**in vivo** *23*: 49-54 (2009)

# Correlation Between MCM-3 Protein Expression and Grade of Malignancy in Mammary Adenocarcinomas and Soft Tissue Fibrosarcomas in Dogs

MARCIN NOWAK[1], JANUSZ A. MADEJ[1] and PIOTR DZIEGIEL[2]

[1]*Department of Pathological Anatomy, Pathophysiology, Microbiology and Forensic Veterinary Medicine, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw;*
[2]*Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland*

**Abstract.** *Background: Minichromosome maintenance proteins (MCM), due to their involvement in DNA replication in the course of mitosis, may provide sensitive markers of cell proliferation. Localization of MCM-3 and the extent of its expression were evaluated in mammary adenocarcinomas and soft tissue fibrosarcomas in dogs. The obtained results were compared to grades of malignancy (G) of the studied tumours. Materials and Methods: The research material was sampled in the course of surgery in 71 dogs of various breeds, aged 4 to 14 years (50 cases of mammary adenocarcinoma and 21 cases of soft tissue fibrosarcoma). The tumours were verified by histopathology and immunohistochemistry was used to evaluate MCM-3 expression. The preparations were photographed and the images were subjected to computer-assisted image analysis using MultiScanBase Ver. 14.02 software. Results: Nuclear expression of MCM-3 was detected in 70% adenocarcinomas and in over 71% of fibrosarcomas. Augmented expression of MCM-3 was observed in samples of tumours manifesting higher grade of malignancy. Statistical analysis demonstrated strong positive correlation ($r=0.71$ for fibrosarcomas, $r=0.52$ for adenocarcinomas; $p<0.05$) between MCM-3 expression and grade of malignancy in the studied tumours. Conclusion: MCM-3 may provide a sensitive and useful marker of proliferative potential in various histological types of neoplastic tumours.*

*Correspondence to:* Marcin Nowak, Ph.D., VMD, Department of Pathological Anatomy, Pathophysiology, Microbiology and Forensic Veterinary Medicine, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Norwida 31, 50-375 Wroclaw, Poland. Tel/Fax: +48 713281567, e-mail: marcin.nowak@up.wroc.pl

*Key Words:* MCM-3, adenocarcinomas, fibrosarcomas, dog.

An increase in the incidence of neoplastic tumours, including their malignant forms, continues to be observed in both humans and animals. Out of the malignant epithelial tumours, mammary gland adenocarcinoma is the most frequent form, both in women and in bitches. Despite increasingly effective oncological prevention and diagnosis, this malignancy represents one of the principal causes of death due to neoplastic disease. Soft tissue sarcomas (STS) represent another form of the disease. In contrast to the relatively frequent carcinomas, in humans they account for just 1% and in animals for 14% of all diagnosed malignant tumours (1, 2). Even if relatively rare, this group of malignancies of mesenchymal origin pose a significant problem in oncology. This reflects, first of all, their incompletely recognised etiopathogenesis and their treatment, which usually manifests low efficacy (2). Fibrosarcomas form a prevalent subgroup of canine soft tissue sarcomas while adenocarcinomas are the most frequently diagnosed type of mammary cancer in bitches (2-6).

Both in veterinary medicine and in human therapy, problems arise due to relatively late detection of the neoplastic disease. The late diagnosis reflects, among other causes, the lack of tumour-induced complaints (*e.g.* in soft tissue sarcomas) (7). In the case of mammary adenocarcinomas in bitches, the tumours are unnoticed by owners of the dogs at the preliminary stages as, similarly to sarcomas, they induce no pain or motor disturbances in the dog. In such a situation, an accurate evaluation of a tumour's malignancy grade (G) represents a very important element of histopathological diagnosis since it allows prediction of the prognosis of the disease to be attempted. Nevertheless, grading of the tumour on the basis of subjective appraisal of cellular pleomorphism, mitotic activity or extent of necrosis in the tumour is insufficient. In such a situation, attempts are made to take advantage of additional markers of cell proliferation (such as proteins of MCM family), which would permit a more precise prognosis.

A key element in cell proliferation involves DNA synthesis. Initiation of DNA replication in both normal and neoplastic cells represents a complex multi-stage mechanism. Principles of the process include cyclical association of initiation factors to the so-called pre-replication complexes (pre-RC) (7-10). The main component of pre-RC involves the hexameric minichromosome maintenance complex (MCM), consisting of strongly conserved proteins, namely MCM-2, MCM-3, MCM-4, MCM-5, MCM-6 and MCM-7 (10, 11). Association of MCM complex to pre-RC takes place as early as the G1-phase but at this stage its conformation is inactive. It is not until the S-phase of the cell cycle that MCM complex becomes phosphorylated, forming an enzymatically active helicase. The latter becomes linked to DNA and subsequently dissociates out of pre-RC. Its effects include disentangling of double DNA strands and gradual association of proteins, which participate in DNA replication (10). It should be noted that association of MCM complex to pre-RC is spaced in time from its activation so that DNA replication occurs only once in every cell division. Even if MCM proteins bind to chromatin before DNA replication and undergo gradual separation in the course of replication, remaining in the cell nucleus throughout the cycle of the replicating cell (12). It should be added that, in contrast to neoplasms, MCM proteins undergo degradation in cells which pass into quiescence stage or undergo terminal differentiation, as occurs in the majority of normal tissues (13-16).

