Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Immunohistochemical and Molecular Studies of p53 and KRAS Protein and Their Relations to Colorectal Carcinoma

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ABSTRACT

The study included 50 tissue blocks embedded in paraffin wax (16 females and 34 males), obtained from a patients group with colorectal cancer (CRC), as well as 35 tissue blocks that were embedded in paraffin wax from normal colon (ulcerative colitis) as controls. A relatively few oncogenes and most prominently tumor-suppressing genes, Kirsten rat sarcoma virus (KRAS), and P53 genes have been mutated into a significant part of CRCs, and a broad collection of mutated genes has been defined in CRC subsets. Current findings showed very significant differences between patients and control subjects in the p53-positive rate ($P < 0.001$). TP53 Pro/Pro genotype positivity was higher in the control group than in the patient group and this was a significant difference ($P < 0.001$) with an odds ratio of <1. The genotype Pro/Pro was considered to be protective against colorectal carcinoma preventively fractured 0.767. The positive rate of p53 Arg/Arg genotype in patients was more frequent and statistically significant ($P < 0.01$), because the odd ratio was more than one. The genotype Arg/Arg would be considered a colorectal carcinoma risk factor. We conclude that p53 overexpression is used as an indicator of p53 mutation (as identified by immunohistoric chemistry) and KRAS protein expression was negatively impaired for all the patients in the current study.

Keywords: Kirsten rat sarcoma virus, carcinoma, stage, colorectal, histology

INTRODUCTION

Carcinogenesis and colorectal cancer (CRC) development are a multistage process where colorectal epithelial cells are transported through small adenoma, large adenoma, and subsequently into adenocarcinoma.[1] The development of CRC involves several steps, which occur through the development of several genetic changes, including chromosome defects, gene mutations, and the epigenetics of various genes which control and differentiate cell growth, apoptosis, and angiogenesis. CRC generally develops from a benign polyp called an adenoma and the subsequent abnormal cell growth, leading to carcinoma, which can spread to other areas of the body.[2,3]

Tumor suppressing genes and oncogenes mutations and probably several pathways lead to lesion pathology transitions and tumor drive toward malignancy and metastasis.[3] CRC is not one disease; it involves a heterogeneous complex of diseases with various genetic and epigenetic changes.[4] Modifications of various genes include activating K-ras oncogene, mutating, and removing p53 anti-oncogene.[5]

In particular, mutations of the TP53 tumor suppressor gene and KRAS oncogene contribute to more than 80% of cases of CRC.[6] TP53 encodes the tumor suppressor protein p53, which is a cell cycle regulator; mutations in TP53 leading to CRC commonly occur in exons 5-8 .[7] In mitogen active protein kinase signals, KRAS encodes a GTPase; CRC-related KRAS mutations are common in codons 12 and 13, both within exon.[8] Then, the aim of this study is, therefore, to clarify the proposed role for mutations in CRC of the p53 and the KRAS genes through the determination of frequent CRC mutation of KRAS and p53 by estimating the rate of protein over-expression employing immunohistochemical (IHC) staining and anti-KRAS antibodies and anit-p53, respectively.

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Received: May 16, 2021
Accepted: June 13, 2021
Published: May 20, 2021

DOI: 10.24086/cuesj.v5n1y2021.28-33

MATERIALS AND METHODS

This study was conducted in the Faculty of Science, University of Kufa, Molecular Laboratories and Al-Sadar Teaching Hospital in Al-Najaf Province. Each paraffin block was processed using hematoxylin and eosin stain for histopathological assessment, mutations in p53 (exon 4 codons 72) were detected by polymerase chain reaction (PCR), mutations detected throughout restriction fragment length polymorphism-PCR (RFLP-PCR) in Krason (exon 2 codon 12), protein expression had been detected in p53 and the immunohistochemical method of KRAS.

