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EXPRESSION OF SMAD2TUMOR SUPPRESSOR GENE IN COLORECTAL CANCER: RETROSPECTIVE STUDY

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ABSTRACT

Background: Colorectal cancer (CRC) defined as the cancerous growths in the colon, rectum and appendix. It is a commonly diagnosed cancer in both men and women and represents the third most common form of cancer and the second leading cause of cancer-related death in the western world, SMAD2 (SMAD family member 2) is a protein-coding gene. It is a tumor suppressor gene located at 18q21 a region commonly deleted in colon adenocarcinomas, The role of Smad2 gene as an important tumor-suppressor gene emphasizes the complexity of rate-limiting check points in human tumorigenesis. Most of Smad2 gene mutations in human cancer are missense, nonsense, and frame shift Mutations at the mad homology2 region (MH2) which interfere with the homo-oligomer formation of Smad4protein and hetero oligomer formation between Smad4 and Smad2proteins, resulting in disruption of TGF-b signaling to thousands of adenomatous polyps in colon and rectum. Authors aimed to investigate the clinical significance of *SMAD2* in colorectal cancer progression. **Materials and methods:** Expression of *SMAD2* was detected in 50 formalin fixed paraffin using quantitative PCR (QPCR) and their levels were analyzed versus clinicopathological factors of CRC patients. **Results:** Significant relation was reported young female and CRC as compared to their counterparts from male individuals. *SMAD*2 Expression was increased significantly with old CRC and reported significant correlation with advanced stage and high grade tumors. **Conclusion:** *SMAD*2 expression was significantly related to differential grading thus pointing out their potential role as predictive markers for CRC prognosis.

KEYWORDS: Colorectal cancer; tumor suppressor genes; *Smad2*; progression, prognosis.

INTRODUCTION

Colorectal cancer is a major cause of morbidity and mortality thorough the world with large geographical differences.[1] Colorectal cancer in Egypt, like most of the developing countries, is lower than that of developed countries with western lifestyle. In Egypt, it is the 6th ranked cancer representing about 4% of total cancers in both sexes compared to the 3rd rank and about 11% for US.[2] Variation in environmental risk factors particularly the higher content of dietary fibers, more physical activity and lower obesity rates can explain for the different incidence rates.[3]

The development of a colorectal cancer is thought to occur in stages. Normal mucosa yields an adenoma that progresses to become cancer. There is strong experimental evidence that abnormal colonocytes with certain genetic alterations are able to proliferate excessively and acquire additional mutations. Eventually, as this process continues, the cells assume malignant characteristics of invasiveness and metastatic potential.[4]

Diet and lifestyle factors are implicated risk factors for the disease. Fruit and vegetable-deficient diet, calorie-dense foods, physical inactivity, obesity, and smoking increase the risk for developing colorectal cancer.[5]

Mutations in specific genes can lead to the onset of colorectal cancer, as happens in other types of cancer. Those mutations can appear in oncogenes, tumour suppressor genes and genes related to DNA repair mechanisms.[6] Depending on the origin of the mutation, colorectal carcinomas can be classified as sporadic, inherited and familial.

Familial CRC refers to patients who have at least one blood relative with CRC or an adenoma, but with no specific germline mutation or clear pattern of inheritance.[7] Point mutations, which appear during life, are not associated with inherited syndromes and only affect individual cells and their descendants. Cancers derived from point mutations are called sporadic cancers, and account for 70% of all colorectal cancers. The

molecular pathogenesis of sporadic cancer is heterogeneous as mutations can target different genes.[6]

Inherited cancers account for just 5% of all CRC cases. Those cancers are caused by inherited mutations that affect one of the alleles of the mutated gene, meaning that a point mutation in the other allele will trigger the apparition of the tumour cell and, subsequently, the carcinoma.[8]

Multiple tests are available to screen for CRC, including stool-based tests (e.g., guaiac-based fecal occult blood testing [gFOBT] or immunochemical-based testing [FIT], fecal DNA testing), endoscopy (e.g. Flexibl sigmoidoscopy [FS] or colonoscopy), and imaging tests (e.g. double contrast barium enema [DCBE], magnetic resonance colonography [MRC], capsule endoscopy).[9]

SMAD2 (SMAD family member 2) is a protein-coding gene. It is a tumor suppressor gene located at 18q21 a region commonly deleted in colon adenocarcinomas.[10] Previous studies have only identified a small number of *SMAD2* mutations in primary CRCs, leaving open the question as to whether these genes contribute to colorectal tumorgenesis,[11] Colorectal cancers (CRCs) frequently harbor somatic mutations in the pathway member SMAD4, but to what extent mutations in SMAD2 contribute to tumorgenesis is unclear.[11]

The study was conducted to investigate the expression of SMAD2 tumor suppressor gene among colorectal cancer patients to detect their role in colorectal cancer progression and their predictive significance for CRC patients. Also their correlation with other clinicopathological factors will be determined.

