented for the first time complaining from colorectal problems (diarrhea, bleeding per rectum, abdominal pain, passage of mucus with or without blood, etc.), proved to be due to colorectal diseases whether malignant or benign. Patients then underwent colonoscopy and biopsy. A signed consents were taken from all patients participated in the study and the work was approved by Medical College Ethical committee. All cases that have received any form of specific cancer treatment prior to blood sample collection such as radical surgery, chemotherapy and/or radiation were excluded from the study. Cases known to have a second primary tumor other than colorectal cancer and those with uncertain diagnosis were excluded as well.

### Samples collection

Venous blood was aspirated and first 2 milliliters were discarded to reduce contamination with skin. Fifteen milliliters of venous blood was aspirated from each participant into a labeled nuclease free tube containing EDTA to overcome blood clotting. Aspiration was performed under strictly aseptic condition wearing of Powderless (nitrate) nuclease free gloves and facemask.

### Extraction of total RNA and purification

Prior to total RNA extraction, lysis of RBCs was performed using lysis solution from QIAGEN containing saccharose, Tris-HCl, Triton X-100, and MgCl<sub>2</sub> was used. Forty milliliter (40 ml) of solution was added to 15 ml of blood. The steps of lysis were completed according to manufacturer's instructions [26]. After lysis of RBCs, extraction of total RNA was started by a readymade kit "mirVana™ miRNA Isolation Kit with phenol from Ambion". Total RNA extraction was completed according to the manufacturer's instructions [27]. Extracted total RNA then purified from DNA contaminants by the use of "DNA-free kit from Ambion" and according to manufacturer's instructions [28]. Purity and concentration of total RNA was measured by Nano-drop spectrophotometer and a ratio of A260/A280 (1.9 - 2.0) was considered as a good purity [29].

### Reverse transcription into cDNA

High CapacityTaqMan Reverse Transcription Kit from Biosystem of life technologies was used for reverse transcription of total RNA to corresponding coded DNA (cDNA) according to manufacturer's instructions [30]. The concentration and purity of cDNA was also checked with Nano-drop spectrophotometer. Realtime qRT-PCR amplification GAPD reference gene was performed on samples from cDNA to assess their quality as template for realtime qRT-PCR of studies genes [31].

### QRT-Realtime PCR amplification

Realtime PCR amplification of mRNAs P53, KRAS, hTE, and GAPDH genes on samples taken from patients with

colorectal cancer and controls was achieved by realtime MxProP3000 Agilent technologies qRT-PCR [31] using two steps SYBRE GREEN I dye master mix from Biosystem [32] and specific optimized primers. The amplification was performed in duplicate to reduce errors and according

to machine manufacturer's instruction [31].The thermal prolife was designed according to machine manufacturer's instructions with adding some modifications [31]. The mean of Ct value for each specimen was taken for gene expression study.

Table 1: Sequences of primers for qRT-PCR amplification.

	Forward primer	Reverse primer
<b>GAPDH</b>	TGCACCACCAACGGT-‘5 ‘TGC-3	GGCATGGACTGTGGTCT-‘5 ‘GAG-3
<b>P53</b>	GTTCCGAGAGCTGAATG-‘5 ‘AGG-3	TTATGGCGGGAGGTTAGA-‘5 ‘CTG-3
<b>KRAS</b>	AAGGTACTGGTGGAGTA-‘5 ‘TTG-3	GTACTAGAAAAGGTGGTCA-‘5 ‘GAG-3
<b>hTE</b>	TGTCACAGCCTGTTTC‘5 ‘TGGA-3	‘GTTCTTGGCTTTCAGGATGG-3‘5

