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Original Article

Expression of FGF19 Gene as an Effective Gene for the Invasive Behavior of Cancer Cells in Patients with Colorectal Cancer

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ABSTRACT

Introduction Colorectal cancer is the second and third most common cancer in women and men respectively. Early diagnosis of illness will reduce pain and costs for patients. In this study, the expression of fgf19 gene in different individuals with colorectal cancer, and also the relationship between the expression of this gene and the degree and stage of cancer cells, will be evaluated.

Methods A total of 60 samples were collected by a surgeon from cancerous tissue and healthy marginal tissue of patients with colorectal cancer. Extraction of RNA was accomplished by a Trizol solution. In the next step, cDNA molecule was synthesized using reverse transcriptase enzyme (RT), and gene-specific primers were designed and synthesized. Then the expression of FGF19 gene was evaluated by Real-time PCR technique. Finally, the data obtained from cancerous tissue and healthy marginal tissue were analyzed by spss software.

Result The expression of fgf19 in tumor tissue and healthy marginal tissue was measured quantitatively, which increased by 56.7 percent.

Conclusion The results of this study indicate that fgf19 as a molecular biomarker plays a significant role in the progression of cancer, and therefore can be considered as a factor in the screening, early detection, prognosis and predictor of the tumor.

Key words: Colorectal cancer, FGF19, Tumor tissue, RT-q PCR, Tumor marker.

Introduction

We live in an era with improved worldwide average living standards and increased access to adequate healthcare that has considerably improved the diagnosis and treatment of diseases. These measures have had an impact on average life expectancy in most regions of the world. However, although death rates from communicable diseases have improved globally as a result of these medical improvements, cancer-related mortality has increased by almost 40% over the past 40 years. A further 60% increase is expected in the coming 15 years, with 13 million people estimated to die of cancer in 2030 (Kuipers *et al.,* 2013). The

main causes of cancer-related mortality have also changed, attributable to alterations in disease incidence, introduction of screening programs and therapeutic improvements. Colorectal cancer was rather rare in 1950, but has become a predominant cancer in Western countries, now accounting for approximately 10% of cancer-related mortality. Reasons explaining this increased incidence include population ageing and the preponderance of poor dietary habits, smoking, low physical activity and obesity in western countries. The change in incidence is not only apparent in the rates of sporadic disease, but also in some familial cancer syndromes. Indeed, given that rates of Helicobacter pylori infection (a causative factor of gastric cancer) have fallen dramatically, colorectal cancer is now the predominant presentation of Lynch syndrome (a hereditary non-polyposis type of colorectal cancer), whereas carriers of this syndrome used to be predominantly affected by gastric cancer (Warthin, 1913; Vasen *et al.,* 2015).

Epidemiology

Colorectal cancer is the second- and third-most common cancer in women and men, respectively. In 2012, 614,000 women (9.2% of all new cancer cases) and 746,000 men (10.0% of new cancer cases) were diagnosed with colorectal cancer worldwide (Globocan, 2012). Combined, in both sexes, colorectal cancer is the third-most common cancer and accounts for 9.7% of all cancers excluding non-melanoma skin cancer. More than half of the cases occur in more-developed regions of world. The age-standardized incidence rate (ASRi) of colorectal cancer is higher in men (20.6 per 100,000 individuals) than in women (14.3 per 100,000). The majority of patients with sporadic cancer are >50 years of age, with 75% of patients with rectal cancer and 80% of patients with colon cancer patients being ≥60 years of age at the time of diagnosis. Incidence varies geographically, with the highest incidence in Australia and New Zealand (ASRi 44.8 and 32.2 per 100,000 men and women, respectively), whereas Western Africa (ASRi 4.5 and 3.8 per 100,000) has the lowest incidence (Figure 2). More-developed regions (Europe, Northern America, Australia, New Zealand and Japan; combined ASRi 29.2 per 100,000) have a higher incidence than less-developed regions (all regions of Africa, Asia (excluding Japan), Latin America and the Caribbean, Melanesia, Micronesia and Polynesia; ASRi 11.7 per 100,000) 10. The seven world regions can be ranked according to increasing ASRi, from Africa (6.3 per 100,000), Asia (13.7 per 100,000), Latin America and Caribbean (14.0 per 100,000), Micronesia/Polynesia (15.0 per 100,000), North America (26.1 per 100,000), Europe (29.5 per 100,000), to Oceania (34.8 per 100,000) 10. Within each of these regions, the ASRi can show marked variation. In Europe, Albania (8.4 per 100,000) and Ukraine (23.4 per 100,000) have a lower incidence, whereas Slovakia (42.7 per 100,000), Hungary (42.3 per 100,000) and Denmark (40.5 per 100,000) have a high incidence. Asia has the greatest diversity with regard to the ASRi of colorectal cancer. The incidence is high in Korea (45.0 per 100 000), Singapore (33.7 per 100,000) and Japan (32.2 per 100,000), but much lower in Nepal (3.2 per 100,000), Bhutan (3.5 per 100,000) and India (6.1 per 100,000). These variations are associated with different socioeconomic levels (Rohani-Rasaf *et al.,* 2013).