Mouse monoclonal anti-human KRAS, p53, and p53 were used to detect KRAS as primary antibodies, and p53 proteins were described by Al-Juborii(20l5).[9]

Staining Results: A visual scoring system was used by a light microscope based on the number of positively stained neoplastic cells in each sample of tissue. The intensity and staining pattern was assigned to each slide. Total intensity ratings were evaluated by counting the percentage of positive cells in 100 malignant cells at 40× total objective magnification.

The immunostaining was calculated as a percent by the number of malignant cells immunostaining (semi-quantitative scoring). The intensity of stain was measured by counting the positive cell percentage in 100 malignant cells in 25 fields representing the most positive area with an objective magnification of 40×. The staining pattern (qualitative assessment) was either faint (staining pattern that could only be detected using higher magnification, ×40 objective) or dense (a diffuse cytoplasmic staining pattern and easily be seen by lower power objective ×4).

KRAS Tissue Evaluation: Three scoring scales were applied at ×40 objective,[10,11] as follows:

Score 0: None of the cells revealed positively for tumor marker stains

1- Score l: 10% of positive tumor cells (Weak).
2- Score 2: <50% of positive cells (Moderate).
3- Score i3: ≥50% of positive cells (Strong).

P53 Tissue Evaluation: P53 nuclear protein expression scored according to: [12] Score 0: 0–l0% positive staining of tumor cells considered negative staining; score l+: 11–25% I positive staining of tumor cells, mild or weak staining; score 2+: 26–50% positive staining of tumor cells, moderate staining; and score +3i: >50 positive staining of tumor cells, strong staining.

RESULTS

Immunohistochemical Expression of p53 and KRAS

The present findings showed a highly significant difference in p53-positive rate between patients and control subjects ($P < 0.001$), Table l. However, the result was limited to patients with colonic carcinoma (46%) whereas none of the control subjects exhibited a positive p53 expression (0%). The p53 IHC expression was detected as brown nuclear dots, as shown in Figures l-3.

The score of p53 was as follows: Score l accounted for 2 (9%) of total positive cases; score 2 was seen in 18 (78%) of

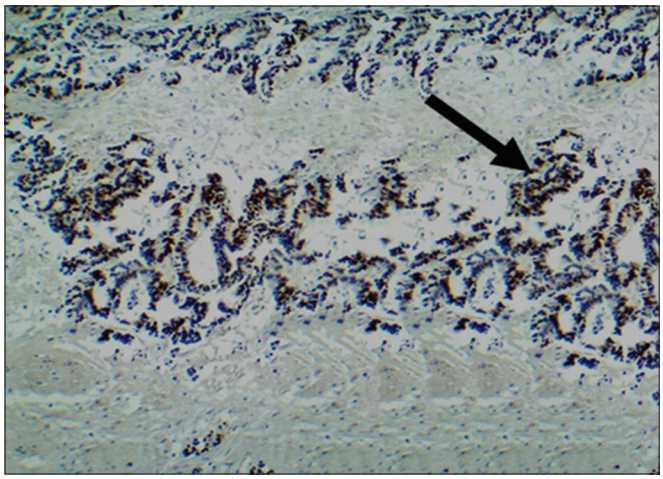

Figure l: Immunohistochemical section p53 expression in the form of brown nuclear stain score 1 (arrow). Notice that <10% of the cells are stained (10×)

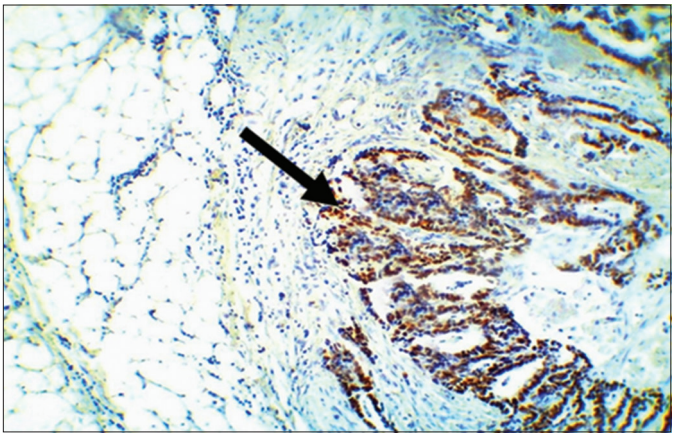

Figure 2: Immunohistochemical section p53 expression in the form of brown nuclear stain score 2 (arrow). Notice that around 10–50% of the cells are stained (10×)

