Expression of p16^{INK4a} Protein in Colorectal Cancer Patients.

-*Eqbal D. Chalap Al- Grawi Al- Ma'amon University College iraq.*

!! ! ! !Œ

أجريت الدراسة الحالية على ثلاثة وأربعون مريضاً راجعوا إلى المستشفى لأجراء جراحة إزالة أورام المستقيم والقولون للمدة من تشرين الأول 2002إلى آذار 2004 ظهرا ربعة35وم وجود الورم الخبيث عند أجراء الفحص النسيجي المرضي بعد مقارنته مع الأنسجة الطبيعية المأخوذة من نفس المريض بوصفها سيطرة لكفاءة التلوين وتقييم الكيمياء النسيجية المناعية IHC أظهرت النتائج أن الفحص المناعي للجينP16IN4a أورام المستقيم والقولون لم تتجاوز نسبتها و5%في حين أظهرت 32الة إيجابية للجين 43الة من اصل 32 سلبية في عدت سالة إيجابية للجين P16^{IN4a}.

Abstract

This study carried out on Forty-three patients attending hospitals for elective surgical resection of colorectal cancer (CRC) from October 2002 to March 2004. Thirty-five resection margins that histopathologically proved free of malignancy taken from the normal tissue of the same patients used as controls of staining efficiency and evaluation for immunohistochemistry (IHC). p16^{INK4a} immunoexpression in the CRN are less than 5% and regarded as negative while 32 of 43 CRC patients had positive reaction for p16^{INK4a}.

Introduction

The cyclin-dependent kinases (CDK) activity is regulated by a number of small proteins that physically associate with cyclins, CDKs, or their complexes, these are CDK inhibitors and they are exist at least in two distinct families of CDK inhibitors in mammalian cells: the p21^{Cip1}/ p27^{Kip1} family and the p16 ^{INK4a}/p18 family.The p16^{INK4a} family of CDK inhibitors specifically interacts with the D-type cyclin-dependent kinases 4 and 6 (CDK4 and CDK6). Unregulated phosphorylation of pRb by

CDK4/6 due to loss of functional $p16^{INK4a}$ could lead to uncontrolled cellular proliferation *(Vassilis et al., 1998)*.

Materials and methods

Selection of patients and controls

Patients attending hospitals for elective surgical resection of colorectal cancer from October 2002 to March 2004 were suitable for this study.From these patients were The Gastroenterology and Hepatology Diseases Center, Baghdad Teaching Hospital as well as private hospitals. Ethical permission to conduct the research was obtained from these hospitals and from all participants in this study. Selections of the patients were accomplished with the assistance of surgeons in the above mentioned hospitals. This study carried out on 43 patients with colorectal carcinomas, 22 men (51.2%) and 21 women (48.8%) with an average age of 54 years and a range of 21 to 82 years. Those patients were 29 (67.4%) above 50 years old and 14(32.6%) patients below 50 years old. Clinical information were collected through direct interview with the patients, and by seeking his /her hospital record as well as previous medical history.In addition,Thirty-five resection margins that histopathologically proved free of malignancy taken from the normal tissue of the same patients used as controls of staining efficiency and evaluation for IHC. Specialized consultant histopathologist examined the sections with heamatoxylin and eosin (H & E). All these cases were subjected to immunohistochemical (IHC) staining protocols were carried out for $p16^{INK4a}$ protein immunoexpression.

Principles of the test

(Shi et al., 1988; Rainer et al., 1997; *George et al., 1998; Angela et al., 2000)*: This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using specific monoclonal antibodies, i.e. primary antibody for specific epitope, (mouse anti-human monoclonal antibody). That then binds to cytoplasmic or nuclear-targeted protein. The bound primary antibody then detected by secondary antibody

(goat anti-mouse), which contain specific label (Peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins). The secondary antibody is then detected by a detection system specific for the label 3, 3`-diaminobenzidine (DAB) in a chromogen solution. A positive reaction will result in a brown-colored precipitate at the antigen site in the tested tissue.