## Results:

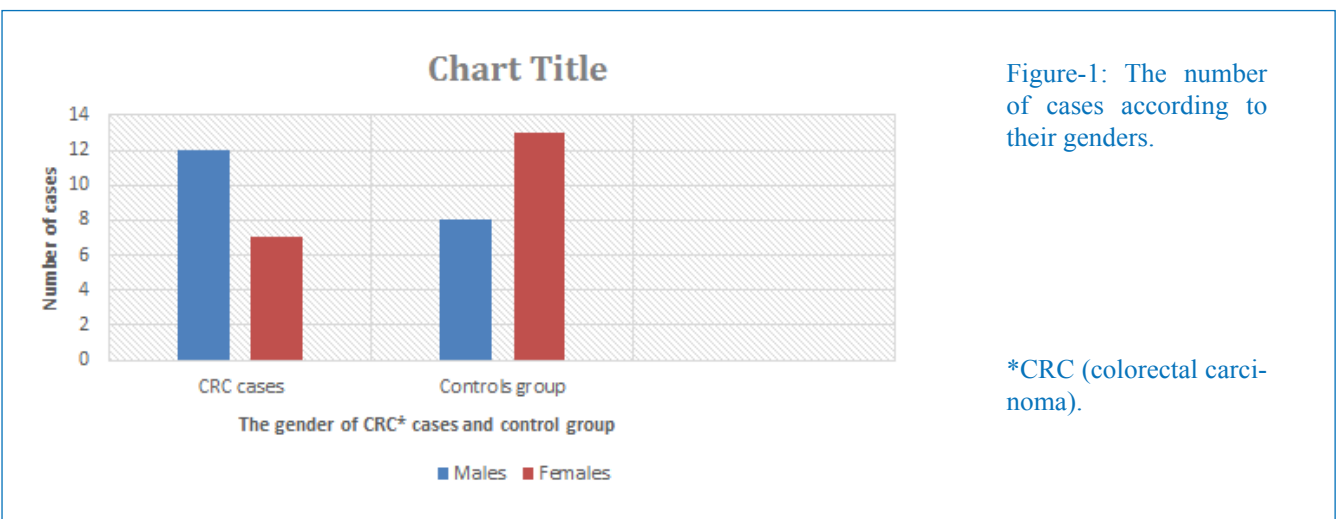
Results of colonoscopy and histopathology on biopsies from CRC cases and controls were considered as a gold standard for the other tests. The details of clinical and

histopathological results were present in (tables 2, and figure-1&2). On comparing results between men and women the P- values were 0.362 for patients and 0.138 for controls, indicating that patients and controls had similar variables in age and sex.

Table 2: Number of cases, mean, range, and SD of age according to gender.

Cases	Sex	Number	Mean age	SD of age
Colorectal ca.*	Males	(60%) 12/20	(45-87) 64.5	9.54 ±
	Females	(40%) 8/20	(39-84) 60.6	11.82 ±
Controls group	Males	(35%) 7/20	(44-88) 63.9	9.85 ±
	Females	(65%) 13/20	(37-85) 59.85	8.93 ±

\*Ca. (cancer).



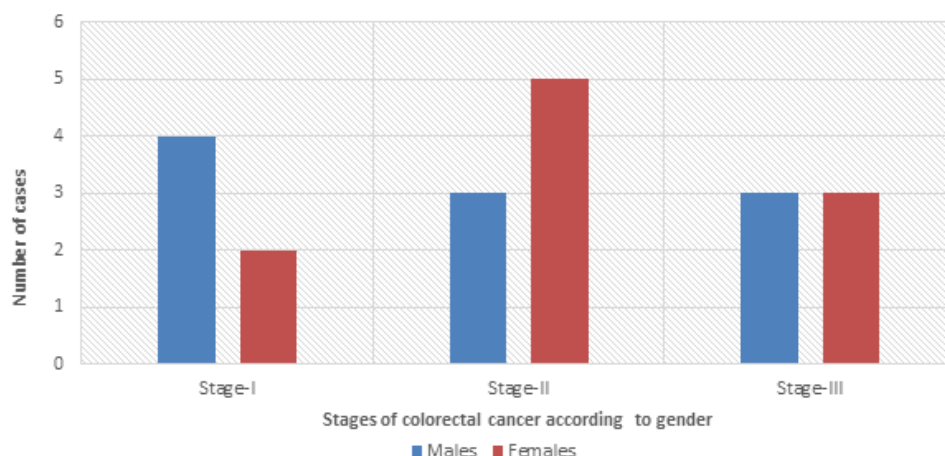


Figure-2: Number and gender of patient according to tumor stages.