SUBJECTS AND METHODS

Sample selection

Fifty formalin fixed paraffin embedded (FFPE) tumors from Egyptian patients diagnosed with primary colorectal cancer were enrolled in the study, all samples were tissue blocks obtained from patients after surgical resection. Exclusion criteria will be patients with pre-surgically treated cancer, recurrent colorectal cancer, or other known malignancies. Before RNA extraction, representative sections were stained and analyzed and samples with tumor percentage more than 80% were only included in the study. Tumor staging were performed according to TNM classification using classification of the International Union Against Cancer[12], and the analyzed pathological feature as defined by the Collage of American Pathologists consensus declaration[13] were lymphatic invasion, tumor pattern, histological grading.

Purification of RNA

Total RNA was isolated from FFPE samples following the manufacture instruction protocol (Cat. no. 73504, Qiagen, USA). Briefly, deparaffinization treatment for the FFPE tissue samples was carried out using deparaffinization solution (Cat. no. 19039, Qiagen, USA). Then samples were incubated at 56°C with lysis buffer containing protinase K to release RNA from the paraffin sections. Then DNase treatment was carried out to eliminate of genomicDNA, And ethanol was added to provide binding conditions for RNA. After words the samples were applied to RNeasy Min Elute spin columns to wash away any contaminants and total RNA was eluted using RNase-free water. Total RNA concentration was detected using Q-5000 spectrophotometer nanodrop (Quawell Technology, Inc., San Jose, USA) at A260/A280. The ratio of purified RNA was ranged between 1.8-2.0, then they were divided into aliquots and stored at –80°C for complementary DNA (cDNA) synthesis.

Reverse transcription to synthesize cDNA

Reverse transcription process was carried out using QuantiTect reverse transcription kit (Cat no. 205311, Qiagen, USA) and cDNA was synthesized according the manufacturer instruction by adding 1µg of RNA template to reverse transcription master mix (reverse transcriptase, RT primer mix and RT buffer)forming a total volume of 20µl and PCR thermal cycler (SureCycler 8800, Agilent Technologies, Germany) was adjusted as following: samples were incubated for 30 minutes at 45°C, then 3 minutes at 95°C. Synthesized cDNA was divided into aliquots and stored at –80°C for gene expression analysis.

Gene expression analysis

SMAD2 expression was carried out using quantitative real-time PCR (QPCR) (Stratagen 3005MxP, Agilent Technologies, Germany) and their primers, as listed in Table (1) with SYBR Green chemistry according to the manufacture's recommended protocol of Quanti Tect SYBR Green PCR (Cat. no. 204143, Qiagen, USA). In brief, 500ng/reaction from cDNA was add to tubes each containing SYBR green master mix, primers for SMAD2 gene (5′-ACCGAAATGCCACGGTAGAA-3′ and an antisense primer, 5′TGGGGCTCTGCACAAAGAT-3′) and RNase free water to form a total volume 50µl, the thermal conditions were: initial activation for 15 minutes at 95°C followed by 40 cycles of: denaturation for 15 seconds at 94°C, annealing for 30 seconds at 54°C, then extension for 30 seconds at 72°C. The internal control used to normalize the expression of the investigated genes was *GAPDH*primer (forward 3\-ATGGGGAAGGTGAAGGTCG-5\, and reverse 3\GGGTCATTGATGGCAACAATATC-5\[14] and calculations of the gene expression analysis were conducted using comparative CT ($2^{-\Delta CT}$ as ΔCt = Target gene − Reference gene).[15]

Statistical analysis

The results were analyzed using Statistical Program for Social Science version 16 (SPSS). Also chi-square analysis was used to compare between qualitative parameters. *P*-value was two-tailed test and it was considered significant if less than or equal 0.05.

RESULTS

The clinical data enrolled individuals

The samples were belongs to 27 males and 23 females patients with different age range 43 - 67 years with mean age 55 years. The patients of early stage were 17 patient (34%) and the remaining were 33 patients with late stage. In this study the site of tumor were detected in 28 patients in the colon and the remaining n=22 patients were at the rectum. the grade of tumor were 26(52%) patients at low grade while 24 (48%) patients at high grade, Demographic data table (1).

Table (1): Demographic data of the patients.