Type of Mouse Anti-human Monoclonal Antibody	Specificity	Clone	Iso-type of Monoclonal Antibody	Recommended Dilution	Applied Dilution	Positive Control
Anti-Human $p16^{INK4a}$ Protein Code No./ K5336	**Clone E6H4 4a**	**$p16^{INK4a}$**	**IgG1, kappa**	**Ready to use**	**1:25**	**Human Lymphocytes *(Jung et al., 2001).***

Immunohistochemistry procedures

Paraffin embedded sections for the patients and positive control were cut into 5 µm thickness, then placed on positively charged slides and left overnight to dry at room temperature. The slides of the patients and the positive controls placed in a vertical position in a drying incubator (hot air incubator) at 65ºC over night.Then the slides immersed sequentially in the following solutions at room temperature for 5 minutes for each of the following: Xylene, Absolute ethanol, 95% ethanol, 70% ethanol and Double distilled water *(Baukelien et al., 2000)*.

The slides were autoclave for 2 minutes at 121º C^{o} in Epitope Retrieval Solution. Then the slides removed and placed in diluted Washing Buffer jar for 5 minutes after cooling *(Bartek et al., 1999)***.** Two to three drops of peroxidase block were applied to cover the whole specimen, and then the slides were placed in a

humid chamber and incubated at room temperature for 30 minutes. 100 µl of the diluted primary antibody or negative control reagent applied onto the sections at the time the slides placed in a humid chamber and incubated at 37ºC for 1 hour. After that, the slides rinsed gently with washing buffer and placed in fresh washing buffer bath for 1 minute. One to two drops of biotinylated link secondary antibody applied onto the sections then the slides placed in the humid chamber and incubated at 37ºC for 30 minutes. One to two drops of the Streptavidin-HRP reagent applied which placed in the humid chamber and incubated at 37ºC for 30 minutes. After rinsing of slides DAB-substrate chromogen solution was applied to the slides which then incubated in darkness at room temperature for 20 minutes. Counter-stained with Mayer's heamatoxylin stain for 1 minute. The sections dehydrated by immersing the slides sequentially in ethanol and xylene containing jars. 1-2 drops of DPX applied onto the xylene-wet sections, and the sections quickly covered with coverslips and left to dry overnight *(Angela et al., 2000)*.

Evaluation of the immunostaining

(Judith et al. 2000; Zhao et al., 2003; Sarkis et al., 2001)

We count the stained cells with the assistance of histopathologist who has experienced in order to avoid non-tumorous areas in the sections. For each set of test conditions we included two types of control specimens: Positive tissue control and negative control (containing monoclonal mouse IgG2a anti *Aspergillus niger* glucose oxidase) contains an antibody that exhibits no any specific reactivity with human tissues. Scoring: reactivity was evaluated by counting the number of positive and negative cell nuclei in several randomly selected fields in each section. Tumor reactivity was expressed as the marker percent (i.e., the number of stained tumor cells per 1000 cells in each section). More than 1000 cells evaluated under 40 X high power field and the percentage of positive cells was calculated *(Nakamura et al., 1998)*.

The $p16^{INK4a}$ intensity of positivity scored as *(Zhao et al., 2003):* Less than 5 % expression as negative (cutoff value), 5-25 % expression as (+), 26-50 % expression as (++), 51-75 % expression as (+++) and More than 75 % expression as (++++)

Results

The immunoexpression of $p16^{INK4a}$ in the thirty-five normal colorectal sections were below the used cutoff value (< 5%) and they regarded as negative *(Zhao et al., 2003)*. The descriptive statistic of the normal colorectal tissue immunoexpression for $p16^{INK4a}$ is shown in table 1 & Figure 1.

Thirty-two of 43 CRC patients (Table 2 & Figure 2) had positive reaction for $p16^{INK4a,}$ Mann-Whitney test was used (table 3 & figure 3) to detect the degree of significance in immunoexpression of $p16^{INK4a}$ in CRN and CRC, their expression in CRC more than in CRN tissue by means of p value <0.001

Table 1: The Descriptive Statistic of The Immunoexpression of $p16^{INK4a}$ Expression in The Colorectal Normal Tissue (CRN) and Colorectal Cancer (CRC) .

Descriptive data for $p16^{INK4a}$		
Markers	**CRN n = 35**	**CRC n = 46**
Mean	**1.91**	**31.93**
Std. Error of Mean	**0.24**	**4.50**
Std. Deviation	**1.42**	**29.53**

Table 2: The Percentages of The Immunoexpression of $p16^{INK4a}$ InThe Colorectal Cancer (CRC) Patients.

$p16^{INK4a}$ *Immunoexpression*	Pos.	%	Neg.	%

Total (n=43)	32	74.4	11	25.6

Table 3: The Mean Ranks of the Immunoexpression of $p16^{INK4a}$ in the Colorectal Normal Tissue (CRN) and Colorectal Cancer (CRC) According to Mann-Whitney Test.

