

# A clinicopathologic study of Ki-67 proliferation index in colorectal carcinoma

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## ABSTRACT

**الأهداف:** معرفة تعابير البروتين كّي أي - 67 في سرطان القولون والمستقيم وتحليل أي علاقة ممكنة مع مختلف العوامل التنبؤية السريرية والمرضية.

**الطريقة:** أجريت هذه الدراسة المقطعية في معامل الأنسجة التابعة لمستشفى ريزغاري التعليمي، إربيل، العراق، وذلك خلال الفترة من يناير 2010م إلى يوليو 2011م. لقد قمنا بتحليل المقاطع النسيجية لحوالي 50 نموذج من الأورام المثبتة في الفورمالين والمصبّة في البارافين. إن عامل التوالد (كّي أي - 67) تم حسابه مناعياً باستعمال الجسم المضاد (أم أي بي 1-) وقاعدة ستريبتوفايدين-بايوتين بيروكسيديس المناعية المعروفة. وبعد ذلك قمنا بتحليل العوامل السريرية المرضية والتنبؤية إحصائياً.

**النتائج:** أشارت نتائج الدراسة إلى زيادة تعبير بروتين التوالد كّي أي - 67 في 31 حالة (62%). وأظهر التحليل الإحصائي علاقة مميزة بين بروتين التوالد كّي أي - 67 ونوع الورم النسيجي ( $p=0.005$ ) ودرجة الورم ( $p=0.018$ ). بالمقابل لم نجد أي علاقة مميزة مع باقي العوامل السريرية المرضية كالعمر، والجنس، وحجم الورم، وموقع الورم، وعمق الورم، مرحلة انتشار الورم وحالة العقد اللمفاوية، ودخول الورم للأوعية الدموية ( $p>0.05$ ).

**خاتمة:** أظهرت الدراسة بأن الزيادة في التعبير المناعي لبروتين التوالد كّي أي - 67 هي ظاهرة موجودة عموماً في حالات سرطان القولون والمستقيم، ولكن لا يكفي الاعتماد عليها فقط للتنبؤ بالمستقبل المرضي لسرطان القولون والمستقيم.

**Objectives:** To investigate Ki-67 immunoeexpressions in colorectal cancer and analyze possible correlations with variable clinicopathological prognostic factors.

**Methods:** A cross-sectional study of tissue sections from 50 formalin-fixed and paraffin-embedded tumor specimens were examined at the Histopathology Laboratory of Rezgary Teaching Hospital in Erbil, Iraq between January 2010 and July 2011. Ki-67 labeling index is calculated immunohistochemically using the monoclonal antibody MIB-1, and the standard streptavidin-biotin

immunoperoxidase method. The clinicopathologic and prognostic features were statistically analyzed.

**Results:** Over-expression of Ki-67 proliferation protein was found in 31 (62%) cases. Statistical analyses revealed a significant relation between Ki-67 proliferation index and histologic type ( $p=0.005$ ) and tumor grade ( $p=0.018$ ); but no significant relation was calculated with the other clinicopathological parameters such as age, gender, tumor's size, site, depth, stage, nodal status, and vascular invasion ( $p>0.05$ ).

**Conclusion:** Ki-67 immune overexpression is a frequent finding in our colorectal cancer cases; but it is not enough to monitor Ki-67 proliferation index alone for prognosis in colorectal cancer.

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Colorectal cancer (CRC) is a major public health problem worldwide,<sup>1</sup> accounting for 10-15% of all cancers. It is the second leading cause of cancer-related death in industrialized countries<sup>2</sup> and the fourth leading cause of cancer death globally.<sup>3</sup> According to Iraqi Cancer Registry in 2006,<sup>4</sup> CRC ranks the 7th most common cancer with increased incidence from 25 to 50% during a 30-years period.<sup>5</sup> The vast majority of CRC cases are sporadic; this means they are not related to

