

Correlation of Ki-67 and MCM-2 proliferative marker expression with grade of histological malignancy (G) in ductal breast cancers

Andrzej Wojnar¹, Christopher Kobierzycki², Anna Krolicka², Bartosz Pula²,
Marzena Podhorska-Okolow², Piotr Dziegiel^{1, 2, 3}

¹Lower Silesian Oncology Centre, Wrocław, Poland

²Department of Histology and Embryology, Medical University, Wrocław, Poland

³Department of Histology and Embryology, Medical University, Poznań, Poland

Abstract: The study aimed at examining a relationship between expression of Ki-67 antigen and minichromosome maintenance 2 protein (MCM-2) and a grade of histological malignancy G in ductal breast cancers. The function of widely used marker of proliferation Ki-67 is still not clear. In contrast, the MCM-2 protein is well known to play an important role in controlling the cell cycle. Both proteins represent small protein molecules, which manifest nuclear expression only during cell division of normal and neoplastic cells. Their expression is noted in several malignant tumours. These studies were conducted on 56 archival paraffin blocks of ductal breast cancers. Immunohistochemical reactions were performed using monoclonal Ki-67- and MCM-2-specific antibodies. Statistical analysis demonstrated a positive correlation between expressions of two proteins ($r=0.6$; $p<0.05$). The most intense expression of these two markers was demonstrated in G3 grade cancers. Statistical analysis showed more pronounced expression of Ki-67 antigen in G3 grade cancers as compared to cancers of G1 and G2 grades ($p<0.001$) and, in the case of MCM-2 protein, a more pronounced expression in G3 grade cancers, as compared to those of G1 ($p<0.05$) or G2 grade ($p<0.01$). The results obtained in our study suggest that MCM-2 could be used as a marker of proliferation in breast carcinomas.

Key words: Breast cancer, Ki-67, MCM-2.

Introduction

In 2006, 429 900 cases of breast cancer were diagnosed in Europe (the most frequent malignant tumour, 13.5% of all cancers), and the respective mortality reached 131 900 cases [1]. In 2008, in turn, 182 460 cases of breast cancer were detected in the United States, with the mortality of 40 480 cases. In parallel with pulmonary cancer and colorectal carcinoma, this was one of the three most frequent malignant tumours, accounting together for, around, 50% of neoplastic diseases in USA, representing the most frequent cause of death of women 20 to 59 years of age [2]. In view of the above, an early diagnosis and effective treatment of disease are immensely important. Intensive studies are

being carried out on determining reliable prognostic factors of a high specificity and sensitivity which could increase efficiency of treatment in such cases. Markers of a significant prognostic value manifest a strict correlation between intensity of their expression and prognosis. In clinical practice cases frequently occur, which manifest similar pathological characters but differ from each other in the course of the disease and in sensitivity to the applied treatment procedures. While evaluation of Ki-67 antigen expression is common in prognostic analysis of various tumour types (including soft tissue tumours, breast cancers, lung or prostate, brain tumours), examination of MCM-2 protein expression in such tumours, including breast cancer, still remains in its preliminary phase [4,7].

Ki-67 belongs to the group of most frequently estimated proliferation markers but its value as a prognostic marker has not been sufficiently proven and studies which dealt with the relationship between cell proliferation and clinical course of a disease fre-

Correspondence: Piotr Dziegiel, Dept. of Histology and Embryology, Medical University, Chalubinskiego 6a, 50-368 Wrocław, Poland; tel.: (+4871) 7841354, fax.: (+4871) 7840082, e-mail: piotr@hist.am.wroc.pl

Table 1. Clinical and pathological characteristic of studied patients.

Clinical/pathological parameter	N (%)
Mean age in years (range)	54.93 (35-83)
Tumour size	
T1	35 (62.5%)
T2	18 (32.1%)
T3	3 (5.4%)
Lymph nodes	
pN0	34 (60.7%)
pN1	9 (16.1%)
pN2	8 (14.3%)
pN3	5 (8.9%)
Clinical advancement	
I	34 (60.7%)
II	9 (16.1%)
III	13 (23.2%)
IV	0
Histological differentiation (Grade)	
G1	7 (12.5%)
G2	28 (50%)
G3	21 (37.5%)

