



Alterations of Tumor Suppressor Genes Expression in Colorectal Cancer: Their Impact on Progression and Prediction of Patients Outcome

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Authors' contribution

This work was carried out in collaboration between all authors. Authors MS and TEM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MA and LREEA managed the analyses of the study. Authors MS, MA and MDEA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Deregulation of tumor suppressor genes as *APC*, *DCC* and *SMAD2* are related to tumorigenesis thus we aimed to investigate their expression among colorectal cancer to validate their relation with clinicopathological factors and the clinical outcome for CRC patients.

Materials and Methods: Formalin-fixed paraffin samples from 115 colorectal cancer were investigated for *APC*, *DCC* and *SMAD2* gene expression using quantitative PCR (QPCR) and their levels were analyzed versus clinicopathological factors and the overall survival (OS) of colorectal cancer patients.

Results: A significant relation was reported between *DCC* and *SMAD2* gene expression with clinical

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stages as they reported decrease expression among those with stage III. The three investigated genes were decreased significantly with poor histological differentiation colorectal cancer patients. The correlations between the expressions of the investigated genes revealed a significant correlation between *SMAD2* and *APC* as well as between *SMAD2* and *DCC*. Moreover patients with mean levels below and equal their expression values showed a considerable difference with OS. **Conclusion:** Gene expression of tumor suppressor genes *APC*, *DCC* and *SMAD2* were significantly related to differential grading and patient's outcome thus pointing out their potential role as predictive markers for prognosis of colorectal cancer.

Keywords: Tumor suppressor genes; colorectal cancer; *APC*; *DCC*; *SMAD2*; progression; prediction.

1. INTRODUCTION

Colorectal cancer is the third common cancer worldwide and its geographical dissemination in the developing countries varies with a high proportion rate [1,2]. In addition to exposure to unsafe environmental agents, unhygienic dietary behaviors and late diagnosis [3], colorectal cancer emerges generally as a consequent accumulation in the deregulation of oncogenes and tumor suppressor genes that lead to imbalance in proliferation and apoptosis processes [4]. As reported by Ting and his colleagues [5] nearly 90% of early diagnosed colorectal cancer patients have reached the 5-year survival on the contrary less than 10% reached this survival rate when diagnosed in metastatic stages.

In the current study, the expressions of three tumor suppressor genes were studied: Adenomatous Polyposis Coli (*APC*), deleted in colorectal cancer (*DCC*) and *SMAD2*. It has been reported earlier that *APC* is "gatekeeper" gene for CRC [6] and its altered expression occur in 90% of familial adenomatous polyposis (FAP), autosomal dominant disease, affecting individuals and developing benign colorectal adenomas that may progress to malignant adenomas [7].

Tumor suppressor gene Deleted in colorectal cancer (*DCC*) is located on chromosome 18q which has been diminished in colorectal cancer [8], and it has been reported to encode a transmembrane protein [9] and its expression is reduced in colorectal cancer [8] as it targets the loss of heterozygosity (LOH) on 18q chromosome with the other two suppressor genes (*SMAD2* and *SMAD4*) [10].

SMADs proteins are related in their structure, and their terms are derived from both *Drosophila* and *Caenorhabditiselegans* proteins; *MAD* (Mothers Against Decapentaplegic) and *SMA*

(Small body size), respectively [11] and they are categorized into three subgroups: inhibitory, common-partner and receptor regulator *SMADs*. Nine *SMADs* (*SMAD1-9*) have been discovered among them *SMAD2* which belongs to receptor regulator *SMAD* and activated through activin, *ALK-4*, *-5*, and *-7* and mainly by *TGF β* - receptors. Mutations in *TGF β* pathway affect genes participate in this pathway leading to tumorigenesis of colorectal cancer [12]. Earlier, it has been reported that *SMAD2*, *SMAD4* and *DCC* targets LOH on chromosome 18q as a part of their function as tumor suppressor genes thus present in colorectal cancer [13].

Some studies reported significant discrepancies in the clinical outcome among colorectal cancer patients although they have received same medications [14,15] which emphasize the significance of cancer genes related to tumorigenesis of colorectal cancer and focus on their altered expression.