Parameter	Number (n)	Percentage %
Age		
≤ 55 years	21	42%
>55 years	29	58%
Gender		
Male	27	54%
Female	23	46%
Stage		
early	17	34%
late	33	66%
Grade		
low	26	52%
high	24	48%
Tumor location		
Rectum	22	44%
Colon	28	56%

As shown in Table (2), the level of SMAD2 gene was reported regarding investigated clinicopathological factors. SMAD2 gene expression was investigated in a FPEE samples using real-time PCR as sensitive and applicable technique for accurate quantitation for gene expression[16], and their levels was normalized against GAPDH as house-keeping gene. The relation between investigated gene and clinicopathological factors were assessed Significance difference between level of SMAD2 and age of enrolled individuals as the level was higher in older CRC patients (>55 years) as compared to those Lower than 55 years. Age is amongst the main risk factors that lead to colorectal cancer.[17] In this study the age range for the enrolled samples were 43-67years and those with age less than 55 years represented (42%) indicating that it is colorectal cancer is a major risk in younger ages as in older ones, but its detection among those with young ages represent a percentage higher than reported in West countries which may concern the epidemiologic all trends among Egyptians these results agreed with previous reports.[18]

In this study the median level of SMAD2 was similar in both gender which disagree with other studies reported increase of CRC among males as compared to women.[19] This discrepancies may be attributed to the different selected population in both.

Table (2): Smad2 level with clinical factors.

Clinical pathological of date	Median	Range(Min-Max)	Mean ±SD	Statistics
Age				
≤55 year(n=21)	12.3	10 - 13.4	50.42±5.622	F Sig.
55year (n=29)>	5.9	5.7 -9.8	53.70±5.622	211.193 0.001
Gender				
Male (n=27)	6.3	5.7 - 13.4	37.70±1.47	F Sig.
Female (n=23)	6.3	5.7 - 13.4	41.43±1.68	4.261 0.44
Clinical stage				
Early(n=17)	24	22-27	24.29±1.53	F Sig.
Late(n=33)	33	30 – 34	32.57±1.22	431.582 0.0001
Grade				
High(n=24)	32	22-34	30.20±3.85	F Sig
Low(n=26)	31	23-34	29.34±4.48	0.526 0.472
Tumor location				
Rectum (n=28)	32	23-34	30.35±3.76	F Sig.
Colon (n=22)	30.5	22-34	29 ±4.62	1.308 0.258

Statistical analysis using ANOVA test.

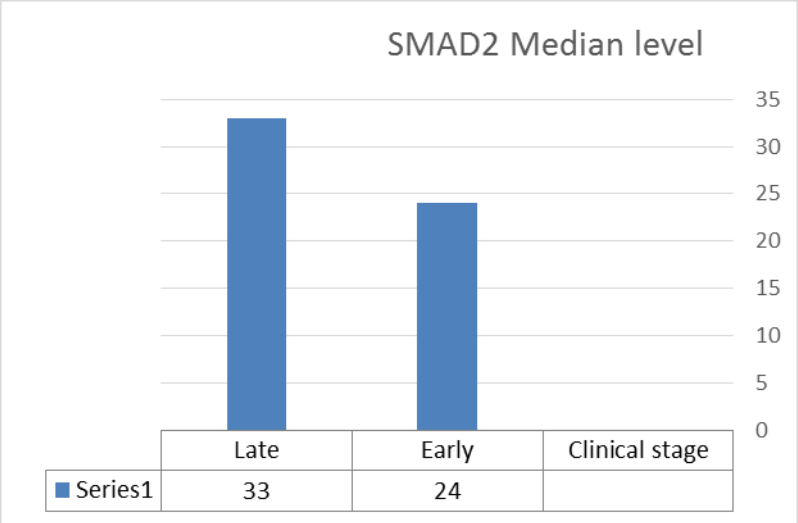

Fig. (1): The median level of Smad2 among Stage.

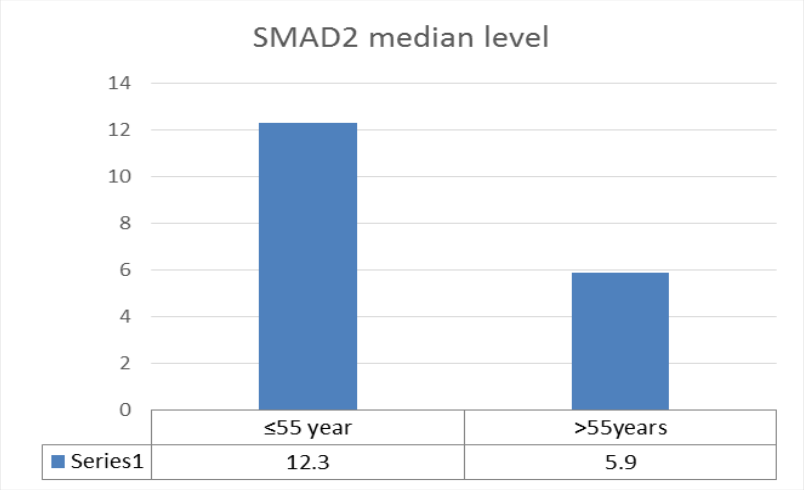

Fig. (2): The median level of Smad2 among Age.