### Results of studied markers

In controls group lower limit of each studied gene was determined and taken as a cut-off point [33]. The  $\Delta Ct$  values lower than cut-off point were considered as background fluorescence (false positive), and higher than it as a true results [33]. Differences between calculated  $\Delta Ct$  of patients and healthy groups (controls) in P53, KRAS, hTE were statistically significant ( $P < 0.05$ ). The interval confidence was 95% on calculating the mean  $\Delta Ct$  values in controls group. Not all of the samples from patients with CRC submitted for amplification by realtime qRT-PCR, revealed amplification curves for genes included in the study. The studied genes that were negative for amplification might be deleted especially for p53 tumor suppressor gene [14]. The mRNA of P53 was overexpressed (true positive) in 11 samples of CRC, and in 3 of controls (false positive). It was underexpressed (false negative) in 9 cases of CRC, and in 17 of controls (true negative). The mRNA of KRAS gene was true positive in 15 samples of

CRC, and without any false positive results in controls. It was underexpressed (false negative) in 5 samples of CRC, and true negative in all control samples. The mRNA of human telomerase gene (hTE) was overexpressed (true positive) in 17 samples of CRC, and without any false positive in controls. It was underexpressed (false negative) in 3 samples of CRC, and true negative in all 20 samples from controls. The combination of markers was overexpressed (true positive) in 18 of CRC, and false positive in 1 of controls. The combination of markers was underexpressed (false negative) in 2 samples of CRC, and true negative in 19 of controls, (table-3, and figure-4).

The qRT-PCR amplification of studied genes was performed in duplicate and the mean of Ct values were taken. The  $\Delta Ct$  values were calculated according to equation ( $\Delta Ct = Ct \text{ value of marker} - Ct \text{ value of reference}$ ). The expression, mean of  $\Delta Ct$  values, and range of studied markers are present in (table-3).

Table-3: Expression, mean of  $\Delta Ct$  values and the range in patients and controls.

Marker result		Mean $\Delta Ct$ value	Range	Expression
P53	CRC	8.46±3.4	12.87 – 3.62	181.02
	Controls	15.96±1.22	18.1 – 16.35	
KRAS	CRC	8.12±2.9	11.51 – 2.35	39.39
	Controls	13.42±1.4	12.95 – 9.66	
hTE	CRC	7.93±3.72	11.94 – 2.15	158.68
	Controls	15.24±2.12	17.25 – 10.46	

