

## CLINICAL STUDY

# The expression of MCM-2 in invasive breast carcinoma: a stereologic approach

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**Abstract:** *Objectives:* The aim of this study is to examine the expression of MCM-2 and conventional proliferation marker Ki-67 in breast carcinoma by stereologic technique and to compare it with various clinicopathologic parameters.

*Methods:* The expression of MCM-2 and Ki-67 on paraffin-embedded tumor tissue sections of patients with invasive breast carcinoma was analyzed immunohistochemically. Stereologic method was used for evaluation of the percentage of positively stained tumor cells.

*Results:* Significant positive correlation was found between the expression of MCM-2 and that of Ki-67 ( $r=0.74$ ,  $p<0.001$ ). MCM-2 and Ki-67 expression was significantly associated with histologic grade ( $p<0.05$ ), and negative correlation was observed between MCM-2 or Ki-67 expression and estrogen status ( $p<0.05$ ). No significant association was observed between MCM-2 or Ki-67 expression and patient age, tumor size, lymph node status, clinical stage and menopausal status.

*Conclusion:* Our results suggest that MCM-2 expression is significantly associated with histologic grade of breast carcinoma and with cell proliferation capacity (Ki-67 labelling index). Additional studies are required using the stereologic method to compare and understand the utility of Ki-67 and MCM-2 expression in invasive breast carcinoma (Tab. 1, Fig. 4, Ref. 34). Full Text (Free, PDF) [www.bmj.sk](http://www.bmj.sk).

Key words: MCM-2, Ki-67, invasive breast carcinoma, cell proliferation.

Breast carcinoma is the most common type of cancer for women in the world. Although several prognostic factors have been proposed, the Nottingham Prognostic Index (NPI) is widely used to predict survival in patients with breast carcinoma. Histologic grade, involving mitosis counting is one of the components evaluated in NPI. Several studies have shown that increased proliferation correlates strongly with poor prognosis (1–4).

Various immunohistochemical proliferation markers such as Ki-67, proliferating cell nuclear antigen (PCNA), and cyclin A are tested to get more accurate results in assessment of the proliferation index. Minichromosome maintenance (MCM) proteins are essential for the initiation and elongation of DNA replication. MCM-2 is a member of MCM proteins (MCM2-7). Each member has the same effect in forming prereplication complex to start the replication (3). In vitro studies demonstrated that these proteins (MCM2-7) are expressed in proliferative cells but not in quiescent or differentiated cells making them specific indicators of cell proliferation. Ki-67 is nuclear antigen that is present

only in cycling cells. Ki-67 is absent in cells passing from G0 to G1, whereas G1 cells after mitosis are consistently positive for Ki-67. Recent studies have proposed that MCM proteins could be more sensitive proliferation markers and provide information about the prognosis of neoplasms (4–6).

To our knowledge, there is no stereologic study on MCM-2 immunohistochemical expression in breast carcinoma. In this study, the immunohistochemical expression of MCM-2 of the invasive breast carcinomas was investigated using stereologic technique; we also evaluated the correlation with Ki-67, a traditional proliferation marker, and various clinicopathological parameters.

## Material and method

In our study, we examined tissue sections of 76 patients with primary invasive breast carcinoma. The clinicopathological features (age, menopausal status, lymph node status, histologic grade, tumor size, clinical stage) were obtained from medical files. Histologic type and histologic grades were confirmed by light microscopic examination. Histologic grading of tumors was based on recommendations made by Elston and Ellis (7).

## Immunohistochemistry

Sections were stained with primary antibody Ki-67 (Neomarkers, RM-9106, SP6, Labvision, prediluted) and MCM-2 Ab-1 (Neomarkers, MS-1726-R7, Labvision, prediluted). Briefly five-