DISCUSSION

Colon cancer or large bowel cancer includes cancerous growths in the colon, rectum and appendix. CRC is a commonly diagnosed cancer in both men and women.[20]

TGF-b is a member of a family of proteins that signal a wide variety of biological responses through transcriptional regulation of genes encoding critical determinants of cell fate, cell-cycle progression, differentiation, extracellular matrix association, motility, and death.[21] TGF-b signaling has been implicated in the transformation of normal melanocytes to melanoma and in the progression of several other human tumors.[22] Inactivation of this pathway, by mutation of the TGF-b receptor or Smad proteins, has been observed in a variety of human cancers.[21]

In this study (58%) of cases were ≥55 years these results consistent with Atkin and his collages and reported that Colorectal cancers typically a disease of the elderly, with over 90% of cases in old patients.[23] The vast majority of cases in young patients are sporadic, as only 16% of young patients have been reported to have a predisposing factor and 23% to have a positive family history.[24] In this study (34)% of patients who≤ 55 years were at early stage, However Fancher and his collage reported that Young age (≤ 55 years) has also been considered a predictor of poor survival, However investigation of these features in sporadic tumors occurring in young patients have led to controversial results, as there are studies reporting that they have similar histopathological features and rates of advanced stage when compared to older patients. In this study the incidence of CRC in rectum was more than the incidence in colon in young age (≤ 55 years). These results were consistent with Bailey and his colleagues and reported that An increasing incidence rate was also observed for patients with rectal cancer aged 43 to 67 years.[25] In this study (50%) of patient were ≤ 55 years with rectal carcinoma and the remaining were > 55years.

In this study gene expression for a tumor suppressor genes SMAD2 was investigated in FPEE samples using real-time PCR as sensitive and applicable technique for accurate quantitation for gene expression.[27] And their levels were normalized against GAPDH as house-

keeping gene. The relation between investigated gene and clinicopathological factors were assessed. Age is one of risk factors that lead to colorectal cancer[28], in this study the age range for the enrolled samples were 43-67years and those with age less than 55 years represented (42%) indicating that it is colorectal cancer is a major risk in younger ages and its rate is higher than reported in West countries which may concern the epidemiological trends among Egyptians these results agreed with previous reports.[18] In addition abnormal expression of the investigated gene was significantly higher in younger ages and for this reason severe consciousness of the possibility intended for colorectal cancer amongst younger individuals should be concerned.

In this study the expression of SMAD2gene Differs from rectum to colon ,while its median level was 32 in rectum and was 30.5 in colon so the expression of SMSD2 affected by the site of tumor. The enrolled patients were categorized according to their clinical stage into stage I-II and stage III and the expression of *SMAD2*gene was significantly correlated with staging. In this study the median level of *SMAD2*of at early stage 24 and at late stage 33 that indicated that the level of *SMAD2* increase with CRC progression. The median level of *SMAD2* of at high grade 32 and at low grade 31 that indicated that the level of *SMAD2* increase with CRC progression. The expressions for *SMAD2*gene of interest were investigated with the enrolled individuals and hence significant correlation was reported between the increased expression of *SMAD2* among the whole survival which points out its efficacy as a predictor for the prognosis of colorectal cancer, In this study the expression of SMAD2 gene Differs from rectum to colon, While its median level was 32 in rectum and was 30.5 in colon so the expression of SMSD2 affected by the site of tumor. The enrolled patients were categorized according to their clinical stage into stage I-II and stage III and the expression of *SMAD2*gene was significantly correlated with staging. In this study the median level of *SMAD2* of at early stage 24 and at late stage 33 that indicated that the level of *SMAD2* increase with CRC progression. The median level of *SMAD2* of at high grade 32 and at low grade 31 that indicated that the level of *SMAD2* increase with CRC progression.

In conclusion, detection of *SMAD2* expression using quantitative PCR on FFPE samples may provide a better insight into CRC progression.

Conflicts of interest

The authors have nothing to disclose.

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