Elevation of Survivin correlated with *APC* mutation in Iraqi Patients with Colorectal Adenocarcinoma

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Abstract

Objective: *To investigate expression and elevation of Survivin protein and expression of mutation in APC gene in colorectal cancer, and analyze the correlation between these two parameters.*

Method : *Immunohistochemistry was employed to detect expression of Survivin protein and PCR technique for detection of mutation in APC gene in 50 patients with CRC lesions. All patients were admitted to Gastroenterology and Hepatology Diseases hospital, and Baghdad Teaching Hospital, between periods from March 2013-November2013*

Results: *Positive rates of Survivin expression were detected in 21(42%). Positive frequency of survivin were detected in 4(19%), 9(42.9%) and 8(38.1%) in well, moderate and poorly differentiated tumour respectively. No significant differences in scoring between the three grade of tumour.*
High positive rates of the expression of APC gene mutation detected in 33 (66%) for in CRC also the high percentage of mutation appeared in well 14 (42.5%), 17 (51.5%) in moderate differentiated and low 2(6.1%) in poorly differentiated tumour p value < 0.05. The results showed high percentage 18(85.7%) of patients have mutation in APC gene with expression of Survivin protein with P value 0.012.

Conclusion: *The expression rates of survivin higher and increased with progression of tumour suggesting decrease the chance of apoptosis in cancer cells. The rate of mutation in APC gene was higher in sporadic colon cancer suggesting that the expression of this mutation in tumour suppresser gene (APC) play an important role in tumourgenesis and malignant transformation.*

Key words: Survivin, Mutation, APC, Colorectal Adenocarcinoma, Wnt, immunohistochemistry.

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INTRODUCTION:

Colorectal cancer (CRC) is one of the fourth most common malignancies(Breast ,Lung and Prostate cancer) in terms of both incidence and mortality worldwide [1].The development of colon cancer is a complex multistep process dependent on both genetic alteration and environmental factors, in which constituent activation of oncogenes and accumulation of molecular genetic alterations causing disorders in cell growth, differentiation and apoptosis, also loss function of tumour suppressor genes as well as genes involved in DNA damage recognition and repair have been implicated [2]. Survivin is one of important member proteins of the inhibitor of apoptosis proteins family. The Function of survivin protein is to inhibit caspase activation in apoptosis pathway, thereby leading to negative regulation of apoptosis or programmed cell death (PCD). This protein highly expressed in most human tumors and fetal tissue, but is completely absent in terminally differentiated cells [3]. Survivin may occur early during malignant transformation or following a disturbance in the balance between cell proliferation and cell death [4]. Expression of survivin was significantly higher in adenomatous polyps and adenocarcinoma as compared to normal colorectal mucosa [5]. Because of its role in malignancy and its key role in proliferation, angiogenesis and apoptosis survivin is currently attracting considerable attention as a good and new target for anti-cancer therapies [6].

APC gene is an important tumour suppressor gene for colorectal cancer. APC mutations are responsible for familial adenomatous polyposis and up to 85% of sporadic colorectal cancers. APC is an essential component of the Wnt/β-catenin signalling pathway, which plays a key role in intestinal homeostasis and colorectal cancers [7].

APC down- regulates expression of survivin via beta-catenin/TCF4, which, in turn, modulates Aurora-B kinase (ABK) activity [8], [9]. APC, which is a tumour suppressor gene, not only helps in mitosis but also promotes both differentiation and apoptosis in the colonic crypt [10].

In colonic crypts that containing mutant *APC*, survivin expression may become constitutive, thereby inhibit apoptosis. In this case, mutant stem cell progeny would tend to maintain their natural, stem-cell-like phenotype as they migrate up the crypt. Such cells would be more likely to proliferate. Thus, constitutive expression of survivin prevents Apoptosis, contributes to cellular immortality, and may be a key contributing mechanism in early colonic tumorgenesis [11].

The aim of this study is to evaluate the expression of Survivin protein by immunohistochemistry (IHC) Correlate with mutation of *APC* gene in patients with colorectal cancer.