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micron tissue sections attached onto the adhesive glass were deparaffinized and rehydrated. Sections were microwaved in 0.1 M citrate buffer (pH 6) for 20 minutes for antigen unmasking. After cooling and washing, the endogenous peroxidase activity was blocked by incubating 3 % H<sub>2</sub>O<sub>2</sub> for 10 minutes. The sections were then washed in running tap water for 10 minutes. Ultra-V-Block Nonspecific Blocking Reagent (Lab Vision, Westinghouse, CA) was used to prevent non-specific bindings. The sections were incubated with primary antibodies for 60 minutes in room temperature. After rinsing the sections with Tris-buffered saline (TBS), the steps of biotinylated goat anti polyvalent (Lab Vision, Westinghouse, CA) secondary antibodies, streptavidine peroxidase solution and aminoethyl carbazole chromogen were performed. All sections were counterstained using Mayer's hematoxyline. Positively stained tumor cell nuclei were counted. The labelling index (LI) of each proliferation marker was determined by percentage of positively stained cells based on a count of at least 500 tumor cells. The cell numbers in two sections of MCM-2 staining were less than 100 so the evaluation of MCM-2 expression was based on 74 cases. Reactive lymph node sections served as positive control. Negative controls (ommission of primary antibody) were included in each run.

### Stereology

Stereology is a method that is concerned with the three dimensional interpretation of planar sections of tissues. It provides practical techniques for extracting quantitative information about a three dimensional material from measurements made on two dimensional planar sections of the material (8). In our study, cell counting was performed by using stereologic analysis method. In this study, the images obtained from stained sections were transformed to computer screen by video-camera mounted on a light microscope and analyzed by a stereological workstation (Stereo Investigator 6.0 -microbrightfield; USA). This workstation had some features such as personal computer and computer controlled motorized specimen stage (BioPrecision MAC 5000 controller system), and a light microscope (Leica DM4000 B). Immunoreactive tumor cells stained with primary antibody Ki-67 and MCM-2 Ab-1 were separately counted using a 20x Leica Plan Apo objective (NA=1.40) which allowed accurate recognition. Immunoreactive tumor cells were counted according to the unbiased counting rules of optical fractionator (8). The optical fractionators approach is a combination of performing counting with the optical disector, and with fractionator sampling for the estimation of population size (9). In our study, a pilot study was done in the beginning of the stereological analysis. It was found that one of 2.250.000 m<sup>2</sup> (in X, 1.50 mm; in Y, 1.50 mm) step size for microscopic sampling would be suitable to perform stereological analysis in our study. In all steps, an unbiased counting frame sizing of 4900.00 m<sup>2</sup> (70.0 mm X 70.0 mm) was used. According to the optic disector counting rules, each disector probe that means three-dimensional counting box has lower height than section thickness. The height of the disector probe was 70- $\mu$ m. Thickness sampling fraction was disector height (16  $\mu$ m)/mean section thickness. All immunoreactive tumor cells were counted

in each sampled disector probe on the sections during stereological analysis (10). While the focus plane approaches to section, images taken from the top of the section to the bottom are changed, the first optical section is seen in optical section plane (OPS). Upper surface of section determined if the type of tissue could be clearly distinguished. After coming to the upper surface of section, focal plane of lens was gone into the section by the bottom surface of it. After determination of the upper surface of section, 5- $\mu$ m-thickness of a guard zone from the surface of section was left. Counting of immunoreactive tumor cells nucleus was performed after OPS. In this study, a coefficient of error value for the optical disector application was estimated. Generally accepted highest limit of CE was 5 % to be adjusted same value in our study (11).

### Statistical analysis

The normality of distribution was tested with the Kolmogorov-Smirnov test. Mann-Whitney U test was used for menopausal and estrogen receptor status. Kruskal Wallis test was performed for histological grade (1, 2 and 3), lymph node stage (1, 2 and 3) and clinical stage (1, 2 and 3). LI values were compared using the Wilcoxon signed rank test. Spearsman's rank correlation (denoted r) was used to test the associations between Ki-67 and MCM-2. Data were expressed as mean $\pm$ SD. A *p* value of 0.05 or less was considered indicative of a statistically significant difference. SPSS v13.0.1 (serial no: 9069728) was used for statistical tests.

### Results

In our study, 76 cases of invasive breast carcinomas were examined. Ten (13 %) cases were classified as invasive lobular, 66 cases (87 %) as invasive ductal carcinoma. Twenty of the tumors (26.3 %) were classified as histological grade 3 (50 %) as grade 2, and 17 (23.7 %) as grade 3.