Marker	CR group	No.	Mean ranks	P value
$p16^{INK4a}$	CRN	35	27.13	<0.001
	CRC	43	49.57	

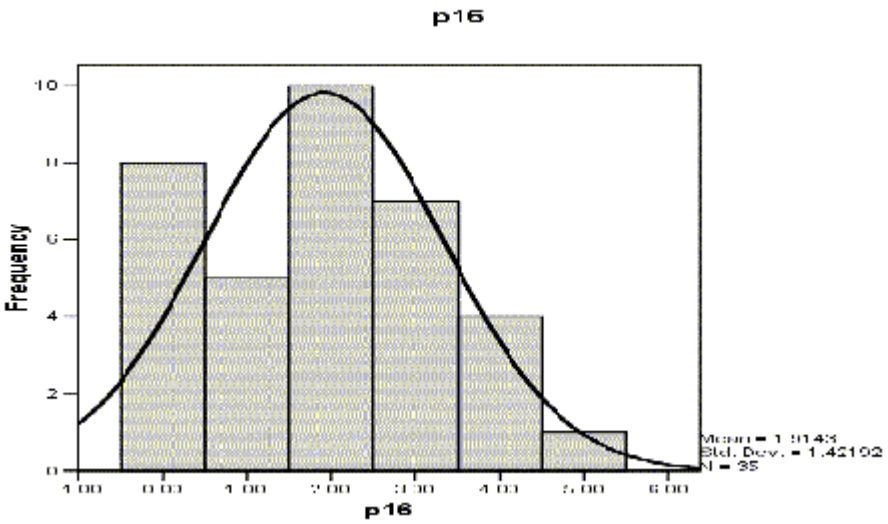

Figure 1: The Distribution of $p16^{INK4a}$ Immunoexpression in The Colorectal Normal (CRN) Specimens.

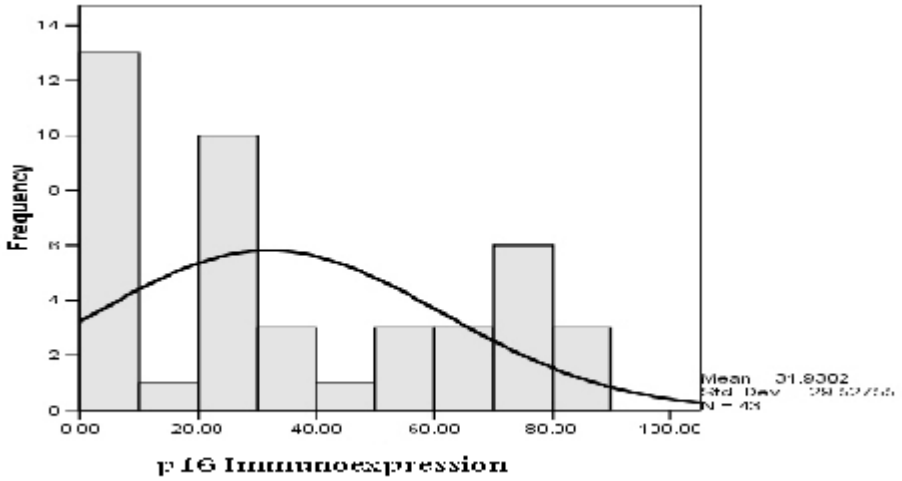

Figure 2: The Distribution of $p16^{INK4a}$ Immunoexpression in The Colorectal Cancer (CRC) Patients.

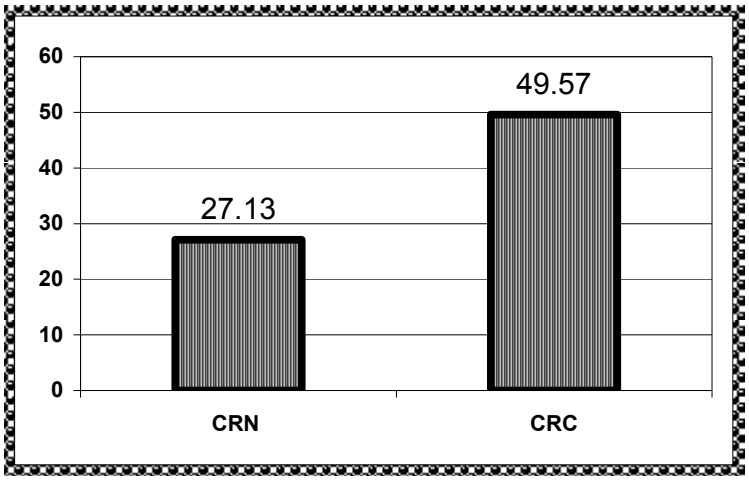

Figure 3: The Mean Ranks of $p16^{INK4a}$ Immunoexpression in Colorectal Normal (CRN) and Colorectal Cancer Tissue According to Mann-Whitney Test.