Diagnosis

A diagnosis of colorectal cancer either results from an assessment of a patient presenting with symptoms, or as a result of screening. The disease can be associated with spectrum of symptoms, including blood in stools, change in bowel habits and abdominal pain. Other

symptoms include fatigue, anemia-related symptoms such as pale appearance and shortness of breath, and weight loss. The predictive value of these symptoms for the presence of colorectal cancer in an elderly patient is limited, but they do warrant further clinical evaluation. With the widespread introduction of population screening for colorectal cancer, many individuals are diagnosed at a pre-clinical stage. In symptomatic patients, colonoscopy is the preferred method of investigation, but other endoscopic methods are also available or being developed (Box 2). For population screening, a range of other methods can be used for primary assessment, followed by colonoscopy in case of a positive test. One of the most common methods used in the diagnosis of cancer involves laboratory tests using biomarkers of colorectal cancers (Salehi Nodeh *et al.,* 2008).

Biomarkers of colorectal cancer

Molecular detection of colorectal cancer offers a noninvasive test that is appealing to patients and clinicians as samples of multiple patients can be analysed in batch. The ideal molecular marker should be highly discriminating between cancer and advanced adenomas from other lesions, be continuously released into the bowel lumen or circulation, and disappear or reduce after the lesion is removed or treated. Indeed, assays using proteins, RNA and DNA in the blood, stool and urine have been developed but with varying degrees of success (Table 1).

Fibroblast Growth Factor

The FGF19 subfamily of fibroblast growth factors (FGFs), consisting of FGF19, FGF21, and FGF23, is a novel group of endocrine factors that have been implicated in the regulation of many metabolic processes (Jones, 2008; Fukumoto, 2008; Kharitonenkov *et al.,* 2011). The subfamily members FGF19 and FGF21 share the ability to regulate glucose, lipid, and energy homeostasis. Both FGF19 and FGF21 transgenic mice are resistant to diet-induced obesity, have decreased adiposity and improved insulin sensitivity, glucose disposal, and plasma lipid profiles (Tomlinson *et al.,* 2002; Inagaki *et al.,* 2007)**.** Administration of recombinant FGF19 or FGF21 protein to diabetic mice resulted in the reduction of serum glucose and insulin levels, improved glucose tolerance, and reduced hepatosteatosis and body weight (Xu *et al.,* 2009, Coskun *et al.,* 2008; Fu *et al.,* 2004; Xu *et al.,* 2009; Wu *et al.,* 2009; Li *et al.,* 2009).

Real Time PCR

Real-time PCR is a strong and cost-effective technique, previously used to identify solid tumors and infections, and routinely for the diagnosis of cancers and infectious diseases. In this study, we used this technique to detect the (FGF19) gene from cancerous tissue and healthy marginal tissue of patients with colorectal cancer, which has several advantages. In addition, with the use of this technique we could avoid the need for bone marrow aspiration (Bernard et al., 2002).

Method and Materials

Tissue Samples processing

A total of 60 samples (30 tumors and 30 healthy margins) by surgeon were collected from cancerous and healthy margins of patients referring to Imam Khomeini Hospital, where 8 were treated with chemotherapy drugs and 22 patients were not treated. (Those whose pathologic outcome was adenoma or cancer) was followed by ethical principles. Their pathological outcomes were collected from the Cancer Institute of the Hospital in order to finally examine the results of the study with the pathological characteristics of the specimens. The specimens were immediately disposed of in RNAase-free conditions at the tube and then quickly frozen in the liquid nitrogen, so that the time interval between isolation and freezing was less than five minutes and then the collected samples were transferred to the laboratory with nitrogen tank for extraction of RNA and maintained at-80 ° C.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the tissues using the Total RNA Purification Kit (RNX-PLUS, CINACONE) according to the manufacturer's instructions. All RNA preparation and handling steps were done under RNAse -free conditions. Isolated RNA was stored at –80 °C until use. RNA concentration was quantified Spectrophotometrically at 260 nm and the purity and integrity were determined using the A260/A280 ratio by nanodrop device, model 2000 Thermo Scientific USA company. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denaturated 1% agarose gel. First-strand cDNA was synthesized from the total RNA using with (Thermo Scientific, K1622, US) cDNA synthesis kits according to the manufacturer's protocol. The normalization of cDNA was performed with the housekeeping gene B2M.