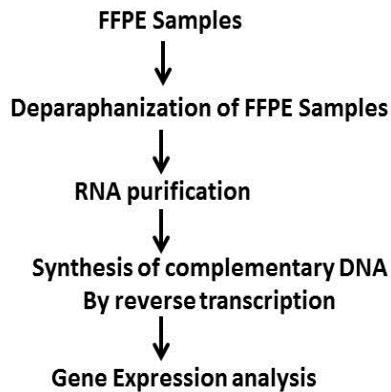
Authors aimed to investigate the expression of *APC*, *DCC* and *SMAD2* tumor suppressor genes in colorectal cancer patients in an attempt to identify their role in colorectal cancer progression and their predictive significance for colorectal cancer patients. Also, their correlation with each other and with other clinicopathological factors will be determined.

2. MATERIALS AND METHODS

2.1 Subjects of Research

After obtaining ethical Approval from the Medical Ethical Committee, this retrospective study was conducted on one hundred- fifteen formalin fixed paraffin embedded (FFPE) non-metastatic Egyptian colorectal cancer were collected from the Department of Pathology, Ain Shams University, Egypt, from 2012 to 2016 all samples were tissue blocks obtained from patients after surgical resection. Before RNA extraction,

representative sections were stained with H&E and analyzed by pathologists. Tumor staging was performed according to TNM classification using classification of the International Union Against Cancer [16], and the analyzed pathological feature as defined by the Collage of American Pathologists consensus declaration [17] were a lymphatic invasion, tumor pattern, histological grading. Representative flowchart for the used methods was represented below.



Patients were treated following the guidelines of National Comprehensive Cancer Network (NCCN), and survival data for enrolled individuals were obtained. The endpoint for the current study was selected as the overall survival (OS) which resembles the time (months) from diagnosis to the end of the study with a follow-up duration of nearly 3 years (36 months).

2.2 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 proteinase K to release RNA from the paraffin sections. Then DNase treatment was carried out to eliminate of genomic DNA, and ethanol was added to provide binding conditions for RNA. Afterwords the samples were applied to RNeasy MinElute 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.

2.3 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 1 µg of RNA template to reverse transcription master mix (reverse transcriptase, RT primer mix and RT buffer) forming a total volume of 20 µl 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.

2.4 Gene Expression Analysis

The expression of (APC, DCC, and SMAD2) genes 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 QuantiTect SYBR Green PCR (Cat. no. 204143, Qiagen, USA). In brief, 500 ng/reaction from cDNA was add to tubes each containing SYBR green master mix, primers for APC [18], and DCC [19] and SMAD2 [20] as listed in Table 1, then RNase free water to form a total volume 50 µl, 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, 56°C and 58°C for APC, DCC and SMAD2, respectively, then extension for 30 seconds at 72°C. The internal control used to normalize the expression of the investigated genes was GAPDH forward primer : 3/- ATGGGGAAGGTGAAGGTCG -5/ and reverse primer: 5/- GGGTCATTGATGGCAACAATATC -3/ [21] and calculations of the gene expression analysis were conducted using comparative C_T (2^{-ΔC_T} as ΔC_T = Target gene - Reference gene) [22].

2.5 Statistical Analysis

The results were analyzed using Statistical Program for Social Science version 16 (SPSS). Non-parametric analysis using Wilcoxon and

Table 1. Primer sequences for the genes under investigation

Gene	Forward direction	Reverse direction
APC	3/-TCRCAGTCTCTTCGAGCRCTT-5/	5/-TCRC CCT AAC ATA CAG GGT GA-3/
DCC	3/-CTGGCTCAATTATTAGTCGG-5/	5/- TCGGGAGTCACCTGGGATT-3/
SMAD2	3/-ACCACCATGGGTAAGAACATGTCGTCC ATC-5/	5/- TTT CCA TGG GAC TTG ATT GG-3/

Kruskal Wallis tests for two and three groups, respectively. Also chi-square analysis was used to compare between qualitative parameters. Overall survival analyses were investigated versus gene expression using Kaplan Meier curves. P-value was two-tailed test and it was considered significant if less than or equal 0.05.