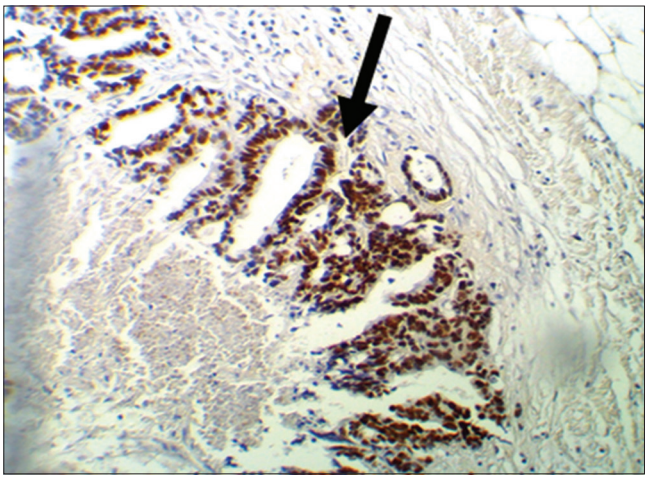

Figure 3: Immunohistochemical section p53 expression in the form of brown nuclear stain score 3 (arrow). Notice that >50% of the cells are stained (10×)

total positive cases; and score 3 was present in 3 (l3%) of total positive cases [Figure 4].

The present results did not show any significant difference in the mean age of patients with positive p53 expression in comparison to patients with negative p53 expression. Moreover, patients with rectosigmoid location showed the highest rate of p53 expression (53.33%). In addition, there was no significant association between the site of colonic tumor, stage or grade, and p53 expression.

KRAS Expression

The immunohistochemical results were negative in all cases of colonic adenocarcinoma and there was not significant correlation between greyscale and stage of tumor (r = −0.l50, $P > 0.05$), [Figure 5].

In addition, these findings did not show any significant differences in mean gray scale between patients with positive p53 expression and patients with negative p53 expression

P53 Genotype Determined by PCR

The rate of positivity for TP53 Pro/Pro genotype was more frequent in control than in patients' groups and this difference was statistically significant ($P < 0.00l$), as shown in Table 2. The risk was estimated by the use of odds ratio statistic which was 0.097 with a 95% confidence interval of 0.034–0.28l, this was due to odds ratio which is <l. Pro/Pro genotype would be considered protective against colorectal carcinoma with a preventive fraction of 0.767, as shown in Figure 6.