METHODS

Patients:

A total of 50 patients (32 men and 18 women with an average age of 51 years and a range of 24 to 78 years) presented with colorectal Adenocarcinoma, were included in this study. The patients were admitted to the Gastroenterology and Hepatology Diseases hospital, and Baghdad Teaching Hospital as well as private hospitals for diagnosis and surgical resection of colorectal cancer. Clinical information was collected through direct interview with the patient, and by seeking his /her hospital record as well as previous medical history

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Also, ethical permission was obtained from these hospitals. Tissues taken from large intestine (biopsy and mass) were fixed in 10% buffered formalin.

Number n=50			Percentage %
Site of CRC	proximal	9	18 %
	Distal	22	44 %
	Rectum	19	38 %
Grade of CRC	Well differentiated	15	30 %
	Moderate differentiated	24	48 %
	Poorly differentiated	11	22 %
Histopathological type of CRC	Adenocarcinoma	45	90 %
	mucinous	5	10 %

Table 1: Table 3.4: Distribution of patients with colorectal cancer according to the site of tumour, grade and histopathological type.

It subjected to routine pathological examination. The site of tumor, histopathological grade and type was done according to the WHO classification [12].G-spin™ Total Kit (Spin type-Intron Biotechnology, Korea) has been used to extract high purity genomic DNA from fresh or frozen whole blood. It is a quick reagent spin type kit. Twelfth normal colorectal tissue and blood samples were taken as control.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry technique is used for the detection of a specific antibody bound to an antigen in tissue sections. Paraffin embedded tissues from patients and negative and positive control samples were cut into 4μm thickness, and then placed on positively charged Silanized slides (Dako, Denmark). Deparaffinising tissue sections was done by immersed slides in sequentially of diluted alcohol and xylene the finally washed with D.W. Antigen retrieval for Survivin: The slides placed in a staining jar containing antigen retrieval solution with citrate buffer PH 6.0. Oven operated for 10 min to boiling tissue section. In our study we used (Expose Mouse and Rabbit Specific HRP/DAB detection IHC Kit (ab80436)) from (abcam, UK) for detection of Survivin using Anti-survivin antibody (Rabbit monoclonal IgG (ab93725 abcam UK)) in dilution 1/100. Immunohistochemistry applied as recommended by the manufacturing Company. Endogenous peroxidase activity was blocked by covering the tissue with H_2O_2 in moist chamber for 10 min, followed by 2 washes in PBS. Protein blocking reagent added to cover specimen, then slides were incubated at room temperature for 5-10 min, and then washed 1 time in buffer. Enough diluted primary Antibody was applied to cover the sections after the slides were placed in humid chamber then incubated at 37 C for 1 hour. The slides washed 3 times in buffer. Complement solution applied and incubated for 10 min at room

temperature the slides washed 2times in buffer. HRP conjugate was applied and incubated for 15 min at room temperature and washed 4 times in buffer. Substrate chromagen solution applied and incubated for 1-10 min at room temperature and then the slides rinsed 4 times in buffer. The slides immersed in bath containing counter stain (Mayer`s Hematoxylin) for 1 min at room temperature and then the slides rinsed gently with D.W and mounting with D.P.X. then examined under light microscope (10X and 40X). The mean percentage of positive cells for the expression of survivin was determined in at least 5 areas at 400-fold magnification, and cases with less than 10 %positively stained cells were defined as negative. Cases with 10 to 29%positively stained cells were defined as " + ", 30 to 59 % as " ++ ", and 60 % or more than 60 % as " +++ "[13].