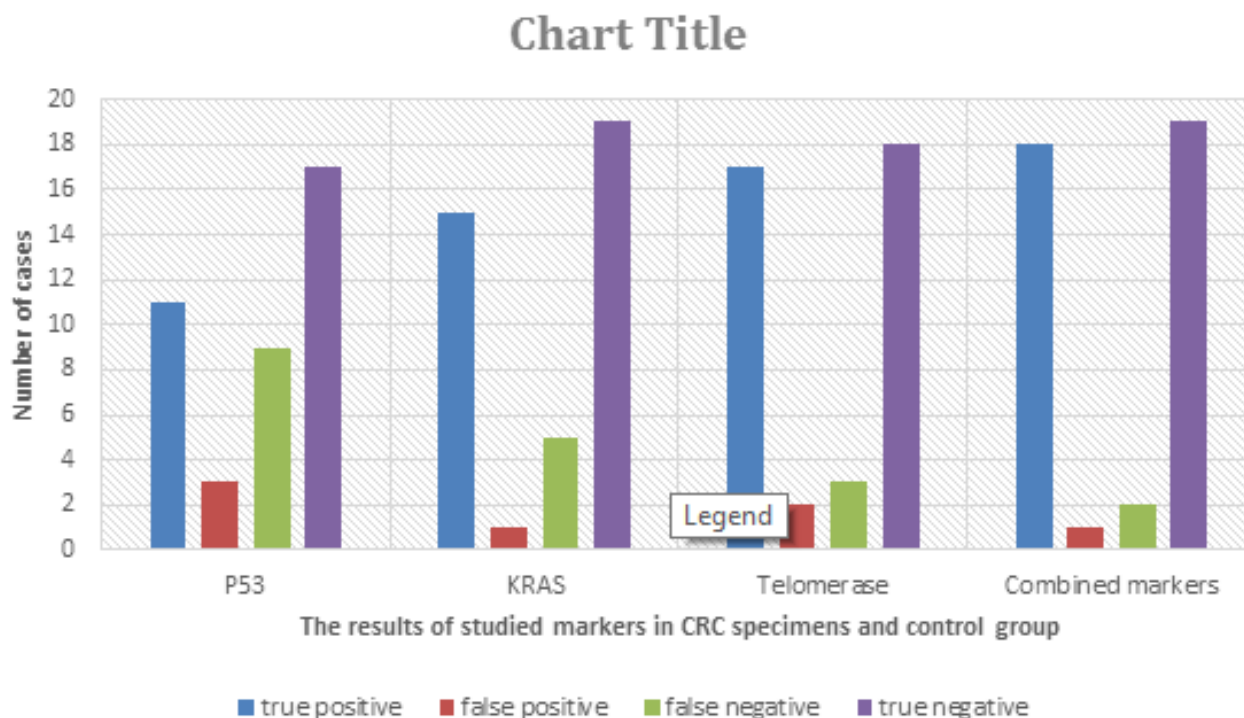


Figure-3: Expression of studied markers in samples from CRCs and controls.

- True positive= the cases of colorectal cancer with positive expression of studied marker.
- False positive= the cases of control group with positive expression of studied marker.
- False negative= the cases of colorectal cancer with negative expression of studied marker.
- True negative= the cases of control group with negative expression of studied marker.

#### Sensitivity and specificity of studied markers

The sensitivity of mRNAs of P53 is 55%, KRAS 75%, for hTE 85%, and for combined markers together 90%. The specificity of mRNAs of P53 is 85%, for KRAS 95%, for hTE 90%, and that for combined three markers together was 95%. The positive predictive values (PPVs) are

78.57%, 93.75%, 89.47%, and 94.73% for P53, KRAS, hTE, and combined markers respectively. The negative predictive values (NPVs) for P53, KRAS, hTE, and combined markers were 65.38%, 79.16%, 85.71%, and 90.47% respectively, (table- 4).

Table- 4: Sensitivity, specificity, PPV and NPV.

Markers	Sensitivity	Specificity	*PPV	**NPV
P53	55%	85%	78.57%	65.38%
KRAS	75%	95%	93.75%	79.16%
hTE	85%	90%	89.47%	85.71%
P53, KRAS, hTE	90%	95%	94.73%	90.47%