genetics or family history, less than 10% of yearly colon cancer are due to a “cancer gene”.<sup>6</sup> Abundant clinical and histopathological data suggest that most, if not all, malignant CRCs arise from preexisting benign tumors. Adenoma carcinoma sequence, is a term that describes the stepwise progression of normal colon epithelium to adenomatous polyps and ultimately invasive cancer that associated with the accumulation of multiple clonally selected genetic alterations as the mutational activation of oncogenes and the inactivation of tumor-suppressor genes.<sup>7</sup> Adenomatous polyposis coli gene (APC gene) is involved in adenoma formation, and K-ras (Kirsten rat sarcoma virus homolog) oncogene is involved in the transition from adenoma to carcinoma in sporadic carcinoma.<sup>8</sup> Since cancer is a disorder of deregulated cell proliferation and cell survival,<sup>9</sup> inhibiting cell proliferation and increasing apoptosis in tumors are effective strategies for preventing tumor growth.<sup>10</sup> Ki-67 is a proliferation-associated nuclear antigen expressed in all cycling cells except resting cells in the G<sub>0</sub> phase, and it reflects cells in the G<sub>1</sub>, S, G<sub>2</sub> and M phases, in particular.<sup>11</sup> The Ki-67 gene is present on the long arm of the human chromosome 10 (10q25). The half-life of Ki-67 protein has been estimated approximately 60-90 minutes. The Ki-67 protein is phosphorylated via serine and threonine with a critical role in cell division. This has been observed from the arrest of cell proliferation when Ki-67 is blocked either by microinjection of blocking antibodies or by inhibition of dephosphorylation. The Ki-67 expression is estimated as the percentage of the tumor cells positively stained by the antibody, with nuclear staining being most common criterion of positivity. MIB-1 is a monoclonal antibody and it recognizes the Ki-67 nuclear antigen in the formalin fixed paraffin embedded tissue sections, and its reactivity is not affected if there is a delay in fixation.<sup>12</sup> The purpose of the present study is to investigate Ki-67 immunexpressions in formalin-fixed, paraffin-embedded tissue sections of CRCs and analyze their possible relations with variable clinicopathologic prognostic parameters as age, histological type, grade, and stage of the tumor.

**Methods.** A cross-sectional study of tissue sections from 50 formalin fixed, paraffin embedded, archival tissue

blocks of CRC, selected by simple random sampling from histopathology laboratory of Rezgary Teaching Hospital in Erbil/Kurdistan of Iraq during the period from January 2010 to July 2011. All patients had been diagnosed to have primary CRC and had undergone surgery. We collected the following information: age, gender, tumor site, tumor size, depths of invasion, and nodal status from the pathology reports. For each case the most represented tumor tissue blocks that contain 80% of tumor sample were chosen, and new sections were made and stained with Hematoxylin & Eosin for histological evaluation (tumor type, grade, stage, nodal status, and vascular invasion).

The pathological tumor staging was performed according to the American Joint Committee on cancer and the Union Internationale Contre Le Cancer (UICC), by grouping the various TNM components.<sup>13</sup> Histological grade was coded as low, moderate, and high.<sup>14</sup> Tumor typing (adenocarcinoma or others), was performed according to recommendation of WHO,<sup>14</sup> the anatomical location of the tumor was divided into proximal colon, distal colon, and rectum by using middle of transverse colon as partition.<sup>15</sup>

The study was approved by the Ethical Committee of the College of Medicine, Hawler Medical University, Erbil, Iraq.

**Immunostaining.** Immunohistochemistry was performed using the avidin-biotin-peroxidase complex according to a previously described protocol by Hsu et al<sup>16</sup> in which primarily monoclonal antibodies raised against Ki-67 (Dako Cytomation, Denmark, clone MIB1) was used & according to Dako Cytomation EnVision<sup>®</sup>+Dual link system-HRP(DAB+) staining protocol for immunostaining. Briefly, for antigen retrieval, deparaffinized sections were pretreated by heating in a microwave oven in 10 mM citrate buffer, pH 6.0, for 20 minutes. After cooling, sections were immersed in phosphate buffered saline (PBS) containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber overnight at 4°C with the following primary antibodies: Ki-67 (clone MIB-1; dilution 1:100; DakoCytomation<sup>®</sup>, Denmark). After rinsing with PBS, slides were incubated with a secondary antibody followed by streptavidin-biotin-peroxidase complex, both for 30 min at room temperature with a PBS wash between each step (LSAB+ system; DakoCytomation<sup>®</sup>, USA Denmark). The slides were developed with diaminobenzidine-H<sub>2</sub>O<sub>2</sub> (DAB+ system; DakoCytomation<sup>®</sup>, USA), counterstained with Harry's hematoxylin and mounted.<sup>16</sup> Negative controls, in which N-universal negative control replaced the

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primary antibody, were run with each batch of stain and breast cancer sections known to stain strongly positive for Ki-67 was included with each run as positive controls.