POLYMERASE CHAIN REACTION

Procedure: *APC* gene analysis was carried out on all extracted DNA samples. The MCR (mutation cluster region) codon A 1260 to 1359 and codon D 1497 to 1596 on Exon 15 were amplified using specific primers(Integrated DNA Technologies, USA), for codon A (F:5`-CAGACTTATTGTGTAGAAGA-3`----R:5`-CTCCTGAAGAAAATTCAACA-3`) 295 bp and For codon D(F:5`-ACTCCAGATGGATTTTCTTG-3` R:5`-GGCTGGCTTTTTTGCTTTAC-3`)300bp . PCR was performed in(20µl) total volume, reaction mixture contain 50-100 ng template DNA, 100 ng of each primer (forward and reverse), 1U DNA polymerase,250µM dNTPs,10 mM Tris – HCL(pH9.0)and 1.5 mM $MgCl_2$. Master Mix (AccuuPower PCR PreMix from (Bioneer,Korea). The conditions of PCR were as follows: initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52-58°C for 1 minute and extension at72°C for 1 minute and final extension at 72 °C for7 minutes. The PCR products were run on 1.5% agarose gel and analysed under an ultraviolet illuminator.

Statistical analysis

Chi sq and correlation coefficient (pearson) were included in statistical processes of all experiments.

RESULTS

As shown in Table 2, the highly percentage of patients with CRC under investigation were recorded in 51-60 years age interval with (30%). Age interval of 41-50 years showed (26%), followed interval of 61-70 years showed (22 %), which reflects the tendency of malignant incidence in older age.

Table 2 :Distribution of colorectal cancer according to age of patients.

Age group	Count	Percentage
21-30	2	4%
31-40	6	12%
41-50	13	26%
51-60	15	30%
61-70	11	22%
> 70	3	6%
Total	50	100%
Average	55.16 years	
P Value	< 0.05	

DETECTION SURVIVIN IN CRC TISSUES

The protein was expressed in cytoplasm of tumour cells as shown in the **Figure (1).** The results showed that survivin was expressed in 21 patients (42%) from CRC patients in present study. According to grades of tumour r, Survivin was highly positive expressed in patients with moderate and poorly differentiated tumour 9 (42.9 %), **8** (38.1 %) respectively, followed by low expression **4** (19 %) in patients with well differentiated tumour. By using statistical analysis (Chi-square test) the results show high significant differences in *p* value (***p*=0.05**) as shown in Table 3. The immune-reactive score of survivin protein expression was determined semi quantitatively respectively and the positive expression was high **9** (42.9%) and **8** (38.1%) in (score 2) and (score 3) followed by **4** (19 %) in (score 1). Chi-square test showed no significant differences in *p* value **(*p*=0.223)** as shown in Table 4.

Table 3 .Expression of Survivin protein according to grade.

Variable	Total n= 50 Frequency (%)	Expression of Survivin Negative	Expression of Survivin positive	*P* Value	χ^2	*dr*
Tumour Histology		n= 29 (58 %)	n= 21 (42 %)			

Lesion Grade	negative	Positive expression (scoring)						
	0	(+) Score 1	(++) Score 2	(+++) Score 3	n= 50	*P* Value	χ^2	df
well	11	1	1	2	15	0.687	0.750	**3**
moderate	15	2	5	2	24	0.223	3.000	**3**
poorly	3	1	3	4	11	0.269	2.625	**3**
Total	χ^2 29(58%)	4 (19%)	9(42.9%)	8(38.1%)	50 100%	***0.223***	3.000	
χ^2		**0.75**	**4**	**1.5**				
P value		***0.687***	***0.135***	***0.472***				
df=		**2**	**2**	**2**				

Table 4. Frequency and score of survivin expression.