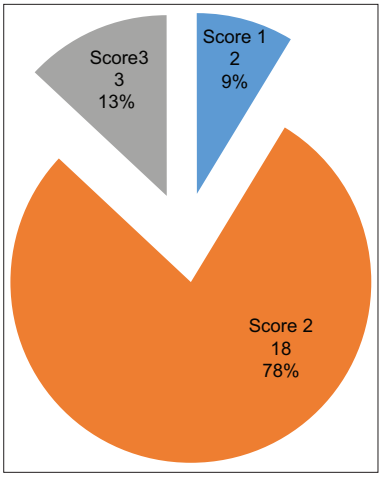

Figure 4: The proportions of p53 scores

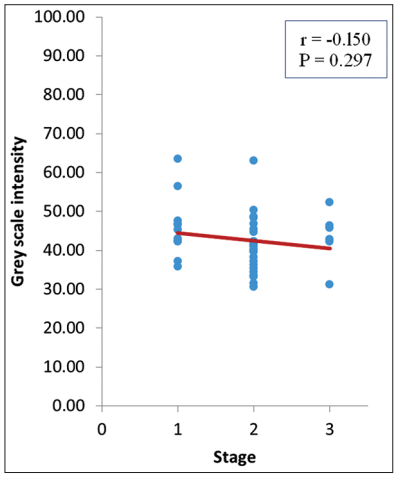

Figure 5: The spearman correlation between gray scale intensity and stage

The rate of positivity for the p53 Arg/Arg genotype was more frequent in the patients group than in the control group and this difference was statistically significant ($P = 0.019$), as shown in Table 3. Risk was estimated by the use of adjusted odds ratio statistic which was 14.2 with a 95% confidence interval of 0.792–245.7 because odds ratio is >1.

Among these findings, the Arg/Arg genotype would be considered a risk factor for colorectal carcinoma. The etiologic fraction was not estimated because one of the cells contains zero counts, as shown in Figure 7.

These results did not reveal any significant association between Arg/Arg genotype, in patients group, and

Table 1: The rate of positive p53 IHC expression in control and patients groups

p53	Control		Colonic carcinoma	
	No.	%	No.	%
Positive	0	0	23	46
Negative	35	l00	27	54
Total	35	l00	50	l00

$P<0.00l$, corrected χ^2=l9.803; DF=I

Table 2: Age and AgNOR in colorectal carcinoma patients in relation to Pro/Pro genotype

Pro/Pro	*n*	Mean	SD	*P*-value
Age				
Negative	34	49.824	18.300	
Positive	16	54.875	12.366	0.323
Total	50	51.440	16.674	
AgNOR				
Negative	34	6.097	0.817	
Positive	16	6.238	0.784	0.569
Total	50	6.142	0.801	

Table 3: Age and AgNOR in colorectal carcinoma patients in relation to Arg/Arg genotype

Arg/Arg	*n*	Mean	SD	*P*-value
Age				
Negative	42	51.405	16.200	
Positive	8	51.625	20.220	0.973
Total	50	51.440	16.674	
AgNOR	42	6.088	0.737	
Negative				
Positive	8	6.425	1.099	0.280
Total	50	6.142	0.801	

clinicopathological parameters including age, gender, site of tumor, grade, stage, and AgNOR count.

The rate of positivity for p53 Arg/Pro genotype was more frequent in patient groups than in control group and this difference was statistically significant ($P < 0.001$), as shown in Table 4. Value was estimated by the use of odds ratio statistic which was 5.236 with a 95% confidence interval of 1.852–14.807 (odds ratio is >l).

Therefore, we can conclude that Arg/Pro genotype would be considered a risk factor for colorectal carcinoma (etiologic fraction was 0.657).

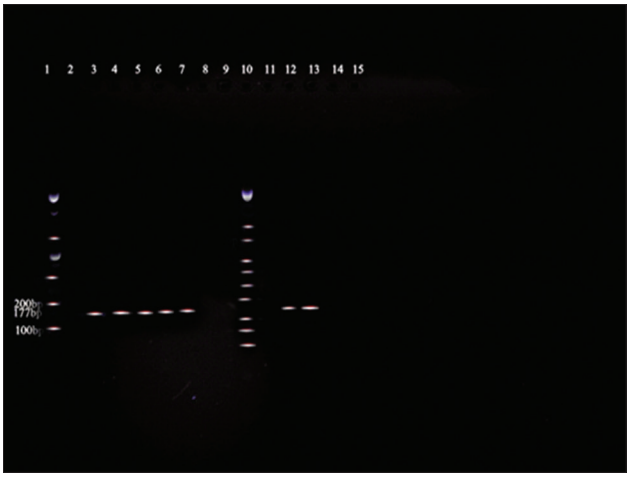

Figure 6: PCR amplification of the TP53 codon 72 (electrophoresis in 2% agarose get) in 13 colorectal adenocarcinoma samples and control samples. Lanes 1 and 10 markers, lanes 3, 4, 5, 6, 7, 12, and 13 positive for Pro allele (177 bp). Lanes 2, 8, 9, and 11 negative for Pro allele. Lanes 14 and 15 negative control