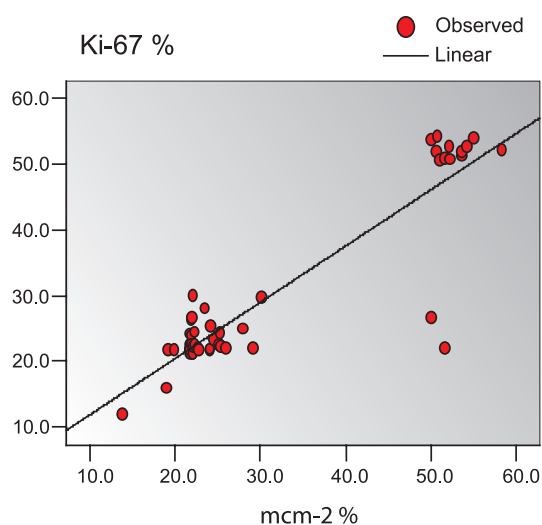


Fig. 1. Spearsman's rank correlation test for correlation between MCM2 and Ki-67 labelling indexes for all cases ( $r=0.74$ ;  $p<0.0001$ ).

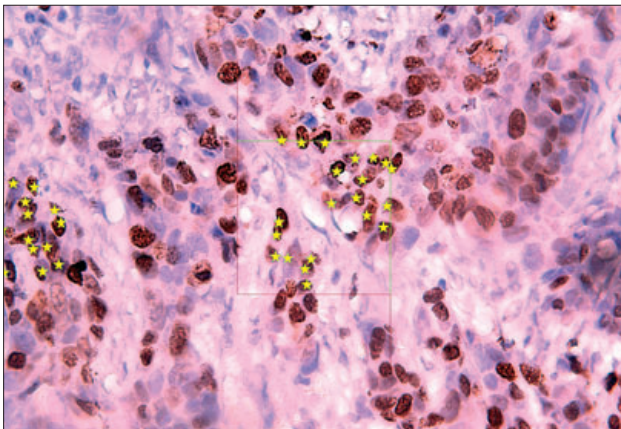


Fig. 2. MCM-2 positive cells (bright yellow stars) that are found in the square are counted in high grade invasive breast carcinoma (immunoperoxidase x 200)

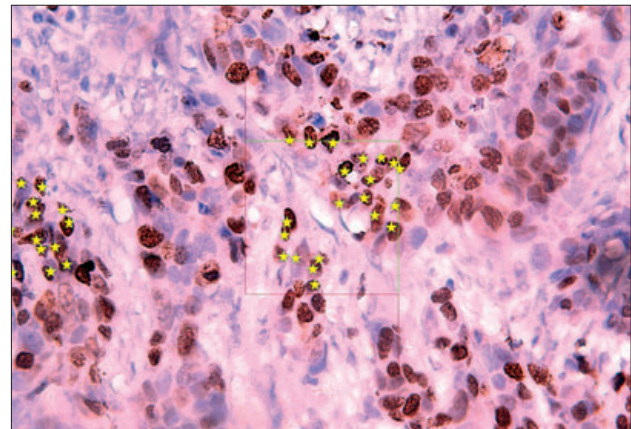


Fig. 3. Ki-67 positive cells (bright yellow stars) that are found in the square are counted in high grade invasive breast carcinoma (immunoperoxidase x 200)

There was a very close correlation between Ki-67 and MCM2 LIs in malignant tumor cells ( $r=0.74$ ,  $p<0.001$ ) (Fig. 1). The expression of MCM-2 (mean LI,  $30.4\pm 13.8\%$ ; range 13.8 % to 58.3 %) was similar to Ki-67 expression (mean LI,  $29.3\pm 12\%$ ; range, 12 % to 54.2 %;  $p=0.198$ ) (Figs 2 and 3). The mean difference in LIs was 1.1 %.

