MCM-2, Ki-67 and PCNA Protein Expressions in pT3G2 Colorectal Cancer Indicated Lymph Node Involvement

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Abstract. *Background: The rate of cancer cell growth depends on proliferative activity and on the tumor cell death rate. The study objective was to assess the expressions of Ki-67, PCNA and MCM2 in colorectal cancer and to analyze the correlation between the expressions and chosen anatomo-clinical parameters (patient's age, tumor location, lymph node metastases). Patients and Methods: The study material included primary tumors pT3 G2 obtained from 55 patients with colorectal cancer. Immunohistochemical investigations were performed using monoclonal antibodies (Ki-67- monoclonal mouse (DAKO) Clone MIB1, PCNA-monoclonal mouse (DAKO) Clone PC10, MCM2-polyclonal goat (Santa Cruz Biotechnology) Sc-9839) directed against human protein Ki-67, PCNA and MCM2. Results: The expressions of Ki-67, PCNA and MCM2 were found to correlate with the presence of lymph node metastases, but not with patient age or tumor location. The expressions of these proteins in the main tumor mass correlated with each other in the majority of patients. Conclusion: The findings allow the assumption that positive expressions of Ki-67, PCNA and MCM2 in the main tumor mass in pathological stage pT3 and degree of histological malignancy G2 may indicate lymph node involvement.*

A tumor's growth rate depends on proliferative activity and the tumor cell death rate. The Ki-67, PCNA and MCM2 proteins appear useful in the evaluation of tumor cell proliferation (1).

The proliferating cell nuclear antigen (PCNA) is a non-histamine nuclear protein which has a molecular mass of 36 kDa. It occurs in two functionally different forms that can be separated due to differences in solubility. One of them, whose expression remains stable within the all cycle, is associated with DNA repair. The role of PCNA in this process lies in its interactions with DNA polymerase epsilon,

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whose basic function is DNA repair. The other form acts as an auxiliary protein for DNA polymerase delta, which is the basic replication enzyme (1). PCNA takes part not only in DNA replication, but also in the reconstruction of the double DNA stands. PCNA starts to accumulate in the G1-phase of the cell cycle reaching a maximum expression in the S-phase, then gradually disappearing till the end of mitosis (2). This antigen functions as a homotrimer, forming a ring of 34L in the internal diameter that ensures embracement of the DNA. This allows DNA polymerase delta to be attached to the DNA chain. Each monomer has two domains, each containing two α helicases, responsible for unwinding of double-stranded DNA helices. The molecular charge is asymmetrically distributed. The internal part has a positive charge stabilizing interactions between PCNA and DNA, while the external surface possesses a negative charge preventing non-specific DNA effects (1). As the PCNA expression occurs in proliferating cells, its level can be a marker of cell cycle kinetics and proliferative activity. A significant correlation has been noted of PCNA level with the degree of tumor malignancy, vascular infiltration, distant metastases and survival (2). Since PCNA expression reflects cell proliferative activity, and proliferation is associated with the formation of neoplasms, including colorectal carcinoma, this antigen has been described as a biomarker of colorectal carcinoma risk (2).