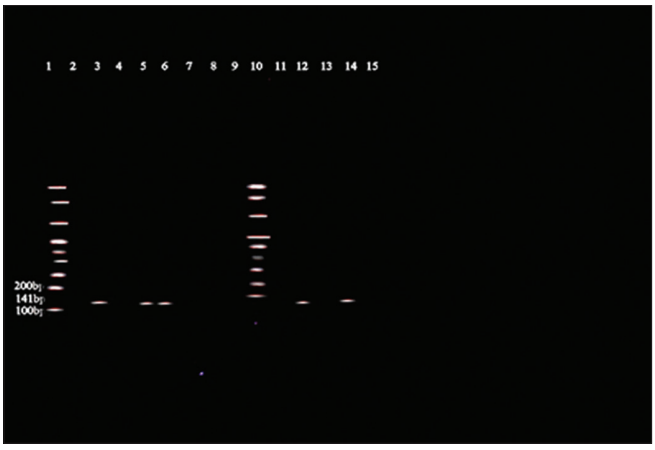

Figure 7: PCR amplification of the TP53 codon 72 (electrophoresis in 2% agarose get) in 13 colorecta1 adenocarcinoma samples and control samples. Lane 1 and 10 markers, lanes 3, 5, 6, 12, and 14 positive for Arg allele (141 bp). Lanes 2, 4, 7, 8, and 9 negatives for Arg allele. Lanes 11, 13, and 15 negative control

Evaluation of KRAS Mutation Using RFLP-PCR

Colonic tissue obtained from all patients with colorectal carcinoma was proved to be negative for KRAS mutation by the use of the RFLP-PCR technique.

DISCUSSION

Role of Immunohistochemical p53 Expression in Colorectal Carcinoma

The present study showed that p53 immunohistochemical expression was not correlated with grade or stage of tumor in patients with colorectal carcinoma. To explain that, one may suppose that p53 mutation is an early event in colorectal carcinoma and hence can be seen in low-grade, intermediate-grade, and high-grade lesions and can be seen in all stages of disease.[13] Another opinion is that p35 occurs very early in the progression of the tumor during time of conversion from adenoma to carcinoma, and hence, it will be a fixed mutation pattern in all grades and stages and eventually no association will be obtained between p53 mutation and grade of tumor and between p53 mutation and stage of the disease.[14]

Importantly, the absence of p53 expression in benign tumors indicated that p53 can be considered as a good marker for malignant colorectal tumors. However, and in harmony with several studies;[13,14] this marker was not useful for the classification of the different histopathological grades and hence prognosis of the disease. The reported pattern of P53 expression in the different stages has been interpreted into different and sometimes contradicting directions. If we consider that only 50% of poorly differentiated tumors expressed P53, p53 might be correlated with poor outcome and bad prognosis. On the other hand, differences between the different histological grades were not significant abolishing the prognostic value of this marker. This might not be surprising if we know that several studies indicated that p53 nuclear staining does not always rule out the presence of mutated malfunctioned p53 protein.[15]

Menezes[14] studied 82 patients with colorectal carcinoma for the immunohistochemical expression of p53, bcl-2, and ki67 and concluded that there was no significant correlation between the expressions of these markers separately or in conjunction,

Table 4: Arg/Pro genotype in colorectal carcinoma patients and control group among this study there was no significant association between Arg/Pro genotype, in patients group, and clinicopathological parameters including age, gender, site of tumor, grade, stage, and AgNOR count