The LI of MCM-2 and Ki-67 showed a significant positive correlation with histologic grade ( $p<0.05$ ) (Fig. 4). The mean LI of MCM-2 in histologic grade 3, grade 2 and grade 1 cases were  $52.4\pm 2.2\%$  (range: 50–58.3 %),  $23.3\pm 2.8\%$  (range: 19–30.1 %) and  $22.9\pm 1.4\%$  (range: 21.7–25.4 %), respectively. The mean LI of Ki-67 in histologic grade 3, grade 2 and grade 1 cases were  $48.3\pm 10.2\%$  (range: 22–54.2 %),  $22.9\pm 2.8\%$  (range: 16–29.7 %) and  $23.3\pm 2.4\%$  (range: 21.3–30 %), respectively.

The LI of MCM-2 and Ki-67 showed a significant negative correlation with estrogen receptor expression ( $p<0.05$ ). The mean LI of MCM-2 and Ki-67 in estrogen positive cases were  $25.1\pm 8.6\%$  (range: 19–53.6 %) and  $23.8\pm 6.6\%$  (range: 16–52), respectively. The mean LI of MCM-2 and Ki-67 in estrogen negative cases were  $37.1\pm 15.4\%$  (range: 19–58.3 %) and  $35.6\pm 15.3\%$  (range: 16–54.2), respectively.

The relationship between the MCM-2 and Ki-67 LI and clinicopathological characteristics are summarized in Table 1. A comparison of LIs and clinicopathological characteristics for each marker revealed no significant association with age, tumor size, lymph node status, clinical stage and menopausal status.

## Discussion

Breast carcinoma is one of the most important causes of death among solid tumors in women. NPI is used to indicate the high risk patients (1). Traditional prognostic factors such as lymph node status and tumor size are not accurate enough. Therefore, to improve the indication of high risk groups, additional predictive and prognostic factors are required. Proliferation rate is the one of the most important prognostic factor yet. However of the different methods to evaluate proliferation, mitosis counting has

been shown most convincingly to provide reproducible and independent prognostic value in invasive breast carcinoma. For last 15 years, the investigators focus on immunohistochemical markers such as Ki-67, cyclins, MCM proteins and PCNA to indicate proliferative cells. LI of these markers strongly correlate with high proliferation and also poor prognosis (3, 4, 12–15).

MCM proteins (MCM2-7) are essential components of DNA replication. When the chromatin combines with the MCM protein, it is competent or „licensed“ for replication. All proliferating cells in cycle have MCM protein. The requirement for MCM proteins in cycling cells but not in quiescent ones may render MCM proteins as specific indicators of cell proliferation (4, 14). Concerning the literature, MCM-2 nuclear expression was found in majority of breast cancer patients (2, 4, 16). MCM-2 expression was noted to be significantly higher in dysplastic cells and premalignant proliferative breast lesions than the normal breast

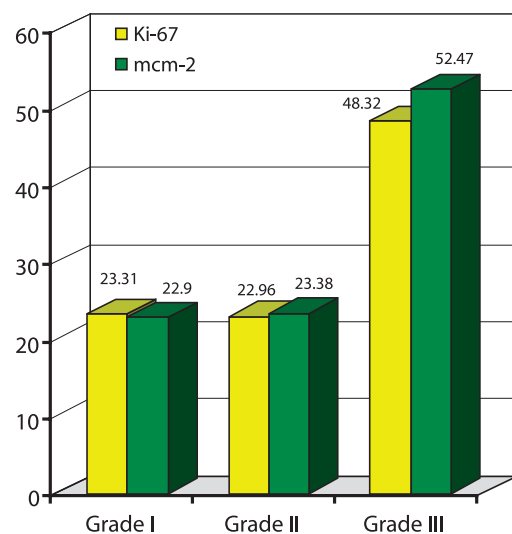


Fig. 4. Histogram comparing MCM2, Ki-67 expression between histologic grades. MCM2 and Ki-67 expression were higher in histologic grade III cases ( $p<0.05$ ).