3. RESULTS

colorectal cancer patients were classified according to their gender status into 51-males (median age 42) and 64 females (median age 45) and the age were (18-67) for both genders. According to colorectal cancer risk factors; patients were staged into stage I (15 cases, 13%), stage II (58, 50.4%) and stage III (42, 36.5%), both stage I and stage II were combined in one stage collectively known as stage I-II. Histological grading were reported as grade I-II (n=97, 84.3%) and grade III (n=18, 15.7%). Lymph node invasion were positive in (n=40, 35.1%) and negative in (n=75, 65.2%). Positive margin were detected in 10 cases (8.6%) while the remaining (n=105, 91.3%) were negative. With respect to tumor location; colon cancer were (66, 57.4%) while the remaining were in rectum (49, 42.6%), according to tumor histological type; 79 colorectal cancer patients were adenocarcinomas and the remaining (n=36) were mucinous type.

The expression of investigated genes (APC, DCC, and SMAD2) was determined in comparison with expression of GAPDH gene i.e. a house keeping gene for all enrolled individuals using real-time PCR.

By analyzing the expression level of investigated tumor suppressor genes with demographic and clinicopathological factors, as reported in Table 1. Significant difference was reported between age of colorectal cancer patients and candidate tumor suppressor genes, as aberrant expression of investigated genes was increased in younger patients than in older ones. DCC expression level was increased in patients with stage II by 1.5 fold than those with stage III and histological grading revealed significant difference with DCC expression. SMAD2 gene expression was

increased 1 fold in colorectal cancer patients below or equal than 51 as compared to those more than 51 years; and 1.2 fold in stage I-II patients as compared to their counterparts with stage III. Regarding histological grading, the expression of APC and DCC and SMAD2 reported significant decrease in GIII tumors followed by G I-II.

Authors investigated the correlation between the expression levels for the genes of interest using linear regression analysis. As reported in Table 2, significant correlation was detected between SMAD2 with both APC (R=0.193, P=0.034) and DCC (R=0.461, P< 0.0001), while no significant correlation reported between APC and DCC.

Also the overall survival (OS) of colorectal cancer patients were studied among investigated tumor suppressor genes regarding both grading and staging. Both APC and SMAD2 reported significant difference with OS as those with decreased levels of gene expression reported as GIII showed worse survival rate, as reported in Fig. (1 and 2, respectively) while DCC did not revealed significance with OS (Fig. 3). On the other hand APC did not revealed significance with OS regarding clinical stage (Fig. 4), while DCC and SMAD2 genes reported significance correlation with OS when concerning clinical staging as patients with decreased expression and stage III showed worse OS as compared to their counter parts with high gene expression, as reported in Fig. (5 and 6, respectively).

4. DISCUSSION

Although several researches are investigating markers to predict the prognosis of colorectal cancer, still there is a need to explore markers that may enhance this issue. Among these markers is the detection of gene expression. In the current study gene expression for three tumor suppressor genes APC, DCC and SMAD2 were investigated in a cohort of 115 FPEE samples using real-time PCR as sensitive and applicable technique for accurate quantitation for gene expression [23] and their levels were normalized against GAPDH as house-keeping gene.

Table 2. Relation between levels of gene expression (Mean ± SE) and clinicopathological factors

Gene	APC	DCC	SMAD2
Factors			
Gender			
Male	8.6 ± 2	10.8 ± 3	30 ± 5
Females	9.4 ± 3	11.6 ± 4.2	31 ± 4.8
Age			
≤ 44 years	11 ± 2	12.4 ± 4	32 ± 5
> 44 years	7.8 ± 2	10.4 ± 4	30 ± 4.8
	F=40, P<0.0001	F=6.2, P=0.014	F=4.8, P=0.029
Clinical Stage			
Stage (I-II)	9.1 ± 2	12.8 ± 3	32.8 ± 3
Stage (III)	8.9 ± 2.1	8.5 ± 4	27.5 ± 5
		F=35, P<0.0001	F=39, P<0.0001
Histological grade			
Grade (I-II)	9.6 ± 2	11.6 ± 4	31.4 ± 5
Grade (III)	6 ± 0.2	9.4 ± 4	28.2 ± 4
	F=28.7, P<0.0001	F=6.1, P=0.014	F=4.2 P=0.04
Lymph node invasion			
Negative	9.2 ± 3	11 ± 4	31 ± 5
Positive	8.7 ± 2.2	11.4 ± 4	32 ± 4
Tumor localization			
Rectum	9.3 ± 2	11 ± 4	31 ± 5.7
Colon	8.8 ± 2.8	11.5 ± 4	30 ± 4.5