In oncology, the Ki-67 antigen is defined as a marker of tumor growth demonstrating the percentage of cells during division. The Ki-67 gene is present on chromosome 10q25 and identifies non-histone protein located in the nucleus, probably bound to the nucleolus (3). Ki-67 is a DNA-binding protein, associated with the proliferative process of the cell, which shows expression in all phases of the cell cycle except for G1. The expression of Ki-67 in the G1-phase is equivocal. Some authors describe an increase in its expression in the late G1-phase, while others only in the S-phase of the cell cycle (3). All the investigators agree, however, that the antigen expression increases during the S and G1 phases, reaching a maximum in metaphase, then decreasing during anaphase and telophase (4). Distribution of Ki-67 in the cell changes within the cell cycle. In the early
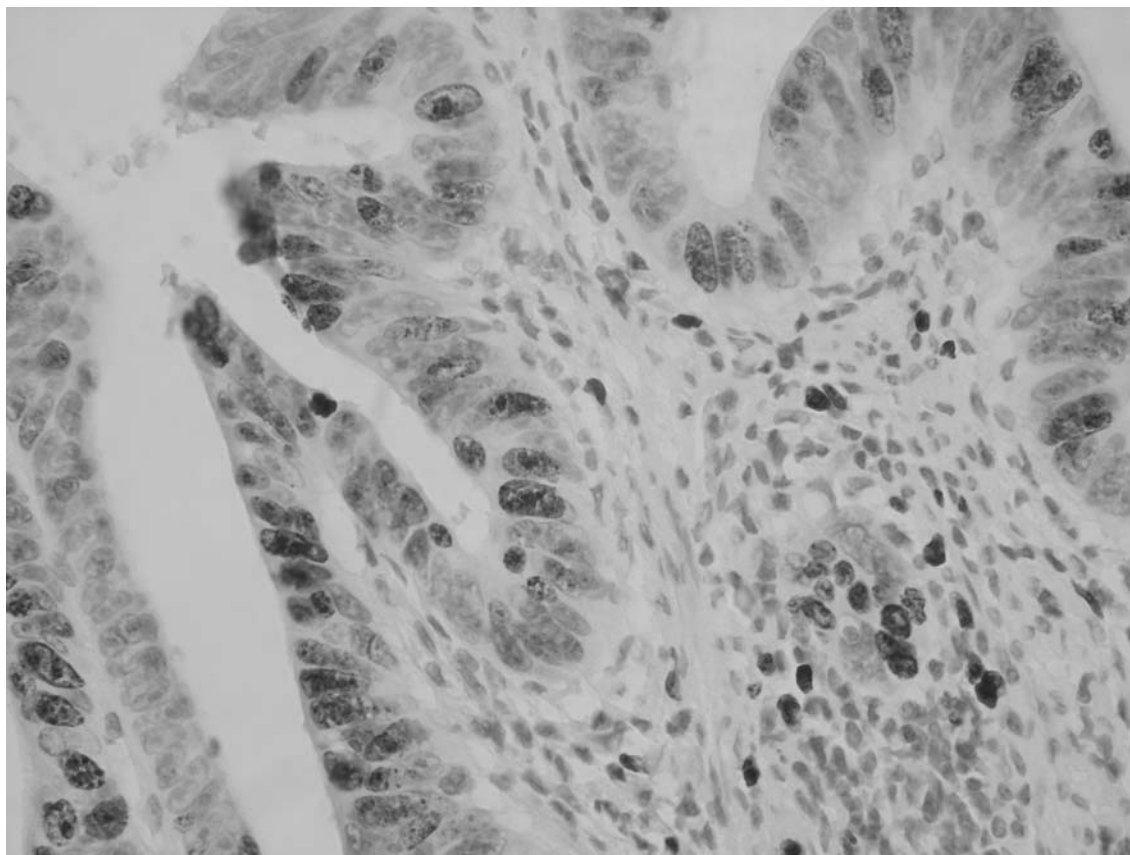
Figure 1. *Nuclear reaction of Ki-67 in specimen of colorectal cancer (x200).*

G1-phase, its location overlaps with satellite DNA regions. In the middle phase of G1 or in the S-phase, Ki-67 is found mainly in the nucleolus. In the G2-phase, Ki-67 expression is confined to the nucleus (5). Following termination of cell division and passage to the interphase, the nucleus undergoes degradation.

The expression of Ki-67 antigen can be used to assess survival, neoplastic progression and can be a marker of disease relapse (6). A large number of Ki-67-positive cells indicates shortened survival also in the carcinomas of the prostate, the breast, the liver, malignant melanoma, malignant lymphomas and lung cancer (3).

MCM2 belongs to a family of ten MCM (minichromosome maintenance) proteins involved in the initiation of replication (7, 8). Six of these proteins, MCM2 to 7, are characterized by a similar structure and function, thus forming the family of DNA helicases. During the cell cycle, MCM2 to 7 form a hexametric complex (~ 600 kDa), the major component of the prereplication complex. It accumulates during the early G1-phase and then binds to chromatin at the site of replication initiation in the late G1-phase (8, 9). The MCM2-7 complex unwinds DNA double helices during the initiation

of replication (9). Being a part of the replisome, a complex of proteins that form a characteristic structure on the replication fork, it also regulates DNA elongation. The MCM2-7 complex is responsible for a single DNA replication during the cell cycle and is removed in the S- and G2-phase (8, 9). MCM protein expression is high in proliferating cells, but low or absent in the G0-phase.