quently yielded divergent results [3-8]. During inter-phase Ki-67 co-localizes with perinuclear located DNA and DNA located near the nuclear envelope what suggests that it is involved in perinuclear chromatin organization [9]. Expression of Ki-67 may develop also in situations of an inhibited DNA synthesis or when the cell undergoes the process of apoptosis [10]. Since both biological functions of Ki-67 and its significance as a prognostic factor remain unclear, it has been decided to include into the prognostic routine another proliferation marker, the MCM-2 protein. Until today, its estimation has been restricted exclusively to investigative purposes and it has not been applied in clinical and pathological evaluations. Its indubitable advantage includes the fact that biology of the factor is well recognized. MCM-2 belongs to the family of MCM (minichromosome maintenance) proteins, which includes the homogenous group of six proteins (MCM-2 to 7), responsible for the start and maintenance of replication [11-12]. At the early G1 phase of the cell cycle, MCM proteins are responsible for formation of the pre-replication complex, what differs them from the Ki-67 antigen, which is mostly present in late G1, S, G2 stages of the cell cycle [14,15]. The contained in the complex MCM proteins manifest activity of helicase, which disentangles DNA threads during replication process [15]. In the course of replication, this provides an appropriate access to all regions of DNA. At the S phase of the cell cycle MCM proteins irreversibly bind to the chromatin, which warrants that exactly one genetic materi-

al replication event occurs per a single cell division [16-17]. In view of the above, expression of MCM family proteins, including MCM-2 proteins, may in future be contemplated to be used as a new proliferation marker since its expression is elevated in rapidly dividing cells [18-20]. This study attempted to examine correlation between expressions of MCM-2 protein and Ki-67 proliferation antigen and to compare their expression in ductal breast cancers of different histological malignancy grades (G).

Material and methods

Patients. The studies were performed on 56 archival paraffin blocks containing fragments of invasive ductal breast cancers, sampled in 2002 during procedures of mastectomy in the Lower Silesia Oncology Centre in Wrocław. Age of the patients ranged from 35 to 83 years (the mean of 54.93±10.87 SD years), all the patients were of female sex. The histological malignancy grade (G) was determined using guidelines suggested by WHO [21]. The clinical and pathological data were obtained from archives of the Lower Silesia Oncology Centre in Wrocław (Table 1).

Immunohistochemistry. Tissue samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. In each case, hematoxylin and eosin-stained preparations were subjected to histopathological evaluation by two pathologists. Formalin-fixed, paraffin-embedded tissue was freshly cut (4 µm). The paraffin sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene and gradually re-hydrated. Activity of endogenous peroxidase was blocked by 30 min exposure to 1% H₂O₂. The IHC reactions were performed using monoclonal antibodies against MCM-2, originating from Novocastra (clone CRCT2.1, 1:50, Novocastra Laboratories Ltd, Balliol Bussiness Park West, Benton Lane, Newcastle upon Tyne, NE12 8EW, UK) and against Ki-67 antibody, originating from Dako (clone MIB-1, 1:50, Dako, Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark). The antibody was diluted in antibody diluent with the background reducing component. All the reactions were accompanied by the respective control reactions in which specific antibodies were substituted by the Universal Negative Control for N-series Mouse Primary Antibodies Kit, N1698 (Dako). The studied paraffin sections were boiled in the Antigen Retrieval Solution in microwave oven in order to unblock antigenic determinants. The investigated antigens were visualized using biotinylated antibodies, streptavidin-biotinylated peroxidase complex (LSAB, Dako) and diaminobenzidine (DAB, Dako). All the sections were counterstained with Meyer's hematoxylin (20 second).

Evaluation of the intensity of IHC reaction. The intensity of Ki-67 antigen and MCM-2 protein expression was evaluated using computer-assisted image analysis. In every section three fields were selected with the highest number of tumour cells yielding positive reaction /Hot Spots/. Percentage of positive cells in Hot Spots was evaluated scoring the brown-labelled cells nuclei, detected after screening of all cell nuclei under ×400 magnification (Olympus BX-42 light microscope with the visual mode AnalySis 3.2 software for computer-assisted image analysis). The general result for every sample was an average of the three hot spot percentages. The intensity of the IHC reactions was independently evaluated in coded preparations by two pathologists (AW, CK); in doubtful cases a re-evaluation was performed using a double-headed microscope and the staining was discussed until a consensus was achieved. The examining pathologists knew no clinical details related to the respective patient.