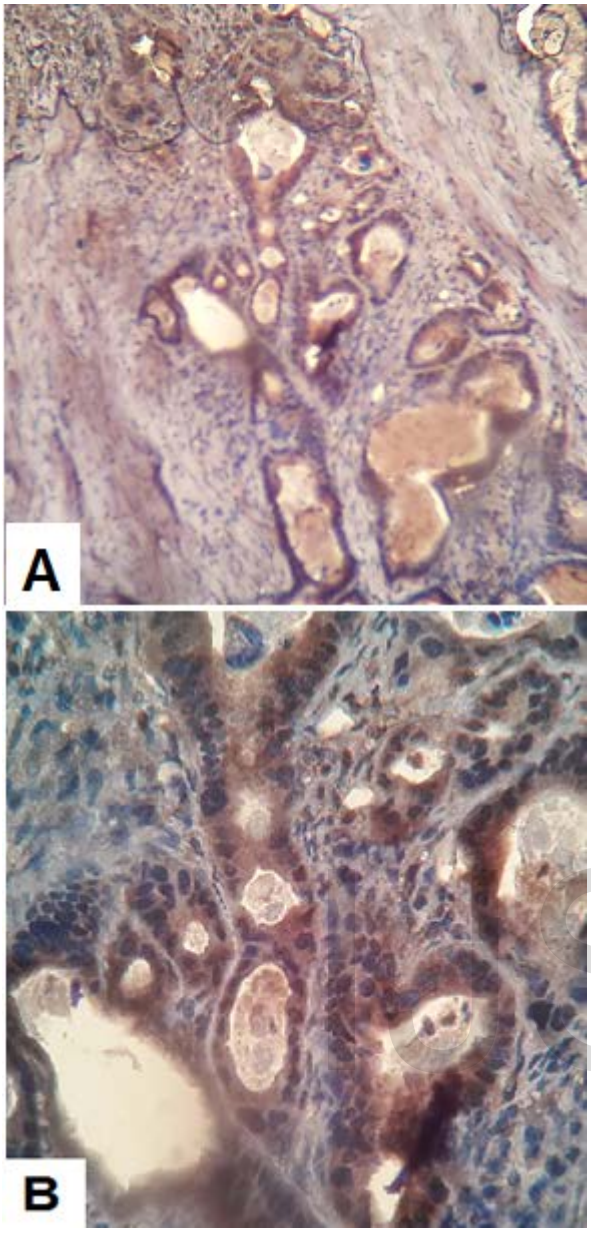


Figure 1: Expression of cytoplasmic survivin. In CRC ,A (10 X) B (40X).

RESULT OF PCR FOR DETECTION OF *APC* GENE MUTATION

Polymerase chain reaction technique was done for t determine of mutation in MCR two primer pairs (forward and reverse) were involved. A positive amplification was resulted for specific primers with certain molecular weight (bp) in addition to internal positive control band.The results revealed presence of mutation in 33(66%) from 50 patients with CRC on exon **15** for *APC* gene which contain **21** exons as shown in Figure **2,3**. Also we examined the amplification in target (1170 bp) region using *APC* AF and *APC* DR primers. The results showed expression of full length in 33 (66%) from 50 patients as shown in Figure 4.