Primer Design

Real-time PCR reactions are required for specific primers; for this purpose, the AlleleID7.6 software was used. Using the NCBI database, the numbers related to the mRNA of each gene were obtained, and by inserting into the software and matching the variety of variants associated with each gene, common points were identified then, the design of the primer was carried out according to various physical-chemical parameters such as the length of the primer, the length of the reaction product, the melting temperature and other items specified by the software. Finally, the primer was designed to be ordered by the Gene Technology Company.

Table 1. Characteristics of primers used in the Real-time -PCR reaction

Gene	Accession number	Forward Primer	Reverse Primer	Product size
FGF19	Chr11	GTGCTTTCGAGGAGGAGATC	CATGGGCAGGAAATGAGAGA	95
β-actin	NM_001101	GATCAAGATCATTGCTCCTCCTG	CTAGAAGCATTTGCGGTGGAC	151

In order to perform Real-time PCR in this study, 2 µL of RT- Reaction Solution (cDNA), 0.4µl of F and R primers, and 10µl of power SYBR GREEN PCR master mix were added to a micro-vial, the reaction volume attained was 20 µL with Rnase-free water. The Real-time PCR reaction was performed on eight- stripe streams, and was repeated at test three time. The temperature schedule for the examined genes was as follows: each complete

amplification step was conducted by a separation step at 95° C for 60° C for 5 minutes, 45 cycles at 95° C for 15 seconds, and 60° C for 60 seconds, which was continued for the melting curve analysis. The SYBR green method was used for performing the melting curve analysis. The Real-time PCR reaction was performed for FGF19 gene as the target gene, and B2M as the reference gene for each patient and healthy sample. In this study, One-Step Real-Time PCR System of developed by the APPLIED BIOSYSTEM Company was used. The data was analyzed using the relative evaluation method. After calculating the amplification efficiency of the reaction primers, in order to use 2-ctΔΔ method. The Real-time PCR data analysis was performed based on the threshold cycle obtained for the target and reference genes in order to use the 2-ΔΔCT method. The difference in between the mean CT of the reference gene from and the mean CT of the target gene was considered as the ΔCT index, calculated for both the test and control groups. Also in addition, the difference between the CTs of the test and control groups was used to calculate the ΔΔCT index, and then the expression ratio between test and control samples and then which changes the ratio of the expression changes between the two tests; and the ratio of the control samples was determined using the formula < Ratio =2-ΔΔCT>.

Results

In order to study the quality of extracted RNA, some of the samples were taken on an agarose gel, thus ensuring the health and non-damage of the RNA during the extraction process. As seen in the Figure (1), there is a mild and uniform smear from the top to the bottom of the gel, indicating the presence of mRNA. Excessive smears indicate mRNA damage. Also, the dual band of 18s, 28s rRNA is clearly seen, and since the 5s rRNA is the smallest, it can be seen at the end. It should be noted that the used notebook is 1 kb.