**Tab. 1. Clinicopathologic Details of Invasive Breast Carcinomas Studied.**

Clinicopathologic Feature	No.	%	MCM2 LI(mean±SD)	P value	Ki-67 LI(mean±SD)	P value
Age				0.70		0.63
<40	10	10.8	29.5±15.2		25.5±11.9	
40–60	49	66.2	32.5±14.7		31.3±13.9	
>60	17	23	26.0±7.8		25.6±8.7	
Menopausal status:				0.49		0.95
Premenopausal	40	52.6	30.6±14.0		29.0±12.6	
Postmenopausal	36	47.4	30.2±12.9		29.6±12.9	
Lymph Node status				0.80		0.38
Negative	26	35.2	31.4±14.0		28.9±12.2	
1–3	28	36.9	29.6±12.6		30.4±13.0	
>3	22	28.9	30.3±14.3		28.2±13.3	
Stage				0.43		0.05
I	9	11.8	22.8±1.6		22.0±0.8	
II	42	55.4	32.0±14.5		31.5±13.2	
III	25	32.8	30.8±13.5		28.7±13.4	
Histologic grade				0.01		0.01
I	20	26.3	22.9±1.4		23.3±2.4	
II	38	50	23.3±2.8		22.9±2.8	
III	18	23.7	52.4±2.2		48.3±10.2	
Tumor size				0.22		0.16
≤2cm	25	32.8	26.0±9.7		25.9±10.8	
2.1–5cm	39	51.5	32.3±14.9		30.5±13.0	
>5cm	12	15.7	32.1±13.3		31.5±14.5	
ER status				0.02		0.01
Positive	31	40.7	25.1±8.6		23.8±6.6	
Negative	45	59.3	34.3±14.9		33.4±14.5	

tissue and it was supported that MCM-2 may have predictive value for malignant potential of the breast lesions (13, 17). Besides breast carcinoma, MCM-2 expression was evaluated in non-small cell lung carcinoma (18, 19), minimally invasive thyroid follicular carcinoma (20), prostate carcinoma (5), colon carcinoma (6), and urinary bladder carcinoma (21). MCM-7 has been studied in gestational trophoblastic disease (22) and metastatic colon carcinoma (23), MCM-5 for hepatitis induced carcinogenesis (24), MCM-3 and 4 for cervix carcinoma (25).

Ki-67 is one of the proteins that regulate cell cycle by phosphorylation and dephosphorylation during mitosis (26, 27). Ki-67 is expressed during cell cycle except for G0 phase (28, 29). Stoeber et al. also reported that Ki-67 was absent in early G1 phase, so it may not be expressed in cells entering G1 from G0 which could subsequently underestimate the number of cells in cycle (30).

In our study, stereologic method was performed to count the positive stained cells immunohistochemically. Stereology is a method that is concerned with the three dimensional interpretation of planar sections of tissues. The images obtained from stained sections were transformed to computer screen by video-camera mounted on a light microscope and analyzed by a workstation (31). Stereology has not been used before for evaluation of MCM-2 and Ki-67 expression in breast carcinoma.

According to our results, MCM-2 LI showed a strong positive correlation with that of Ki-67. In fact, based on the results of previously published comparative studies, MCM-2 protein has been shown to identify significantly more cells in cycle than Ki-67 in breast carcinoma and other several types of neoplasia (4–5). In a recent study, a strong positive correlation was observed between MCM-2 and Ki-67 expressions in colon carcinoma which was consistent with our results (6). The reason of high LI for Ki-67 might be due to sensitivity of the stereologic method that we used in the current study.

We have found a statistically positive correlation between MCM-2 or Ki-67 expression and histological grade. There are several reports indicating similar correlation between MCM-2 expression and histological grade in breast carcinomas (1, 2, 4, 12, 16), and other types of carcinomas such as prostate, urinary bladder, lung, oligodendroglioma and renal cell carcinoma (19, 21, 32–34). We have also found similar significant association between Ki-67 expression and histologic grade.

## Conclusion

Our results suggest that MCM-2 expression is significantly associated with histologic grade of breast carcinoma and with

cell proliferation capacity (Ki-67 labelling index). Additional studies are required using the stereologic method to compare and understand the utility of Ki-67 and MCM-2 expression in invasive breast carcinoma.

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