The function of the MCM2 protein suggests that it can be used as a marker of proliferation, as well as a prognostic factor in neoplastic disease (8). Elevated MCM2 expression is demonstrated by 97% of neoplasms, including breast carcinoma, lung carcinoma, prostate carcinoma, renal adenocarcinoma, cancer of the urinary bladder, glial cell carcinoma and colorectal cancer (2, 7, 8). A comparison between MCM2 expressions in normal tissues and neoplastic lesions has demonstrated that this antigen may be a more reliable marker than PCNA or Ki-67, allowing differentiation of normal cells from the neoplastic ones (9).

The study objective was to assess the expressions of Ki-67, PCNA and MCM2 in the cells of the main tumor mass in correlation with chosen anatomo-clinical parameters in patients with colorectal carcinoma pT3 G2.
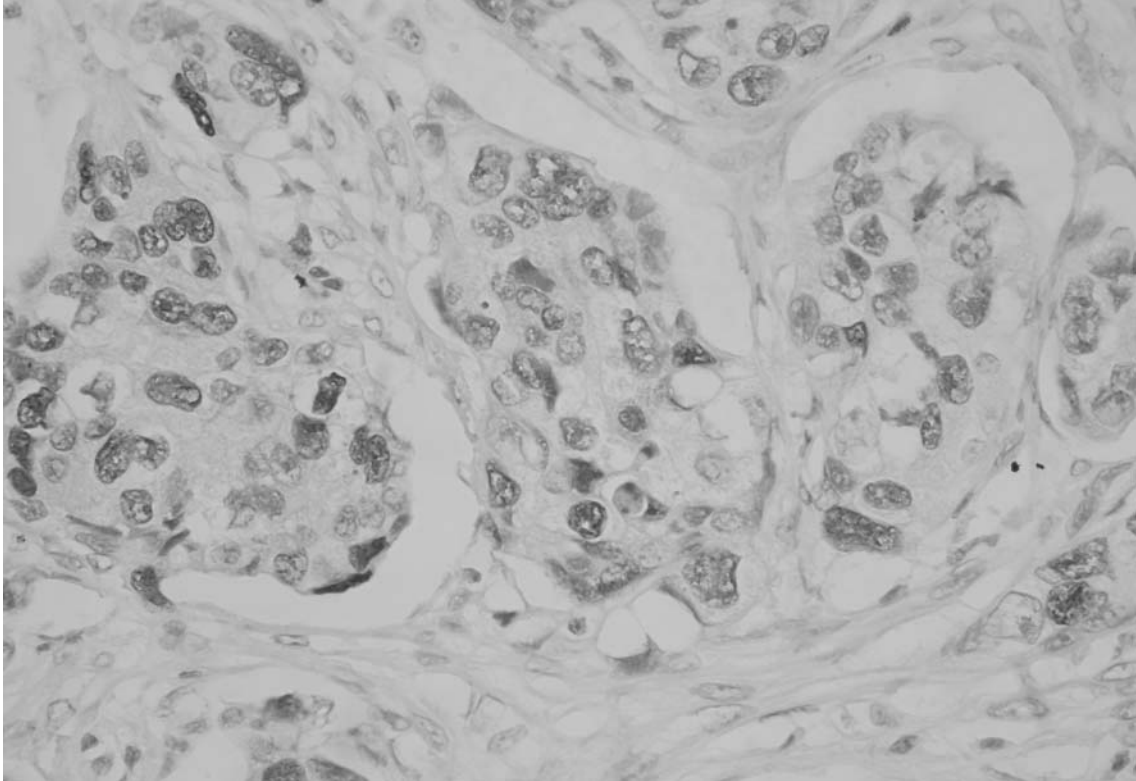
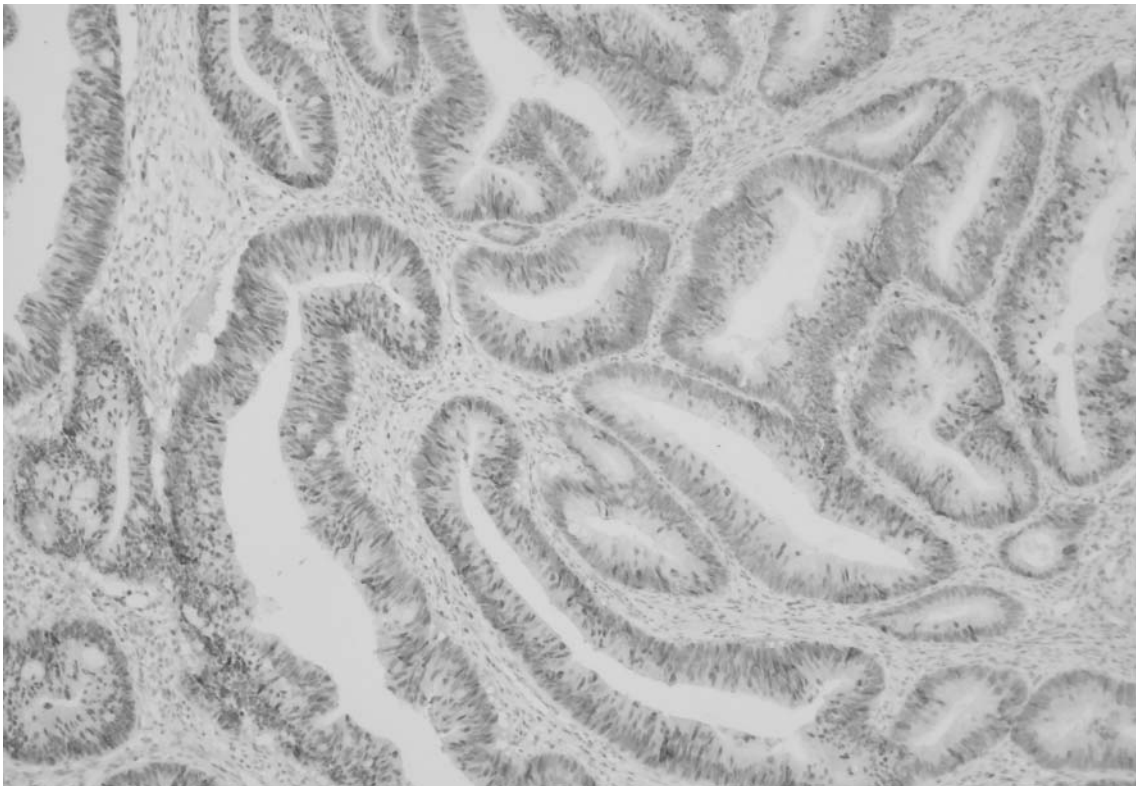Figure 2. *Strong reaction of PCNA in main mass of tumor from colorectal cancer (x400).*