Discussion

Haber in *1997* and *Al-Mohanna et al.* in *2004* understood that $p16^{INK4a}$ consists of 156 amino acids, a major cyclin dependent kinase (CDK) inhibitor, and it is the product of a *tumor-*

suppressor gene that has been found inactivated in different cancer types. The *p16*^{INK4a} gene has been found homozygously deleted, mutated, or transcriptionally inhibited by methylation in a large number of different human tumor types.

We showed that 14.3 % of the normal colorectal tissue had no immunoexpression and the other slides have < 5 percentage immunoexpression and regarded as negative. All the p16^{INK4a} - positive cells have nuclear immunoexpression of p16^{INK4a} while in some of the tumor cells showed residual p16^{INK4a} immunoexpression in the cytoplasm of cells, whereas the rest of the cells (that regarded negative) showed only minimal or no staining at all. This finding goes with other study done by *Richard et al.* in *2000* that did immunohistochemistry (IHC) for colorectal tissue and revealed that all normal samples were essentially p16^{INK4a} negative, with only few scattered positive cells along the crypt axis with no specificity. The absence of staining was not due to methodological problems, since *Jung et al.* in *2001* who studies p16^{INK4a} immunoexpression in colorectal cancer patients observed the same results. In study of CRC patients *Tomlinson et al.,* in *1998* and *Ahuja et al*., in *1997* have observed loss of heterozygosity in the *p16*^{INK4a} locus in 38% of CRCs and hypermethylation of the *p16*^{INK4a} gene in 34% of cases.

The *p16*^{INK4a} methylation causes gene silencing and loss of p16^{INK4} tumor suppressor function in colorectal tumors was associated with proximal location in the gut. Thus, reflect the difference in the loss of p16^{INK4a} protein expression by promoter methylation between left- and right-sided primary colorectal carcinomas *(Regine et al., 2003).*

When we compared this result with the CRC, this study find 74.4% have positive reaction after IHC staining, most of them with high immunoexpression score. In other studies *Nadir et al.,* in *1999* found that the most common alteration involved an increase in the p16^{INK4a} protein immunoexpression in 92% of the adenomas and 91% of the adenocarcinoma.This can be discussed if we know that p16^{INK4a} is a tumor-suppressor protein and increased in relation to increase of proliferation rate in attempt to

over come this increasing cell cycle rate in colorectal tissue, and presence of *p16^{INK4a}* genetic alterations was associated with shorter survival in CRC patients *(Esteller et al., 2001; Isrid et al., 2001)*. In addition, The *CDKN2/ p16^{INK4a}* gene product has been found to them nonfunctional in a high percentage of cell lines (75%) and various malignancies *(Cairns et al., 1994)*.

Richard et al. in *2000* suppose that decrease in the proliferation rate was correlated with a p16^{INK4a} up-regulation and CRC lacking p16^{INK4a} immunoexpression or tumors with other aberrations in the p16^{INK4a} /cyclin D1/pRb pathway had a less pronounced decrease in proliferationThis study was verified the p16^{INK4a} immunoexpression in human colorectal tissue including, colorectal normal (CRN) and colorectal cancer (CRC). An interesting point found that p16^{INK4a} significantly have high immunoexpression in CRC than in normal colon (CRN) by means of p value <0.001.

Toyota et al., in *2000* subdivided colorectal cancer into two major groups that generate molecular diversity using different pathways (genetic or epigenetic). The molecular progression pathway to colorectal cancer starts with a single cell that has acquired a genomic alteration, which provides a growth and survival advantage to that cell and its progenitors. A typical genomic alteration would be the somatic mutation of a gene critical to the control of cell growth or death. Somatic gene mutations are the consequence of DNA point mutations, DNA rearrangements, amplifications, or deletions. *Ahuja et al.* concerned in *1997* that the mutation, whatever the form, might result in the inappropriate activation or deactivation of the gene.