Arg/pro	Colonic carcinoma		Control		*P*-value	95%CI			
	No.	%	No.	%		Odds ratio	Lower	Upper	EF
Positive	26	52.00	6	17.14					
Negative	24	48.00	29	82.86	0.001	5.236	1.852	14.807	0.657
Total	50	100.0	35	100.00					

concerning the grade of tumor. Asaad[16] conducted a study on the role of p53 and Cyclin D1 in 41 patients with colorectal carcinoma and reported that p53 immunohistochemical expression was not correlated to grade or stage of tumor. Another study conducted by Malik[17] on 50 patients with colorectal carcinoma and found no association between grade of tumor and p53 mutation. It was reported that by Ghavam-Nasiri,[18] there was no significant association between p53 protein expression and some common clinicopathologic variables such as age, gender, site of tumor, pathologic type, and stage of the disease. The lack of significance reported herein might indicate that the defensive role of p53 expression is accompanied by other pathways that define the prognosis and grade of the tumor. KRAS mutations, for example, were one of the targets which support that direction.[19]

On the contrary, several works of literature had reported significant associations between p53 expression and grade and stage of tumor: [20,21] From the above discussion, one can conclude that the role of p53 as a prognostic factor in colorectal carcinoma is still controversial and need thorough investigation of the contrary to the proven fact of the prognostic value for p53 expression in several other solid tumors.

Role of AgNOR in Colorectal Carcinoma

The present study showed that AgNOR score was significantly higher the patient group in comparison with the control one, and also, it was significantly correlated with the grade of tumor, being higher with less differentiated tumors. These findings can be attributed to the fact that malignant tumor cells are often characterized by aberrant chromatin patterns such as polyploidy and aneuploidy, and this abnormal chromatin pattern will be reflected in the form of high score nuclear organizing regions.

Arginine and Proline Genotype Polymorphism in Colorectal Carcinoma

The present study showed that Pro/Pro genotype was protective against colorectal carcinoma while Arg/Arg and Arg/Pro genotypes were risk factors for colorectal carcinoma, on the other hands, the present study failed to establish any significant correlation between above-mentioned genotypes and various clinicopathological characteristics of patients enrolled in the study. This may be attributed to the following suggestion: Arg/Arg genotype may be transcribed into less functioning or malfunctioning p53 protein and as it is well known that p53 is the guardian of the genome, the cell will be rendered liable for targeting by many mutagenic agents with the ultimate acquisition of the neoplastic phenotype.

Onrat[22] conducted a study on 35 patients with colorectal carcinoma and reported that individuals homozygous for the Arg allele have a higher frequency than other alleles and that colon cancer may be related to Arg allele frequency. Dastjerdi[23] also studied TP53 gene polymorphism in Iran and stated that a significant difference between cases and controls was found for the arginine/arginine genotype compared with (grouped) arginine/proline and proline/proline genotypes (odds ratio = 1.451 [1.002–2.103], P = 0.048) and that arginine/arginine genotype may be correlated with overexpression of p53 and increased risk for CRC. These findings support the findings of the current study.

In the present study, none of the tissue samples taken from the paraffin block of patients with colorectal carcinoma was positive for KRAS mutation. Smith[24-26] stated the KRAS mutations were significantly more common in rectal than in colon tumors, indicating differences in the pathways of carcinogenesis in these tissues. P53 and KRAS mutations were rarely found together in the same tumor, suggesting different genetic pathways leading to tumor formation, a finding which strongly supported the finding of the present study. It was reported that the KRAS and p53 mutations rarely coexist in the same tumor,[27] indicating that these alterations do not represent a synergistic evolutionary pathway, again these findings are similar to the findings of the present study.

CONCLUSIONS

From the achieved results of this study, we can conclude the following: P53 is an independent prognostic marker and be regarded as an early event in the pathogenesis of CRC. The p53 and KRAS mutations are not found together in the same tumor and neither KRAS gene mutations nor its protein overexpression were detected in colorectal carcinoma. Such findings necessitate the need for conducting a specific study focusing on KRAS genes expression and protein overexpression in colorectal carcinoma.

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