Figure 3. *Reaction of MCM-2 in colorectal cancer cells (x100).*

Patients and Methods

The study group consisted of 55 patients with colorectal carcinoma (pT3; G2), who were operated on in the Surgical Ward of the J. Sniadecki Hospital in Bialystok. Correlations between the expressions of antigens of cell proliferation Ki-67, PCNA, MCM2 and chosen anatomo-clinical parameters (patient's age, tumor location, lymph node involvement) were analyzed. There were 34 women and 21 men in the group, aged 43-89 years (mean 69 years).

Immunohistochemistry. Slides of 4 μm-thick serial sections of the primary tumor were prepared from each patient. The immunolocation of Ki-67 (monoclonal anti-human Ki-67 antigen/ clone MIB-1/DAKO, Gdansk, Poland), PCNA (monoclonal mouse anti-PCNA/clone PC10/DAKO, Gdansk, Poland) and MCM-2 (MCM2/Sc-9839, goat polyclonal/Santa Cruz Biotechnology, Warszawa, Poland) was performed using the labelled streptavidin biotin (LSAB) method protocol, described by DAKO (LSAB+HRP Kit, DAKO, Gdansk, Poland). In brief, the slides from each patient were dewaxed using xylene and transferred to alcohol. They were then placed in citric buffer (pH 6.0) and heated in a microwave oven (700 W) for 10 minutes to expose antigens. Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide in methanol for 10 min. After washing with phosphate-buffered saline (PBS), the slides were incubated at 20˚C for one hour with Ki-67 and PCNA monoclonal antibodies or overnight for MCM-2 antibody. The reaction products were visualised with diaminobenzidine (DAB) (DAKO, Poland). Nuclear immunostaining was observed for all proteins (Figures 1-3).

Immunostaining evaluation. Nuclear accumulation of Ki-67 protein in neoplastic cells was assessed semiquantitatively and defined as: (–) lack of reaction to Ki-67 or reaction <50% of cells, (+) reaction to Ki-67 present in >50% of cells. The reaction to PCNA was evaluated according to the following criteria: (–) lack of reaction to PCNA or reaction present in <60% of cells, (+) reaction present in >60% of cells. The reaction to MCM2 was described as: (–) lack of reaction to MCM2 or reaction in <50% of cells, (+) reaction present in >50% of cells.