Table 3. Correlation between the APC, DCC, and SMAD2 expression levels

	APC		DCC	
	R	P	R	P
SMAD2	0.193	0.034	0.461	< 0.0001
APC	--	--	0.142	0.13

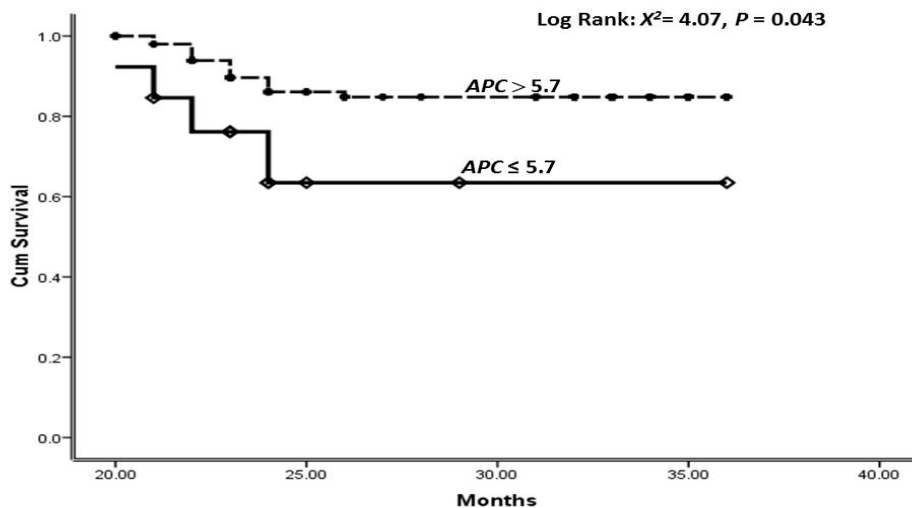


Figure 1: Kaplan Meier curves for comparing between APC and histological differentiation. The poor differentiation is represented by straight line while the well-moderate differentiation is represented by dashed line.

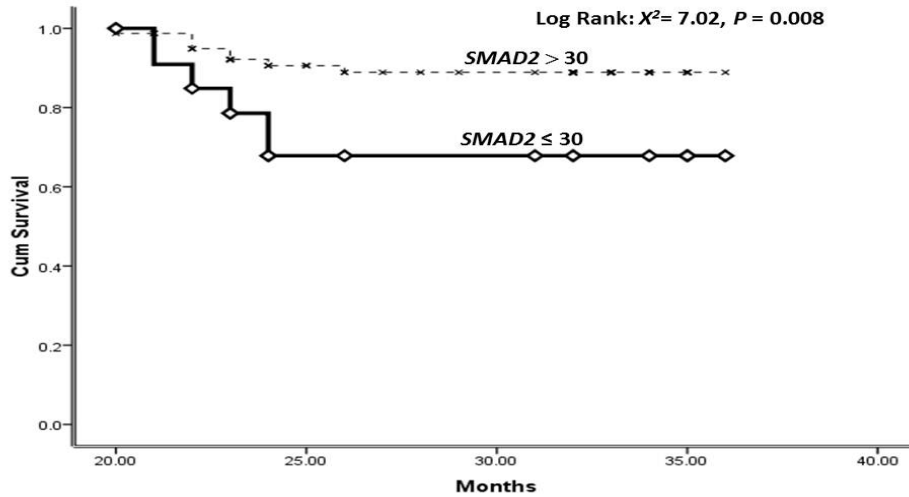


Figure 2: Kaplan Meier curves for comparing between *SMAD2* and histological differentiation. The poor differentiation is represented by straight line while the well-moderate differentiation is represented by dashed line.