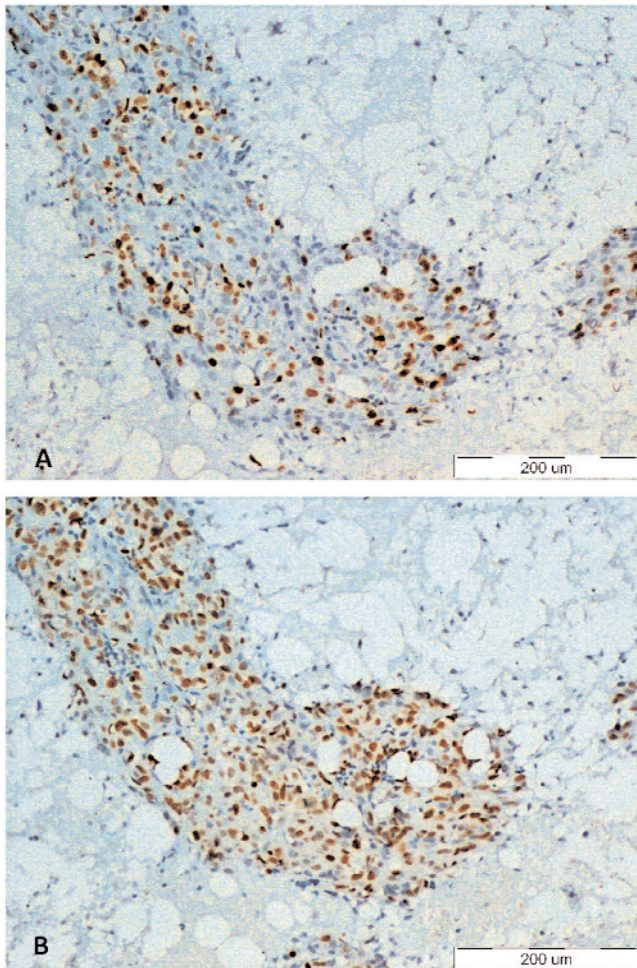


Fig. 1. Nuclear expression of Ki-67 (A) and MCM-2 protein (B) in ductal breast cancer. Serial sections, counterstaining with haematoxylin (original magnification $\times 100$).

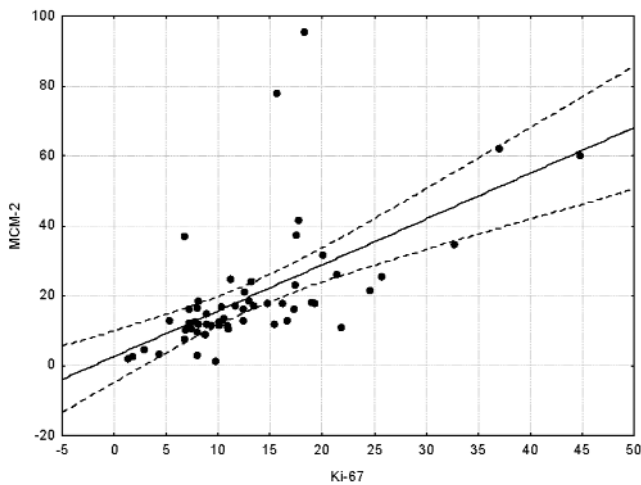


Fig. 2. Correlation of Ki-67 antigen expression with MCM-2 protein expression in ductal breast cancer: $r=0.6$; $p<0.05$.

Statistical analysis. The results were subjected to statistical analysis using Statistica 7.1 PL software (Statsoft, Poland). The relationship between the expression of Ki-67 antigen and MCM-2 protein was