\* Positive predictive values

\*\* Negative predictive values



## Discussion:

Colorectal cancer is the third leading cause of cancer related death in adult life [1]. Early diagnosis followed by radical surgical treatment is the only hope of improving survival rate [2, 3]. Most sporadic cases of CRC are diagnosed at advanced stages because late appearance of worrying symptoms [3]. In order to increase survival rate and improve outcome of patient with CRC a simple noninvasive screening method should be implemented. Diagnostic value of circulating tumor cells (CTCs) in finding of solid human tumors has been increasingly attracting attention of researchers [7]. The CTC are originating from shedding of tumor cells from primary cancer into the circulation and are present in circulation from early stages of cancer [7]. Detection of CTCs in blood samples is performed by finding of specific molecular changes peculiar to them especially in genes that are the cause or the result of cancer development [8]. The Present study up to my knowledge was the first attempt in Iraq for finding three molecular markers in blood of patients for early detection of CRC by employing qRT-PCR method. Finding of CTCs is not an easy task because of their small count compared to accompanying huge number of white blood cells. This problem was overcome in the present study by efficient extraction and purification of total RNA, use of plenty amount of total RNA (1.0 µg) in each amplification tube, and performing amplification of targeted genes in duplicate. A significant number of false positive results appeared in the present study which resulted in a marked reduction in the specificity of the study. In order to reduce the number of false positive expression in samples taken from controls group as a result of background fluorescence a cut-off point was chosen for each marker. Any reading of Ct values of studied markers in samples taken from controls group above the cutting - point was considered as a true negative and that below it was considered as a false positive result. This procedure markedly reduces the false positive results and increases the specificity of studied markers. The use of combination of markers in present study increased the sensitivity but did not increase the specificity of the tests, table 4. The study of P53 gene is an important method in detection of CTCs, because it is an important tumor suppressor gene that was called the "Guardian of genome" [11]. Mutations or deletions were found in most tumors including the CRC especially in transition from colonic adenoma to carcinoma [11, 12]. The presence of mutations or deletion of this gene is an indirect method for presence of circulation tumor cells and for presence of internal cancer including colorectal cancer [13]. In present study 11/20 (55%) of samples of CRC showed high expression of P53 gene (true positive), and 9/20 (45%) of samples were false negative. Samples of CRC that were negative for P53 amplification might be the results of gene deletion which is a common cause of cancer development as a result of loss of tumor suppressor function of this important gene [13]. International studies for the rate of p53 mutations in

CRC and CTCs were lower than present studies by using different methods of assay including RT-PCR. Noa Rivilin et al [9] found p53 mutation in ranging between 10 - 100% of cancer, while Zulfikar A.J.Khan reported these mutations in about 50% of tumor tissues and CTCs. The reason for these discrepancies in results of p53 mutation might be due to efficacy of total RNA extraction and amplification. The reported highly expressed p53 gene was mutated and functionally inactive and this is the reason behind development of CRC in these patients inspite of presence of over-expressed p53 gene. The results of expression of p53 in internal studies by Khan ZA et al [12], Barry Iacopetta [13], and Ying Liu and Walter F [14], were in accordance with present study.

The KRAS protein is encoded by the KRAS gene, and took its name from Kirsten rat sarcoma viral oncogene homolog. The proto-oncogene of normal KRAS gene performs an essential function as a signaling molecule in normal tissues [12]. Mutant KRAS gene is an oncogene with activation mutation resulting in excessive cell proliferation and development of many cancers including CRC [13]. The presence of KRAS mutation in blood sample is strong evidence for the presence of CTCs and indirectly for presence of internal cancer [14]. Mutation of KRAS gene was found in 15 out 20 cases (75%) of blood samples taken from cases with CRC in present study using qRT-PCR, while in study by Mohamad Tag Elsabah, and Imam Adel [35], Cong Tan and Xiang Du [36], and Watanabe T et al [37] it was 42.3%, 34 - 45%, and 31% respectively. The differences between the mentioned results and present study were due to the use of different methods of RNA extraction, purifications and detection.