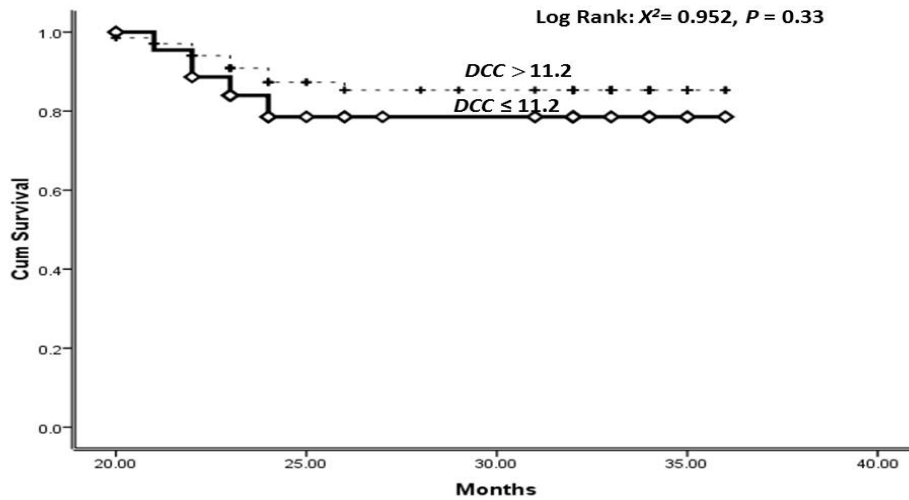


Figure 3: Kaplan Meier curves for comparing between *DCC* and histological differentiation. The poor differentiation is represented by straight line while the well-moderate differentiation is represented by dashed line.

The relation between investigated genes and clinicopathological factors were assessed. Age is among the major risk factors that lead to colorectal cancer [24-25], in the current study the age range for the enrolled samples were 18-67 years and those with age less than 44 years represented (38.3%) 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 [26]. Moreover aberrant expression of the investigated genes was

significantly higher in younger ages and hence excessive awareness of the possibility for colorectal cancer among younger individuals must be concerned.

The enrolled patients were categorized according to their clinical stage into stage I-II and stage III and the expression of *DCC* was significantly correlated with staging. These results are consistent with previous studies reported concerned about the expression of *DCC* protein using immunohistochemistry in all stages [27] which direct the significance of any mutation in

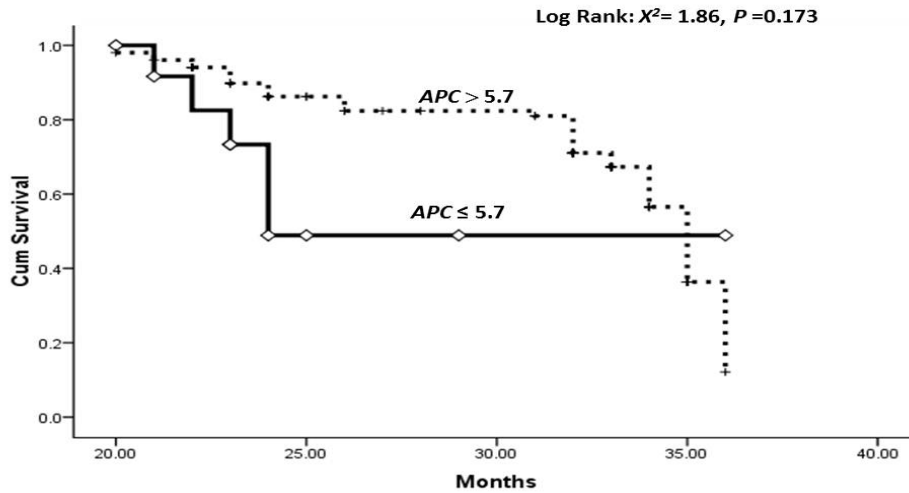


Figure 4: Kaplan Meier curves for comparing between APC and clinical staging. Stage III is represented by straight line while stage I-II is represented by dashed line.