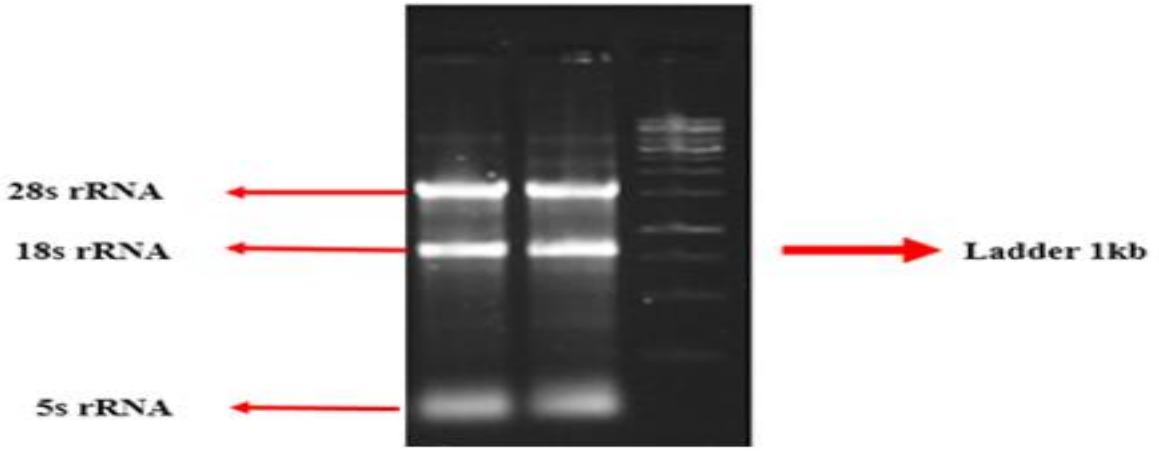

Figure 1. Qualitative study of RNA extracted from colorectal cancer tissue and its normal marginal tissue on Agarose gel 1%**.** The 28s rRNA is detected in front of the band of 1500bp and 18s rRNA versus the band of 750bp. Minimal smear shows the quality of extraction

Results of the analysis of Real Time PCR Reaction

In order to determine the Real Time PCR response, various sample dilutions (1, 0.1, 0.01) were tested Figure (2). Gene amplification curve is shown in Figure (3) and the standard curve slope expresses the efficiency and specificity of primers Figure (4). The efficiency of a

good reaction is between 90% and 110%. The R2 index is an indicator that indicates how far the regression of the obtained graph can be used to obtain the expression of the gene in the unknown graphs.