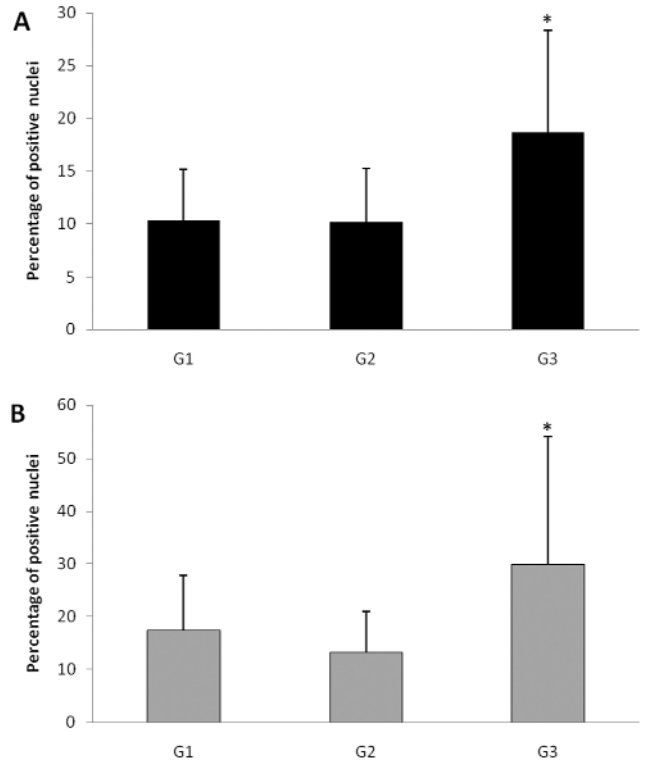


Fig. 3. Intensity of Ki-67 antigen expression * $p<0.001$ G3 vs. G1 and G2 (A) and of MCM-2 protein: * $p<0.05$ G3 vs. G1 and G2 (B) in ductal breast cancers.

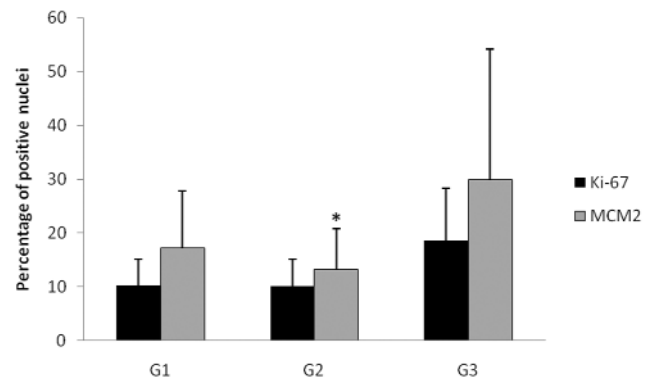


Fig. 4. Comparison between expression of Ki-67 antigen and that of MCM-2 protein in ductal breast cancers manifesting various grades of histological malignancy G; * $p<0.05$ G2: Ki-67 vs. MCM-2.

examined using Spearman's rank correlation. The relationship between expression intensity of the two proteins and histological malignancy grade G was examined using ANOVA rank test of Kruskal-Wallis. The differences were accepted as significant at $p<0.05$.

Results

All the examined tumours manifested nuclear expression of Ki-67 antigen (Fig. 1A) and MCM-2 protein (Fig. 1B). In hematoxylin-counterstained serial sec-

tions intensely brown-stained cell nuclei were noted. Statistical analysis of the relationship between expression of the two markers Ki-67 and MCM-2 demonstrated a strong positive correlation between expressions of the two markers ($r=0.6$; $p<0.05$) (Fig. 2). Also the relationship was examined between expression of the examined proteins and G grade of studied tumours. A more pronounced expression of Ki-67 antigen was demonstrated in G3 than that in G1 or G2 ($p<0.001$) (Fig. 3A) and, in the case of MCM-2 protein in G3 than that in G1 ($p<0.05$) or G2 ($p<0.01$) (Fig. 3B). For either marker the difference between expression in G1 and G2 proved to be insignificant. Also, expressions of Ki-67 antigen and MCM-2 protein were compared between individual grades of histological malignancy (G) of the studied tumours. Only in tumours of G2 grade expression of Ki-67 antigen was significantly lower than expression of MCM-2 protein (Fig. 4). In the case of Ki-67 antigen the average of 11% tumour cells manifested its expression in G1, 10% in G2 and 18% in G3 while in the case of MCM-2 protein the mean percentage of positive tumour cells amounted to 17% in G1, 13% in G2 and 30% in G3.