**Assessment of nuclear accumulation of Ki-67.**

After MIB-1 staining, positive expressions of Ki-67 in light microscope, give distinct nuclear staining of brown color. Also, the positive nuclear staining was observed in the epithelial cells of normal colonic mucosa and in the lymphoid cells and they served as internal positive control. The number of tumor cells with distinct nuclear staining was recorded by manual counting of at least 1000 tumor cells in 10 different consecutive high power fields (x400) in the most reactive areas of the slides. Cells with questionable nuclear staining were discounted. Also, necrotic or thick areas and severely overlapping tumor cells were avoided during evaluation. The percentages of positive tumor cells were then calculated as Ki-67 proliferation index (Ki-67 PI). We considered the tumor positive with significant proliferating activity only if nuclear Ki-67 accumulation was identified in at least 10% of all malignant cells in a tissue section.

**Statistical analysis.** Cross table was performed and association between Ki-67 immuoexpression and different variables were measured using the Fisher's exact test in Statistical Package for Social Sciences (SPSS, Chicago, IL USA) version 16. A  $p < 0.05$  was considered statistically significant.

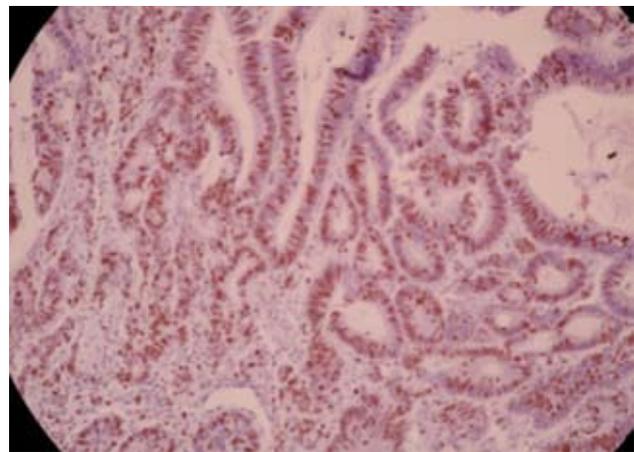
**Results.** A total of 50 formalin fixed, paraffin embedded blocks of colectomy/hemicolectomy specimens from patients with CRC, were included in this study. There were 27 (54%) males and 23 (46%) females. Patient's age ranged from 16-87 years with a mean age of 55 years. Proximal colon constituted 10 (20%), while distal colon and rectum constituted 40 (80%) of cases. Twenty (40%) of the tumors were less than 5 cm, while 30 (60%) of the tumor were more than 5 cm in size. Vascular invasion was seen in 23 (46%) of the cases. Ten (20%) were well differentiated adenocarcinomas, 31 (62%) were moderately differentiated, while 9 (18%) cases were poorly differentiated. All 50 cases were diagnosed histologically as adenocarcinoma among these, 90% (n=45) were non-mucinous adenocarcinoma and 10% (n=5) were mucinous adenocarcinoma and signet-ring cell type carcinoma.

The relationship between mean Ki-67 proliferating index and clinicopathologic variables of our CRC patients is shown in Table 1. Specific staining with monoclonal anti Ki-67 antibody MIB-1 was exclusively confined to the nuclei of the malignant cells (Figure 1). The overall frequency of CRC with positive Ki-67 nuclear staining,

**Table 1 -** Ki-67 immunostaining and clinicopathologic features of colorectal cancer patients.

Variables	N	Positive* n=31	Negative† n=19	P-value
<b>Age (years)</b>				0.371
≤55	19	10	9	
>55	31	21	10	
<b>Gender</b>				0.147
Male	27	14	13	
Female	23	17	6	
<b>Size</b>				1.000
≤5 cm	20	12	8	
>5cm	30	19	11	
<b>Site</b>				0.154
Right colon	10	7	3	
Left colon and rectum	40	24	16	
<b>Depth</b>				0.197
T1 & T2	14	11	3	
T3 & T4	36	20	16	
<b>Nodal status</b>				0.079
Negative	25	19	6	
Positive	25	12	13	
<b>Vascular invasion</b>				1.000
Negative	27	17	10	
Positive	23	14	9	
<b>TNM stage</b>				0.243
Stage I & II	25	18	7	
Stage III & IV	25	13	12	
<b>Grade</b>				0.018‡
I & II	41	29	12	
III	9	2	7	
<b>Tumor type</b>				0.005‡
<b>Adenocarcinoma</b>				
Non mucinous	45	31	14	
Mucinous +signet ring	5	0	5	

\*≥10% of tumor cells showed positive nuclear staining, †<10% of tumor cells showed positive nuclear staining, ‡significant, TNM - tumor, lymph nodes, metastasis



**Figure 1 -** Well-differentiated colorectal carcinoma with positive nuclear staining for Ki-67 (IP-Mayer's Hx. counterstain x100).

namely Ki67 proliferation index (PI)  $\geq 10\%$ , was 31 (62%). A significant relation was observed between Ki-67 PI and tumor's grade ( $p=0.018$ ) and histologic type of tumor ( $p=0.005$ ); but there was no significant relationship between Ki-67 PI expression and other clinicopathologic parameters in terms of gender, age, tumor size, location, and so forth ( $p>0.05$ ).