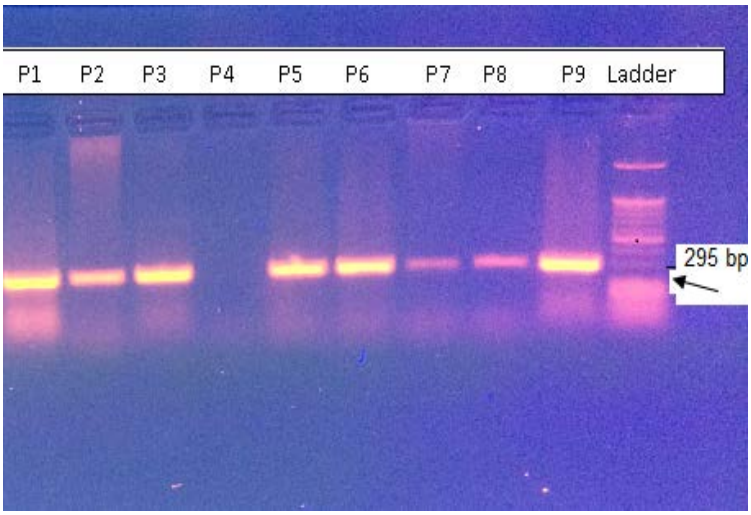
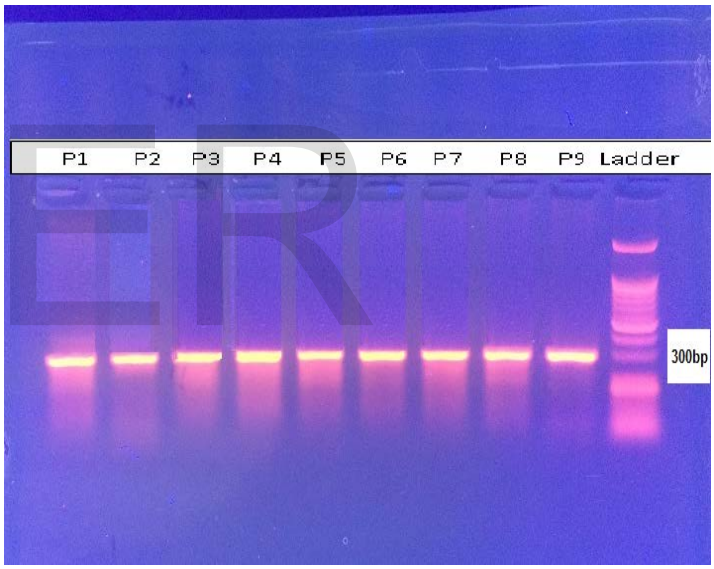
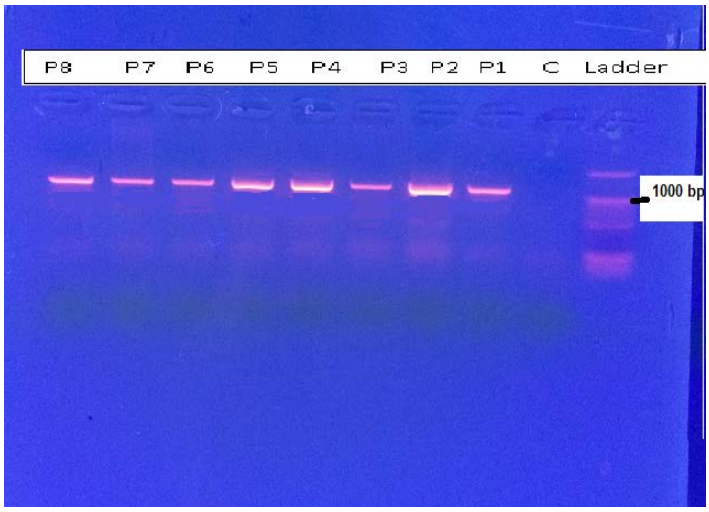


Figure **2 representative gel picture of MCR of**



APC gene Exon 15 APC A (295 bp)

Figure 3 representative gel picture of MCR of APC gene Exon 15 APC D (300 bp).



Tumor HistologyTotal	n= 50 Frequency (%)	Mutation in APC n= 17 (34%) Negative	Mutation in APC n= 33(66 %) positive	P value
Well deferentiated	15 (30 %)	1 (6.7 %)	14(93.3 %)	
moderate deferentiated	24 (48 %)	7 (29.2 %)	17(70.8 %)	
poorly deferentiated	11 (22 %)	9 (81.8 %)	2 (18.2 %)	0.001

Figure 4 representative gel picture of MCR of APC full fragment (1170 bp).

Table 5 expression of APC mutation according to grade of tumour

In present study the results showed high rate expression of survivin in well and moderate differentiated tumour compared to poorly grade with significant differences as show in Table 5

			Survivin
Spearman's rho	APC mutation	r	.354*
		p	.012

Table 6:Correlation between survivin and APC mutation

	Survivin (%)				
	N of patients	NO Expression	Expression of Survivin	*P* value	x
	(50) 100%	29	21		
		58%	42%		
APC mutation					
Non mutation expression	17(34%)	14 (48.3%)	3 (14.3%)		
		82.40%	17.60%		
Mutation	33(66%)	15 (51.7%)	18 (85.7%)		
expression		45.50%	54.50%		
P value				0.012	6.27

Also high correlation between Survivin expression and presence of *APC* mutation; there are 18 (85.7%) of patients with both survivin and mutation with p value < 0.05 (0.012) as show in Table 6.

RELATIONSHIP BETWEEN APC AND SURVIVIN

Also by statistical analysis using spearman's rho the result showed high correlation coefficient between these parameters as show in Table 7.

Table 7: correlation coefficient between Survivin and APC mutation.