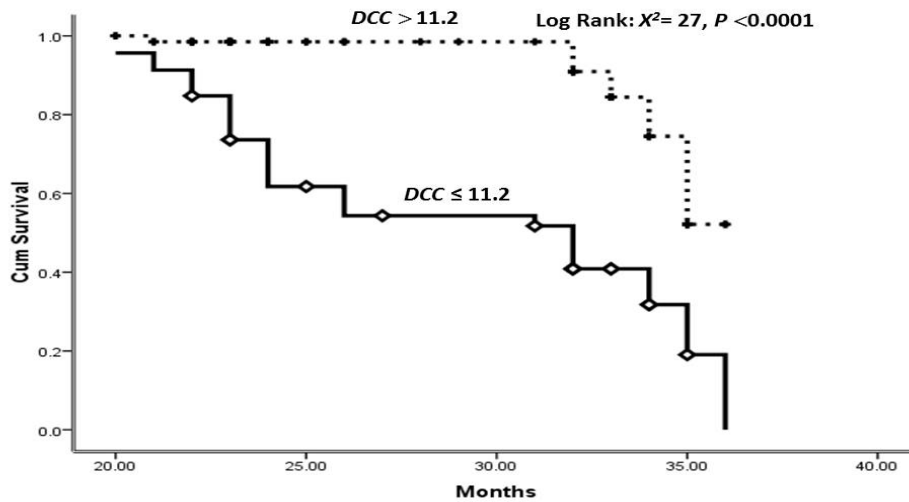


Figure 5: Kaplan Meier curves for comparing between DCC and clinical staging. Stage III is represented by straight line while stage I-II is represented by dashed line.

chromosome 18q21.2]; on which DCC gene is located, to be linked to colorectal carcinogenesis [28]. For SMAD2, categorized as pathway-restricted SMADs, significant difference between its mRNA upregulation with age and clinical stage was detected in the current study. It has been reported that age is among the candidate for colorectal cancer risk factors [29] but its association with SMAD2 is firstly being addressed in the current study and a further research is needed to explore the relation between SMAD2 and age among colorectal cancer patients. Moreover there was a significant

difference in SMAD2 expression level and clinical stages as decreased level of SMAD2 was reported with late stages. In a previous study [30] the loss of SMAD2 was reported in colorectal cancer and it was attributed to the role of SMAD2 as negative regulator for TGF β , hence the role of TGF β is modified into stimulator of tumor growth rather than inhibitor of tumor growth [31-32].

The diverse of histological types reveal the biological character of cancer and hence direct both diagnostic and prognostic criteria. Cellular differentiation of colorectal cancer is closely

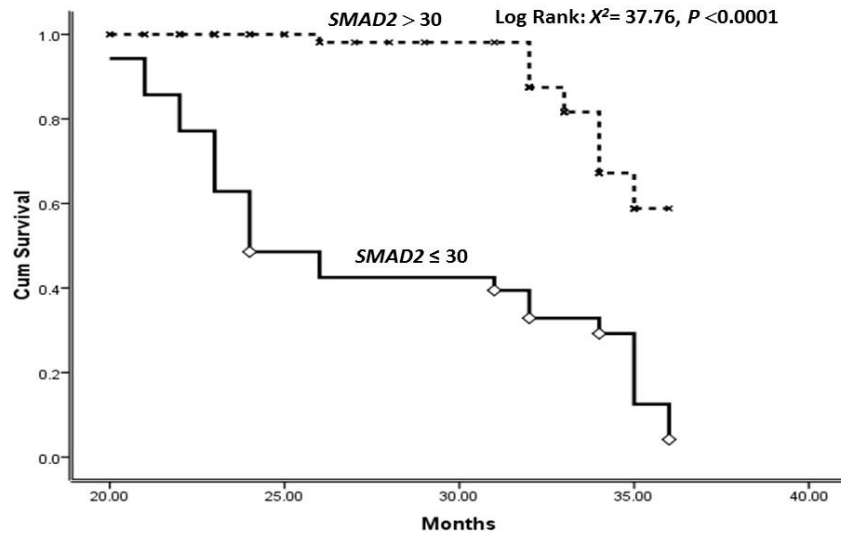


Figure 6: Kaplan Meier curves for comparing between *SMAD2* and clinical staging. Stage III is represented by straight line while stage I-II is represented by dashed line.

related with the diversity of pathologic varieties like involvement of lymph nodes and growth configuration [33]. Standard grading classified colorectal cancer into grade I, grade II and grade III and it is dependent on individual estimation of the histopathologist. Diversity of differentiation in the same cancer samples frequently leads to significant inter-and intra-observer difference in grading [34].