The percentage of reaction-positive cells was calculated in 500 neoplastic cells in each study sample at x400 magnification. Results were subjected to statistical analysis using the Pearson's correlation coefficient.

Results

Statistical analysis showed lack of correlation of Ki-67 expression with patient age and tumor location. However, the Ki-67 expression was found to correlate with lymph node involvement and was statistically significant (Table I).

Statistical analysis demonstrated lack of correlation of PCNA expression with patient age and tumor location. However, a statistically significant correlation was noted between PCNA expression and lymph node involvement (Table II).

Statistical analysis found a lack of correlation of MCM2 expression with patient age and tumor location. However, a statistically significant correlation was noted between the expression and lymph node involvement (Table III).

Table I. *Correlation between expression of Ki-67 and chosen features in colorectal cancer (n=55).*

	Age	Location	Lymph node metastasis	PCNA	MCM2
Correlation coefficient (r)	0.116	0.028	**0.834**	**0.888**	**0.793**
Significance (*p*-value)	0.400	0.840	<0.0001	<0.0001	<0.0001

Table II. *Correlation between expression of PCNA and chosen parameters (n=55).*

	Age	Location	Lymph node metastasis	Ki-67	MCM2
Correlation coefficient (r)	0.124	0.023	**0.829**	**0.888**	**0.775**
Significance (*p*-value)	0.368	0.866	<0.0001	<0.000	<0.000

Table III. *Correlation between expression of MCM2 and chosen parameters (n=55).*

	Age	Location	Lymph node metastasis	PCNA	Ki-67
Correlation coefficient (r)	0.109	0.122	**0.788**	**0.775**	**0.793**
Significance (*p*-value)	0.426	0.375	<0.0001	<0.0001	<0.0001

Discussion

A neoplasm is a tissue that proliferates in a non-coordinated way with adjacent tissues. Neoplastic growth is characterized by unrestrained proliferation predominating over cell death and by a loss of normal cell differentiation. Thus, a neoplasm is a growth disorder showing excessive cell proliferation. Therefore, the assessment of this proliferation is a valuable method used in the diagnosis of neoplastic lesions.

Certain high-molecular substances, *e.g.* Ki-67, PCNA and MCM2, are used for the assessment of proliferation. As a change in their expression reflects the proliferative activity of neoplastic cells, they are referred to as markers of proliferation. Overexpression of these markers may suggest disturbed regulation of division of cells that form an

intensively growing tumor. It can also help evaluate the degree of aggressiveness and metastasizing potential.

Similarly to other researchers, we found no correlation of Ki-67 expression in the main tumor mass with patient's age or tumor location (10, 11). Valera *et al.* (12) have demonstrated that Ki-67 expression correlates with tumor development. Mullerat *et al.* (13) revealed that Ki-67 may serve as a marker of early dysplasia and therefore its expression should be used as a screening test for high-risk individuals.

Numerous reports describe the correlation between Ki-67 expression and tumor advancement (14). Considerable differences have been noted in this expression between normal mucosa and adenoma or adenocarcinoma (15). The expression of Ki-67 is higher in adenoma than in normal mucosa, being the highest in adenocarcinoma (16). The expression of this antigen also reflects the degree of neoplastic cell differentiation. The lower the degree, the higher is the Ki-67 expression in neoplastic cells (16, 17). Dzięgiel *et al.* (17) have found that Ki-67 expression strongly correlates with the degree of histological malignancy (G) and depth of tumor infiltration in the intestinal wall (pT). The deeper the infiltration, the higher the expression. Rubio *et al.* (18) have obtained results suggesting more intensified expression at the invasion front.

We found a correlation between Ki-67 expression and lymph node involvement. The Ki-67 expression was more pronounced in patients with lymph node metastases. According to Valera *et al.* (19), the determination of Ki-67 expression allows classification of patients with similar clinical features and survival prognosis into diagnostic groups.

Many literature reports discuss the application of Ki-67 as a marker to evaluate the survival of patients with colorectal cancer (10, 20, 21). The authors agree that in patients who exhibit high Ki-67 expression, shorter survival can be predicted as compared to those with low levels of this antigen (10, 20). Salminen *et al.* (21) showed that significant prognostic factors were Dukes stage, presence of lymph node metastases, age and presence of Ki-67 (>50%) in colorectal cancer.