DISCUTION

Survivin specifically bind to microtubule and regulate the mitotic cycle, causing inactivation of caspase -3 and caspase -7 to inhibit apoptosis, deferent level of survivin had been detected in various malignant tumour tissues 70.7% in breast cancer [14], 48.2% in gastric tumor [15] and 63.5 in CRC [16]. These studied suggested that survivin plays an important role development of tumor.

In the present study, we describe the detection of survivin protein expression in CRC. The protein was found significantly in CRC than negative expression in normal control tissues and associated with differentiation grade of tumor this was agree with other study that found a high relation of survivin expression and degree of tumor [17]. In comparison results with previous study has been controversial that survivin expressed in normal colon epithelium [11]. Other study found survivin protein in 29% of normal colonic epithelium taken from mucosa adjacent to tumor [18]. Thus, quantitative analysis of survivin may act as a diagnostic marker in CRC. Recently, antibodies to survivin protein have been demonstrated in the sera of patients with CRC [19].

For *APC* mutation the results showed high frequency of mutation (66%). This observed frequency is identical to the 60% reported by Powell et al [20] and 59.1% by other study [21]. Our mutation analysis was restricted to the MCR of *APC* and agreement with several researches that recorded 68--77% of the somatic mutations in APC are found in the MCR, which represents < 10% of the APC coding region [22]. This is contrast with study by Sameer et al who reported low rate of mutation 12.8% in Kashmiri population [23]. High frequency of expression of mutation in well followed by moderate differentiated tumor and low in poorly differentiated, it appear that mutation in MCR in APC may be lead to damage the structure of APC protein. This lead to cytoplasmic and nuclear accumulation of B- catenin which activate the Wnt pathway that promote proliferation leads to generation of tumor [24].

High correlation coefficient between Survivin and mutation in *APC* gene are present in this study and came with previous study that found high rate of both markers in CRC [11] , this finding raise an interesting question: is APC mutation regulate the survivin expression that may effect in apoptosis mechanism and maintain crypt cell renewal.

CONCLUSIONS

We concluded that, in Iraqi patients with CRC increase in survivin implicated in tumour growth. APC mutation occurs in high frequency correlated with survivin expression and this mutation plays a pivotal role in the initial tumorigenesis and enhances the tumour development beside the expression of survivin protein lead to inhibition of apoptosis that advance the tumor progression in advance stages.

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REFERENCES:

[1] World Health Organization Fact Sheet No 297, October 2011 (Accessed December 2011).

[2] S.Narayan , D. Roy" Role of APC and DNA mismatch repair genes in thedevelopment of colorectal cancers" Mol Cancer 2003, 12(2):41.

[3] NK Sah, Z Khan , GJ Khan, PS Bisen . "Structural, functional and therapeutic biology of survivin". *Cancer Lett.* 2006,**244** (2): 164–71.

[4] N. Zaffaroni. et al'' Expression of the anti-apoptotic gene survivincorrelates with taxol resistance in human ovarian cancer''. Cell Mol. Life Sci.,2002, 59, 1406–1412.

[5] H. Kawasaki , M.Toyoda , H. Shinohara , et al. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 2001;91(11):2026–32

[6] D.C.Altieri "Targeted therapy by disabling crossroad signaling networks: the survivin paradigm". *Mol Cancer Ther* 2006; 5:478-82.

[7] J.Yang,,W. Zhang,P.M. Evans, X.Chen, X.He, C. Liu"Adenomatous Polyposis Coli (APC) Differentially Regulates β-Catenin Phosphorylation and Ubiquitination in Colon Cancer Cells". *The Journal of Biological Chemistry, 2006,*281, 17751-17757.

[8] BM.Boman,L.Kopelovich,LD.Siracos.,T.ZhangT,K.Hen dersonK,Z.Cofer,etal. A Tcf4-GFP reporter mouse model for mon- itoring effects of Apc mutations during intestinal tumorigenesis. *MolCarcinog* (2009) **48**:821–31.