In the current study tumor suppressor genes were correlated significantly with tumor variation as their expression was decreased in grade III. Mutations in APC gene result in transcriptional activation through β -catenin; principal cell-cell adhesion molecule, to transcriptional activate a family of transcription factors which further modulate tumorigenesis [35]. Moreover Zhang and Shay [36] have reported that alteration in APC gene results in truncated gene leading to activation of Wnt signaling pathway and upregulation of other multiple cellular processes. Also allelic loss in chromosome 18q may result in mutant expression of three candidate tumor suppressor genes located on this chromosome; DCC, SMAD4 and SMAD2, which further resulted in diminishing their expression in grade III (poorly differentiated) colorectal cancer and lead to tumor progression.

Our results were consistent with these findings as DCC was significantly decreased in grade III tumors. DCC protein dysfunction may be caused by many factors that affect gene expression such

as allelic loss, point mutation, deletion or insertion in the initiator of promoter region of the gene [19] indicating its relation with colorectal cancer progression. SMAD2 expression has been studied among the cohort of colorectal cancer patients, its expression was reduced in grade III colorectal cancer samples which reveal their linkage with colorectal cancer progression as well.

Several molecular signaling pathways are associated with colorectal cancer tumorigenesis, among these pathways are TGF β /SMAD and canonical Wnt/ β catenin pathways. Currently significant relation between APC and SMAD2 was detected (Table 3). It has been reported by Hamamoto and his colleague that heterozygous deletions in both genes (APC and SMAD2) accelerated gastrointestinal tumorigenesis [36], thus mutations of SMAD2 may not act as initiator for carcinogenesis but rather as tumor gene that facilitates cancer progression through its co-activator role of canonical Wnt/ β catenin pathway [37]. Significant correlation was reported between DCC and SMAD2 which may be attributed to the fact that both genes are located on the same chromosome i.e. chromosome 18q which may direct to their combinational effect on progression of colorectal cancer this association was previously reported through "Vogelgram" model of colorectal cancer that highlight the stepwise manner of development of colon cancer [38].

About 40-50% colorectal cancer patients relapsed although early diagnosed. Different chemotherapeutic treatment strategies with (oxaliplatin, irinotecan and fluoropyrimidine) merged with targeted biological regimens (bevacizumab and cetuximab or panitumumab) resulted in improvement of median OS especially for colorectal cancer patients with metastatic evidence [39]. Though this category of colorectal cancer patients should receive initial, second and third line treatments and still the 5-year OS is less than 10%. It has been reported that although colorectal cancer patients are being classified by clinicopathological features, still the treatment response is heterogeneous which may stress for the understanding of the molecular events initiating colorectal cancer [40].

The expressions for the genes of interest were investigated with the OS of the enrolled individuals and accordingly significant correlation was reported between the increased expression of APC, DCC, and SMAD2 with overall survival which points out their usefulness as a predictor for the prognosis of colorectal cancer patients. In this study colorectal cancer patients with grade III and exhibiting decreased tumor suppressor genes expression revealed worse survival as compared to those with grade I-II and high gene expression. Thus with relevance of gene expression analysis it will be applicable to tolerate a better treatment strategy to improve the patient's outcome.

5. CONCLUSION

In conclusion, analyzing the expression of genes involved in the tumorigenesis of colorectal cancer using quantitative PCR on FFPE samples extends our knowledge for combinational expression of APC, DCC and SMAD2 on colorectal cancer progression to achieve a better understanding of tumor development and realize the effective molecular targets for a comprehensive management of personalized colorectal cancer treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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