Critical gene functions also may be deregulated by mechanisms other than mutation, such as inappropriate gene promoter methylation. This epigenetic gene regulation is essential to normal cell growth and differentiation. Abnormal DNA methylation, both hypomethylation and hypermethylation, is common in colorectal cancer. *Chen et al.* in *1998* detected that hypomethylation of a gene promoter will lead to inappropriate over transcription of the gene or elevated mutation rates. Whereas *Thiagalingam et al.* in *1996* found that aberrant, hypermethylation of the promoter will

lead to the silencing of the transcription. *Thiagalingam et al.* noticed in *1996* that microsatellite instability (MSI) positive cancer is linked to DNA hypermethylation. Therefore, hypermethylation of the promoter will lead to transcriptional silencing.

References

- *Ahuja N., Mohan A.L., Li Q., Stolker J.M., Herman J.G., Hamilton S.R., Baylin S.B., and Issa J.P. (1997).* Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res., 57: 3370–3374.
- *Al-Mohanna M.A., Manogaran P.S., Al-Mukhalafi Z., Al-Hussein K.A. and Aboussekhra A. (2004).* The entire tumor suppressor $p16^{INK4a}$ gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. Oncogene, 23: 201–212.
- *Angela K., Bjarne L., and Cornelis J.F.V-N. (2000).* Signal amplification in immunohistochemistry at the light microscopic level using biotinylated tyramide and nanogold–silver staining. Journal of Histochemistry and Cytochemistry, 48: 933-942.
- *Bartek J., Lukas J., and Bartkova J. (1999).* Defects in cell cycle control and cancer. J. Pathol., 187: 95-99.
- *Baukelien V.T., Barbara M.L., Herbert M.P., Harold H.J.B., Pascale S., Frank T., Thea T., Wynne G.A., Cornelius J.V-G., Frans A.N.Z., Babs G.T., Patrick G.J., and Godefridus J.P. (2000).* Thymidylate synthase expression in patients with colorectal carcinoma using a polyclonal thymidylate synthase antibody in comparison to the TS 106 monoclonal antibody. Journal of Histochemistry and Cytochemistry, 48: 755-760.
- *Breivik J., Lothe R.A., Meling G.I., Rognum T.O., Borresen-Dale A.L., and Gaudernack G. (1997).* Different genetic pathways to proximal and distal colorectal cancer influenced by sex-related factors. Int. J. Cancer, 74: 664-669.
- *Cairns P., Mao L., and Merlo A. (1994).* Rates of the p16 (MS1) mutations in primary tumors with 9p loss. Science, 265: 415-416.

- *Chen Y-G., Hata A., Lo S.R., Wotton D., Shi Y., Pavletich N., and Massague J., (1998).* Determinants of specificity in TGF-ß signal transduction. Genes and development, 12(14): 2144-2152.
- *Esteller M., Gonzalez S., Risques R.A., Marcuello E., Mangues R., Germa J.R., Herman J.G., Capella G., and Peinado M.A. (2001).* K-*ras* and *p16* aberrations confer poor prognosis in human colorectal cancer. Journal of Clinical Oncology, 19(2): 299-304.
- *George V.T., Kinga S., Michael M., Giulio D., Michele P., and Massimo L. (1998).* Down-regulation of p27 is associated with development of colorectal adenocarcinoma metastases. American Journal of Pathology, 153: 681-687.$
- *Haber D.A. (1997).* Splicing into senescence: the curious case of $p16^{INK4a}$ and $p19^{ARF}$. Cell, 91: 551-558.
- *Isrid S., Henrik P., Roland V., Matthias L., Silke R., Timo H., Gerhard W., Steffen H., Bernd D., Peter T.D. (2001).* Analysis of p53/Bax/$p16^{ink4a}$/ CDKN2 in esophageal squamous cell carcinoma: high Bax and $p16^{ink4a}$/CDKN2 identifies patients with good prognosis. Journal of Clinical Oncology, 19(8): 2272-2281.
- *Judith A.M., Joy J.D., Val G.R., Stephanie C., Fareeda Y.A., Joseph F.L., Graeme I.M., and Howard L.M. (2000).* Expression of cell cycle control proteins in primary colorectal tumors does not always predict expression in lymph node metastases. Clinical Cancer Research, 6: 1113-1118.
- *Jung A., Schrauder M., Oswald U., Knoll C., Sellberg P., Palmqvist R., Niedobitek G., Brabletz T., and Kirchner T. (2001).* The invasion front of human colorectal adenocarcinomas shows co-localization of nuclear ß-catenin, cyclin D_1, and $p16^{INK4a}$ and is a region of low proliferation. American Journal of Pathology. 159: 1613-1617.
- *Nadir A., Hanina H., Wataru Y., Alfred I.N., Aharon H., Yao Y., Alessandro S., Hirofumi Y., Itzhak S., David R., Ina F., Bernard W., Eiichi T., and Peter R. H. (1999).* Abnormalities in the expression of cell cycle-related proteins in tumors of the small bowel. Cancer Epidemiology Biomarkers and Prevention, 8: 1101-1105.