Telomeres are repetitive nucleotides present at the end of each chromatid their function is to protect the ends of the chromosomes [15]. Telomerase is the name given for enzyme essential for telomere lengthening [15]. Name of telomere is derived from the Greek nouns telos means "end" and meros "part". The telomere sequences of nucleotides are TTAGGG, which are repeated in human about 2,500 times [16]. During chromosome replication the ends of the chromosome are shortened with each cell division [16]. They are replenished by an enzyme, telomerase reverse transcriptase in germ and stem cells [15]. The activation of telomerase will result in a state of unlimited ability of cells for cell division and even carcinoma formation [16]. Activation and overexpression of human telomerase enzyme is seen cancers of solid organs including colorectal carcinoma [16]. So that, detection of hTE gene in blood samples is a strong evidence for presence of CTCs, and indirect evidence for presence of internal cancer including CRC. In present study hTE gene amplification was found in 17 out of 20 (85%) of blood samples taken from patient with CRC which is in accordance with studies the studies by Roberto Bertorelle et al [20, 38] and Nowak J et al [21].

Depending on results obtained from present study a conclusion was reached that, molecular tests employing qRT-PCR are efficient methods for detection of circulating tu-

mor cells in the blood of patient with colorectal cancer by finding of specific molecular changes in certain genes that are the cause or the result of colorectal cancer development namely p53, KRAS, and human telomerase. The tests are a non-invasive, rapid and cost effective and can be used on old peoples whom cannot tolerate colonoscopy. Nevertheless, inspite of efficacy of molecular procedures in detection

of CRC, it should not be considered as a substitution for colonoscopy and biopsy which remains the gold standard method for diagnosis and treatment of CRC with a specificity approaching 100%. Further studies, on large sample seize with more markers and combinations of markers are recommended to enforce the results of present study.

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## استعمال جهاز البلمرة نوع الوقت الحقيقي للتحري عن المعلومات الجينية والمسماة باللغة الانكليزية p53, KRAS, and human telomerase gene واستعمالها كمؤشرات حيوية للكشف المبكر عن سرطانات القولون والمستقيم في عينات الدم

أيسر حميد لطيف

كلية طب/ الجامعة المستنصرية

### الخلاصة:

**المقدمة :** الرغم من ان سرطان القولون والمستقيم هو مرض قابل للشفاء إذا تم اكتشافه مبكراً ومع هذا فإنه لا يزال السبب الرئيسي الثالث للوفاة بسبب السرطان في العالم . والسبب يعزى الى التأخير في اكتشاف المرض مما يجعل تشخيصه في المراحل المتقدمة لكون المرض لا يسبب اعراض الا حينما يصل الى مراحله الاخيرة حيث لا يمكن استئصاله جراحياً . وهذا السبب يستدعي العمل على ايجاد طرق حديثة للكشف عن السرطان وخاصة باستعمال الفحوصات الجينية المتطورة على عينات الدم.

**طريقة البحث :** دراسة تجريبية اجريت على عشرون مريضاً مصابون بسرطان القولون والمستقيم غير المنتشر والمثبت بناظور القولون والمستقيم والفحص النسيجي. وعشرون آخرين غير مصابين بسرطان القولون والمستقيم والمثبت بواسطة ناظور القولون والمستقيم والفحص النسيجي. لتحديد التعبير عن المعلومات الجينية قيد البحث والمسماة باللغة الانكليزية (p53, KRAS, and human telomerase [hTE]) والتي تسبب سرطان القولون والمستقيم ودراستها ومقارنة نتائجها بين المرضى المصابين بسرطان القولون والمستقيم وغير المصابين.

**نتائج البحث :** كانت نتائج التعبير الجيني للمعلومات قيد البحث في عينات الدم المأخوذة من المرضى المصابين بسرطان القولون والمستقيم اعلى بكثير من نتائج العينات المأخوذة من الاشخاص غير المصابين بسرطان القولون والمستقيم ونسب عالية جداً.

**الخلاصة:** اعتماداً على النتائج المذكورة أعلاه، والخاصة بالتعبير الجيني للمعلومات قيد البحث والمسماة باللغة الانكليزية (p53, KRAS, and human telomerase [hTE]) انه يمكن استخدام هذه المعلومات كمؤشرات حيوية في الكشف المبكر عن سرطان القولون والمستقيم غير المنتشر في الدم.