Discussion

Expression of Ki-67 and MCM-2 markers and relationships between the two were examined in relatively numerous studies. Expression of the proteins was studied in cancers of the larynx, oral cavity, thyroid gland, in tumours of salivary gland, colorectal cancers, tumours of adrenal glands, lungs and in renal carcinoma [22-31,33]. Results of some investigators suggested a higher prognostic value of MCM-2 protein than that manifested by currently widely used Ki-67 [23,28,30,34,35].

Chatrath *et al.* [22] examined benign and malignant lesions in the larynx. Their statistical analysis demonstrated a very strong correlation between expression of Ki-67 and that of MCM-2, which pointed to a similar prognostic value of the studied proteins. Szelachowska *et al.* [23] studied spinocellular cancers of oral cavity and demonstrated a higher prognostic value of MCM-2 as compared to that of Ki-67. In the cases of thyroid tumours, Mehrorta *et al.* [24] observed increased expression of both markers in malignant lesions (follicular and papillary tumours). The studies demonstrated also that neither MCM-2 nor Ki-67 could be applied for distinguishing benign from malignant lesions in the thyroid gland. Giaginis *et al.* [25] showed that expression of MCM-2 was significantly linked to clinical and pathological data and manifested strong correlation with cell proliferation in colon carcinoma. In turn, studies of Guzińska-Ustymowicz *et al.* [26] on colorectal cancers demonstrated that an increase in MCM-2 expression might point to devel-

opment of metastases to lymph nodes. In other studies Vargas *et al.* [27] showed in tumours of salivary glands that MCM-2 was a strong marker of differentiation between benign and malignant lesions. Szajerka *et al.* [28] compared expression of MT, Ki-67 and MCM-2 in tumours of adrenal cortex. They demonstrated a very strong correlation between MCM-2 and MT and a moderate correlation between MCM-2 and Ki-67. In view of their results MCM-2 seems to provide a reliable indicator of malignant processes in adrenal cortex. In cases of pulmonary adenocarcinomas Hashimoto *et al.* [29] demonstrated a similar diagnostic usefulness of both Ki-67 and MCM-2. On the other hand, Yang *et al.* [30] suggested that in non-small cell lung carcinomas two markers in parallel, MCM-2 and gesoline should be estimated. In such tumour the authors found that usage of Ki-67 manifested no prognostic value. In renal carcinoma, in turn, Dudderidge *et al.* [31] suggested that MCM-2 should be used as a marker of proliferation and prognosis. Moreover, Kodani *et al.* [32] suggested that an increased expression of MCM-2 and p53 may point to a malignant neoplastic transformation in cells of oral mucosa. They tried to explain it that the MCM-2 expression could involve the p53 protein-dependent pathway and, in parallel, they found that MCM-2 represents a useful marker of proliferation.

Expression of MCM-2 protein was already studied in some papers concerning breast cancer. Reena *et al.* [34] examined MCM-2 expression in benign and malignant lesions of the breast. Similarly to us, they noted a high MCM-2 proliferation index in tumours manifesting grade G3 of histological malignancy and a relatively low index in tumours of G1 grade, pointing to positive correlation between MCM-2 expression and G grade. Gonzales *et al.* [35] obtained very similar results in studies on breast cancers, indicating that expression of MCM-2 was a more frequent and more pronounced than those of the widely used Ki-67. They postulated that MCM-2 labelling index represents a strong independent prognostic factor and for a more reliable prognostic evaluation it should be considered in parallel with parameters of the Nottingham Prognostic Index (NPI). Ki-67 and MCM-2 were also evaluated in advanced gastric cancer. Although this study did not show any correlation between both markers, shorter postoperative survival time was associated with high MCM-2 expression [33].

Results of our studies have demonstrated an increased nuclear expression of both MCM-2 protein and Ki-67 antigen in ductal breast cancers. Results obtained by us and other authors comparing expression of the two proteins point to a comparable value of the two markers, sometimes suggesting a higher significance of MCM-2. Ki-67 antigens represents the widely used proliferation marker in mammary gland cancers but there is lack of explanation related to the mecha-

nisms in which the protein acts suggest a search for other prognostic factors. The difference in expression of both markers between G1 and G2 grades was insignificant. In view of the strong correlation in expression of the studied markers the potential of a more frequent determination of MCM-2 antigen could be considered, as a marker of proliferation in breast malignant tumours. This may be particularly valuable in ductal breast cancers since expression of the protein correlates with its G malignancy grade.

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