- *Nakamura M., Katano M., and Kuwahara A. (1998).* Transforming growth factor-ß1 is a preoperative prognostic indicator in advanced gastric carcinoma. Br. J. Cancer, 78: 1373-1378.
- *Okamoto A., Demetrick D.J., Spillare E.A., Hagiwara K., Hussain S.P., Bennet W.P., Forrester K., Gerwin B., Serrano M., Beach D.H., and Herris C.C. (1994).* Mutations and altered expression of $p16^{INK4}$ in human cancer. Proc. Natl. Acad. Sci. U.S.A., 91: 11045-11049.
- *Rainer M., Hartmut M., Boris H., Evariste G., Britta U.K., and Alfred C.F. (1997).* Constant detection of CD2, CD3, CD4, and CD5 in fixed and paraffin-embedded tissue using the peroxidase-mediated deposition of biotin-tyramide. The Journal of Histochemistry and Cytochemistry, 45: 1665-1672.$
- *Regine Schneider S., Carsten B., Brigitte P., Tino H., Frank M., Hans L., and Albert R. (2003).* Differences in loss of $p16^{INK4}$ protein expression by promoter methylation between left- and right-sided primary colorectal carcinomas International Journal of Oncology, 23: 1009-1013.
- *Richard P., Jorgen N.R., Bela B., Goran L., and Roger S. (2000).* Human colorectal cancers with an intact p16/Cyclin D1/pRb pathway have up-regulated p16 expression and decreased proliferation in small invasive tumor clusters. American Journal of Pathology, 157: 1947-1953.
- *Sarkis H.M., Maria K., Mohamed A., Ayman L., Fawaz H., Bruce J., and Michael E. (2001).* Bcl-2 is a useful prognostic marker in dukes' B colon cancer. Annals of Surgical Oncology, 8(6): 533-537.
- *Shi Z.R., Itzkowitz S.H., and Kim Y.S. (1988).* A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. The Journal of Histochemistry and Cytochemistry, 36: 317-322.
- *Thiagalingam S., Lengauer C., Leach F.C., Schutte M., Hahn S.A., Overhauser J., Willson J.K.V., Markowitz S., Hamilton S.R., Kern S.E., Kinzler W.K., and Vogelstein B. (1996).* Evaluation of candidate tumor suppressor genes on chromosome 18 in colorectal cancers. Nature Genetics, 13: 343-346.

- *Tomlinson I., Ilyas M., Johnson V., Davies A., Clark G., Talbot I., and Bodmer W. (1998).* A comparison of the genetic pathways involved in the pathogenesis of three types of colorectal cancer. J. Pathol., 184: 148–152.
- *Toyota M., Ohe-Toyota M., Ahuja N., and Issa J.J. (2000).* Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. Proc. Natl. Acad. Sci., U.S.A., 97: 710-715.
- *Vassilis G., Panayotis Z., Athanassios K., Triantofillos L., Aspasia K., Marinos V., Antonios R., Thanos D.H., John K.F., and Christos K. (1998).* Alterations of the p16-pRb pathway and the chromosome locus 9p21–22 in non-small-cell lung carcinomas relationship with p53 and MDM2 protein expression. American Journal of Pathology, 153: 1749-1765.
- *Wiencke J.K., Zheng S., Lafuente A., Lafuente M.J., Grudzen C., Wrensch M.R., Miike R., Ballesta A., and Trias M. (1999).* Aberrant methylation of $p16^{INK4a}$ in anatomic and gender-specific subtypes of sporadic colorectal cancer. Cancer Epidemiol Biomarkers Prev., 8: 501-506.
- *Zhao P., Ying-Chuan H., and Ian C.T. (2003).* Expressing patterns of p16 and CDK4 correlated to prognosis in colorectal carcinoma. World J Gastroenterol, 9(10): 2202-2206.