**Discussion.** Colorectal carcinoma is a major cause of morbidity and mortality worldwide.<sup>17</sup> Cellular proliferation is fundamental to maintain tissue homeostasis and is important in oncogenesis. More recently, advances in understanding tumor biology had led to development of targeted therapy, allowing progress in treatment of CRC<sup>18</sup> and quantification of cell proliferative activity in neoplasia is currently the subject of considerable investigation as assessment of tumor cell proliferation may predict tumor behavior.<sup>19</sup> The Ki-67 is a nuclear antigen expressed in highest concentration in all stages of the cell cycle, but not in resting cells and it is widely used in routine pathology as a proliferation marker to measure the growth fraction in human tumors. MIB-1 is a monoclonal antibody that recognizes a fixation resistant epitope of Ki-67 antigen; and has allowed retrospective examination and estimation of the proliferative fraction of neoplasia.<sup>20</sup> This study was designed to evaluate the immunoreactivity of Ki-67 PI in formalin-fixed, paraffin- embedded tissue sections of colorectal carcinomas and to investigate the relationship between the proliferative activity of colorectal carcinoma with variable clinicopathologic prognostic parameters as age, histological type, grade, and stage of the tumor.

Thirty-one (62%) cases of CRC showed 10% or more positive tumor cell nuclei and 19 (38%) cases had less than 10% positive tumor cell nuclei. Worldwide, CRC showed a wide range of Ki-67 PI, ranging from 13-90%,<sup>19-23</sup> indicating a variation in proliferative activity. Explanation for this wide-ranged variation in the proliferative activities of CRC, as measured by MIB-1 antibody, among studies could be due to a difference in epitope preservation, in staining procedures, in methods of evaluation or quantification of Ki-67 immunostaining and in study population.

It was observed, that Ki-67 PI was high in Grade I and Grade II as compared to the Grade III carcinomas with significant statistical relation ( $p=0.018$ ), indicating that proliferating index was low in poorly differentiated tumors than well or moderately differentiated. These findings are similar to a study in Japan,<sup>22</sup> in which they concluded that the positive rate of Ki-67 PI in poorly differentiated adenocarcinoma and mucinous carcinomas was significantly lower than in well differentiated and moderately differentiated adenocarcinomas, suggesting

that proliferative activity is low in cancers with poor differentiation. On the other hand, other studies<sup>20,23</sup> showed the reverse, in which the Ki-67 PI appeared to increase with decreasing degree of differentiation of carcinoma.

Another highly significant relation observed between Ki-67 PI and histological type of CRC ( $p=0.005$ ), in which the proliferative activity was higher in non mucinous tumors than in mucinous and signet ring carcinoma. This finding agreed with other studies;<sup>23</sup> but disagreed with others as that carried out by Lanza et al where mucinous carcinoma showed higher levels of Ki-67 reactivity than non-mucinous adenocarcinomas.<sup>24</sup> Also, it was observed that Ki-67 PI was not significantly related with the TNM tumor's stage. Other studies showed that tumors in advanced stage with subserosa or deeper invasion have a low Ki-67 proliferating index than tumors in an early invasive stage;<sup>22</sup> while others showed the reverse; namely, mean Ki-67 proliferating index increased with advancing tumor stage.<sup>20</sup> The other remaining clinicopathologic parameters as age, gender, tumor location, size, depth, nodal status and vascular invasion showed no statistical significant relationship with Ki-67 PI expression. This is supported by studies from others,<sup>25-27</sup> who reported no relation between Ki-67 immunoreactivity and various clinicopathological and prognostic variables in cases of colorectal carcinomas. Other investigators have suggested that this lack of relation is due to considerable heterogeneity in colorectal carcinomas.<sup>21,28</sup>

In conclusion, immunohistochemical technique for detection the Ki-67 PI is simple and applicable to surgical specimens; and reproducibility with MIB-1 antibody immunostaining is excellent even when paraffin embedded tissue sections are used. Ki-67 immune overexpression is a frequent finding in our CRC cases, but it is not enough to monitor Ki-67 proliferation index alone for prognosis in colorectal cancer as it was not significantly related to variable clinicopathologic parameters a part from histological type and grade of tumor.

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