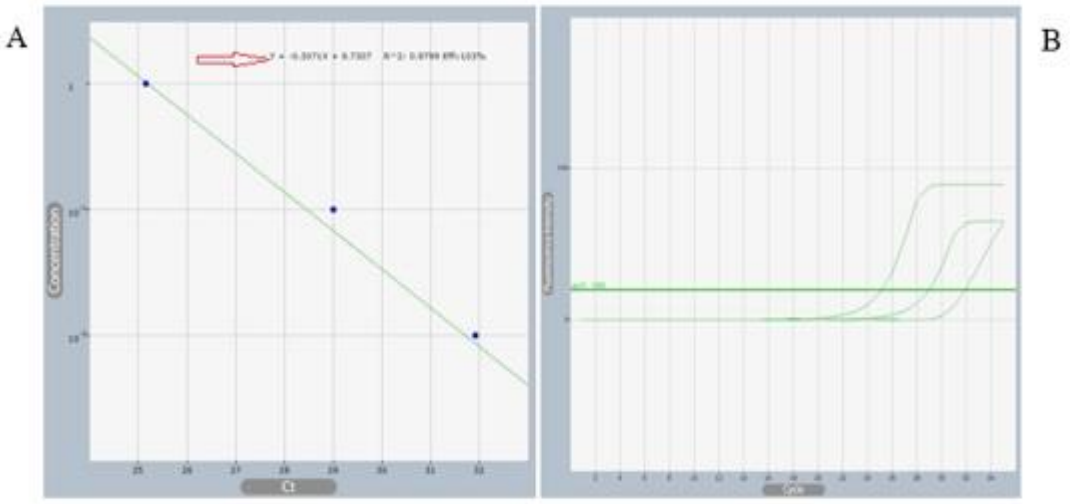

Figure 2. Various sample dilutions curve **Figure 3.** FGF19 gene amplification

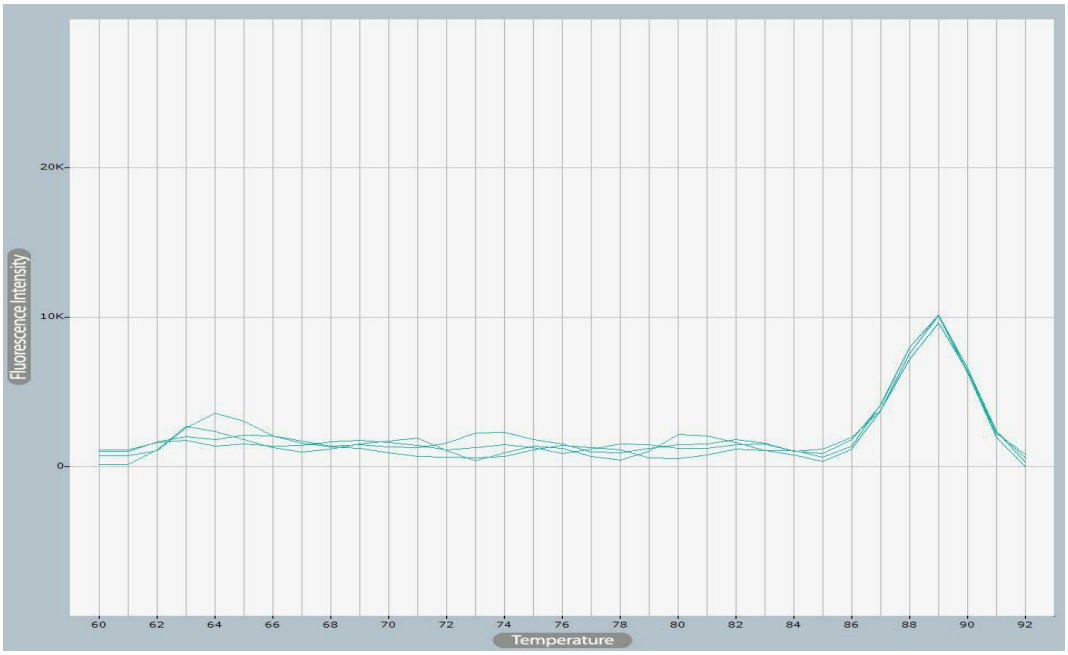

Figure 4. Standard Melting Temperature curve

To evaluate the expression of the genes, the Cycle threshold factor, or so-called Ct, was used. During the Real Time PCR, the device displays the amount of fluorescence variations in each cycle in a curved manner. The higher the amount of product produced, the greater the number of SYBR Green colors attached to it, resulting in increased light emitting fluorescence. The Ct or the threshold line represents the degree of product in which all the samples enter the logarithmic phase and are exponentially multiplied. After analyzing and

measuring the fold change, the average gene expression was 21.49 and showed an increase of 56.7 percent Figure (5).

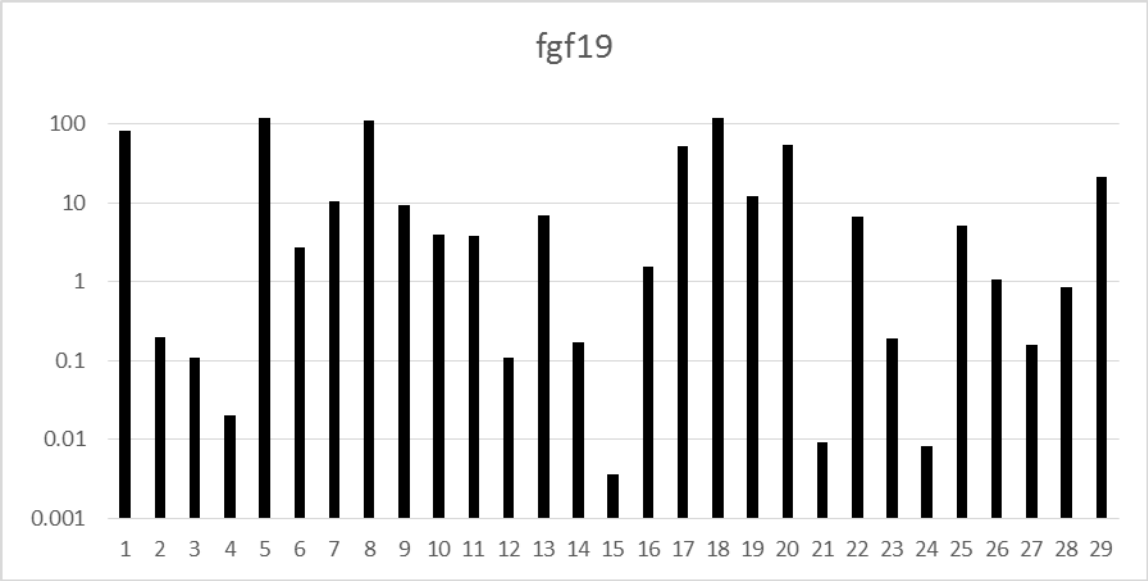

Figure 5. FGF19 gene expression

Conclusion

The results of this study indicate that fgf19 as a molecular biomarker plays a significant role in the progression of cancer, and therefore can be considered as a factor in the screening, early detection, prognosis and predictor of the tumor.

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