[9] T.Zhang,J.Z.Fields,LOpdenakeLTOtevrel,E.Masuda,J.P. Palazzo,et al." Survivin-induced Aurora-B kinase activation–amechanismby whichAPCmutations contribute to increase dmitoses during colon cancer development. *AmJPathol*(2010)**177** : 2816–26.

[10] M.B.Boman1 and J. Z. Fields" An APC:WNT counter-current-like mechanism regulates cell division along the human colonic crypt axis: a mechanism that explains how *APC* mutations induce proliferative abnormalities that drive colon cancer development" Frontieres in Oncology November 2013 ,V 3 ,Article 244.

[11] T. Zhang, T. Otevrel, Z. Gao, et al." Evidence That APC Regulates Survivin Expression : A Colon Cancer possible Mechanism Contributing to the Stem Cell Origin of colon cancer, *Cancer Res* 2001;61:8664-8667.

[12] T.Nakayama , G.Hatachi ,C.Y Wen ,A. Yoshizaki .,K. Yamazumi , D.Niino andI. Sekine ,"Expression and significance of Tie-1 and Tie-2 receptors, and angiopoietins-1,two and four in colorectal adenocarcinoma: immunohistochemical analysis and correlation with clinicopathological factors".World J. Gastroenterol, 200511(7): 964-969.

[13] LJ. Lin.,CQ. Zheng ,Y. Jin ,Y. Ma,W.G. Jiang ,T. Ma ," Expression of survivin protein in human colorectal carcinogenesis". World Journal of Gastroenterology , 2003;9(5):974-977.

[14] K.Tanaka, S.Iwamoto, G.Gon, T.Nohara, M.Iwamoto, N.Tanigawa"Expression of survivin and its relationshipto loss of apoptosis in breast carcinomas" *Clin.Cancer Res*.2000;**6**(1):127-134.

[15] XD.Zhu, G.J.Lin, L.P.Qian, Z.Q.Chen," Expression of survivin in human gastric carcinoma and gastric carcinoma model of rats"*World J. Gastroenterol*.2003 ;**9**(7): 1435-1438.

[16] A.I.Sarela, C.S.Verbeke,J. Ramsdale, C.L.Davies, A.F.Markham, P.J. Guillou, " Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma" *Br. J. Cancer*, 2002; **86**(6):886-892.

[17] Qi-lian LIANG†, Bi-rong WANG, Guo-hong LI "DcR3 and survivin are highly expressed in colorectal carcinoma and closely correlated to its clinicopathologic parameters" *J Zhejiang Univ Sci B 2009 10(9):675-682* ISSN 1673-1581.

[18] A.I.Sarela, R.C.Macadam, S.M. Farmery,A.F. Markham and P.J. Guillou" Expression of the antiapoptosis gene, *survivin*, predicts death from recurrent colorectal carcinoma" Gut, 2000;*46:* 645–650.

[19] R.Megliorino ,FD. Shi ,XX. Peng , X.Wang , E.K.Chan , E.M.Tan,J.Y. Zhang" Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection" Cancer Detect Prev. 2005;29:241-8.

[20] S.M.Powell ,N. Zilz,Y. Beazer-Barclay , et al. "APC mutations occur early during colorectal tumorigenesis"Nature 1992;359:235 – 7.

[21] S.W.Samowitz, M. L. Slattery, C. Sweeney, et al" *APC* Mutations and Other Genetic and Epigenetic Changes in colon cancer."*Mol Cancer Res* 2007;5:165-170.

[22] M. Miyaki,M. Konishi,R.Kikuchi-Yanoshita. et al. "Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors" Cancer Res., 1994;54, 3011--3020.

[23] A. Syed Sameer, Zaffar A. Shah, Safiya Abdullah, Nissar A. Chowdri and Mushtaq A. Siddiqi" Analysis of molecular aberrations of Wnt pathway gladiators in colorectal cancer in the Kashmiri population" HUMAN GENOMICS. JULY 2011,VOL 5. NO 5. 441–452.

[24] R. Fodde, R. Smiits, H. Clevers" APC signal transduction and genetic instability in colorectal cancer" Nat Rev Cancer ,2001;1(1):55-57.