Many authors have also described the significance of Ki-67 expression for therapy prognosis. Micev *et al.* (22), who evaluated the correlation between Ki-67 expression and response to chemotherapy, showed that in individuals with higher expression, therapy was less effective. Freitas *et al.* (15) found that increased expression of PCNA or Ki-67 in adenomatous tissue May be a predictor of adenoma reccurrence.

Statistical analysis of the findings concerning the pT3 G2 carcinomas showed no statistically significant correlation of PCNA expression with patient's age or tumor location. Similar results were obtained by Sun *et al.* (23) and MaKay *et al.* (24). Most authors seem to agree that PCNA expression does not correlate with age, sex or tumor location. However, there are many literature reports that describe the relationship between intensification of PCNA expression in the primary tumor and lymph node involvement (25). Nakae *et al.* (26) estimated the expression of PCNA in colorectal carcinoma in patients without distant metastases as well as in those with liver involvement. Their findings evidently indicate that PCNA is higher in patients who develop liver metastases.

Many authors have also evaluated the correlation between the depth of invasion into the colonic wall and PCNA expression, revealing that the expression grows with increasing infiltration depth (26). According to Teixeira *et al.* (27), there is a statistically significant correlation between PCNA expression in the invasion front and metastasizing potential.

Some literature data refer to the changes in PCNA expression depending on the degree of dysplasia. The expression is less pronounced in low-degree dysplasia, gradually increasing reaching higher values in high-degree dysplasia, being the highest in cancerous lesions. Shimada *et al.* (28) described the correlation existing between PCNA and atypia of neoplastic cells, suggesting a rise in the expression with increasing atypia (28). Intensification of the expression also reflects the degree of differentiation of neoplastic cells. According to literature data, the less differentiated the cells, the higher their PCNA expression (27).

Many investigators have demonstrated a statistically significant correlation between PCNA expression and the degree of tumor advancement (29, 30). The expression grows gradually with the advancement of lesions from normal mucosa, through adenoma to adenocarcinoma, showing a correlation with histological malignancy (31). Changes in the expression from normal mucosa to colorectal carcinoma have been described. In normal intestinal mucosa the expression of PCNA is approximately 30% and is present in the lower part. However, in carcinoma cells, there is a 72% expression, and the PCNA-positive cells are dispersed. It has been also shown that higher-stage carcinomas (pT) exhibit greater PCNA expression. Larezzi *et al.* (32) described PCNA as a marker able to identify more aggressive adenomas that may transform into malignant neoplasms.

The current investigations performed on a homogenous group of pT3 G2 carcinomas revealed a correlation between PCNA expression and lymph node involvement. The expression was higher in patients with metastases to lymph nodes. Our findings seem to confirm the results reported by other authors (25, 27).

There is also much literature concerning the role of PCNA as a marker of survival (26). Choi *et al.* (25) found a correlation between the enhancement of PCNA expression and a chance for 4-year survival, decreasing with growing expression. Qiu *et al.* (33) showed the existence of a statistically significant correlation between PCNA expression and cancer relapse. In our study, the expression

was significantly higher in patients experiencing a relapse. Hence, it can be concluded that more pronounced enhancement of the PCNA expression demonstrates a greater risk of relapse.

Statistical analysis of the results obtained in the study group did not show a significant correlation of MCM2 expression with patients' age or tumor location. Davies *et al.* (7) estimated the differences in MCM2 expression between patients with colorectal carcinoma and a control group. Their findings clearly indicate that intensification of MCM2 expression correlates with the presence of this carcinoma.

The current study showed a correlation between MCM2 expression and metastasis to lymph nodes: the expression was higher in patients with lymph node involvement. Scott *et al.* (34) described a relationship between the intensity of MCM2 expression and tumor advancement. The expression grew gradually with advancement from normal mucosa, through adenoma, reaching a peak in adenocarcinoma.

Statistical analysis of our results showed a significant correlation between the expressions of Ki-67, PCNA and MCM2 in most patients.

Summing up, the findings obtained in this study group of homogenous colorectal carcinoma patients (pT3 G2) show that the expressions of Ki-67, PCNA and MCM2 in the main tumor mass indicate lymph node involvement, and thus greater aggressiveness of the neoplastic process.

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