This study aimed at determining localization of extent of expression of MCM-3 in cells of canine mammary adenocarcinomas and soft tissue fibrosarcomas using immunohistochemical techniques and at comparing the obtained results with grades of tumour malignancy (G). Positive results of the tests might provide a rationale for application of proteins in MCM family as markers facilitating determination of the extent of tumour cell proliferation.

## Materials and Methods

Samples for the study were obtained during surgery in 71 dogs of various breeds, aged 4 to 14 years. The tumours were verified as adenocarcinomas (50 cases) and as fibrosarcomas (21 cases) by histopathology.

Formalin-fixed, paraffin-embedded tissue was cut (4 μm) and the sections were prepared routinely. Detection of MCM-3 antigen expression was preceded by 15 min exposure of the sections in a microwave oven to boiling Antigen Retrieval Solution (DakoCytomation, Denmark) at 250 W. For demonstration of MCM-3 antigen expression, mouse monoclonal antibodies (clone 101) were used at a dilution of 1:50 (DakoCytomation). The sections were incubated with antibody for 1 h at room temperature. Subsequently, incubations were performed with biotinylated antibodies (15 min, room temperature) and with streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB2; HRP, DakoCytomation, Denmark). DAB (DakoCytomation, Denmark) was used as a chromogen (7 min, room temperature). All the sections were counterstained with Meyer's hematoxylin. In every case, controls were included in which the specific antibody was substituted by the Primary Negative Control (DakoCytomation).

Microphotographs of the obtained preparations were subjected to computer-assisted image analysis *via* a computer coupled to Axiophot Optical Microscope (Carl Zeiss, Germany). The set had the potential to record images and to perform their digital analysis.

The measurements took advantage of MultiScanBase Ver. 14.02 software (Computer Scanning Systems, Warszawa, Poland).

Microscope examination allowed for sorting of the tumours into three groups of a variable malignancy grade. The method for the evaluation of the malignancy grade included three parameters scored on a scale from 0 to 3 points: histological differentiation of the tumour; number of mitoses per 10 large visual fields under ×400 magnification and area of necrosis. The sum of the points enabled the distinction of 3 grades of malignancy (G) among the tumours: 2-3 pt, G1; 4-5 pts, G2; 6-8 pts, G3 (1). The expression of MCM-3 antigen was appraised semiquantitatively by evaluating the proportion of positive cells 0-5% : no reaction (–), 6-25% : weak reaction (+), 26-50% : moderate reaction (++), above 50% : intense reaction (+++). The obtained results were subjected to statistical analysis employing Statistica PL software (StatSoft, Krakow, Poland) and using Spearman's correlation analysis.

## Results

The expression of MCM-3 protein was demonstrated both in canine mammary adenocarcinomas and in soft tissue sarcomas (Figures 1, 2). The expression was noted in the cell nucleus.

Evident differences in intensity of the protein expression were detected in each group of the studied tumours. In cases of mammary adenocarcinomas, 28% tumours manifested MCM-3 expression estimated at +, 26% at ++ and 16% at +++. In cases of soft tissue fibrosarcomas, 47% tumours manifested expression estimated at +, over 19% at ++ and almost 5% at +++. It should be mentioned that 30% of adenocarcinomas and over 28% of sarcomas presented no MCM-3 expression. The distribution of MCM-3 expression, as related to G tumour malignancy grade in mammary adenocarcinomas (Figure 3) and fibrosarcomas (Figure 4) presented an interesting pattern. In G1 adenocarcinomas, over 46% tumours manifested no MCM-3 expression, over 28% showed expression of the protein at +, almost 18% at ++ and over 7% at +++. In cases of G2 adenocarcinomas, over 13% of the tumours demonstrated no MCM-3 expression, more than 33% showed expression at + , 40% at ++ and over 13% at +++. In all cases of G3 adenocarcinomas, MCM-3 expression was shown: 14% at +, over 28% at ++ and over 57% at +++. In soft tissue fibrosarcomas of G1 grade, 40% of tumours manifested no MCM-3 expression, over 53% at + and almost 7% at ++, while expression at +++ was not noted. All fibrosarcomas of G2 or G3 grade manifested variable expression of MCM-3: in G2, MCM-3 expression was detected in 50% at + and

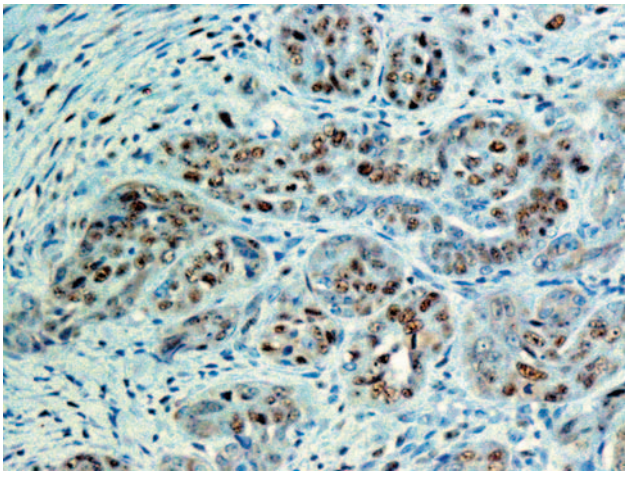

Figure 1. *High expression of MCM-3 (brown nuclei) in cells of canine mammary gland adenocarcinoma (×200, counterstained with hematoxylin).*

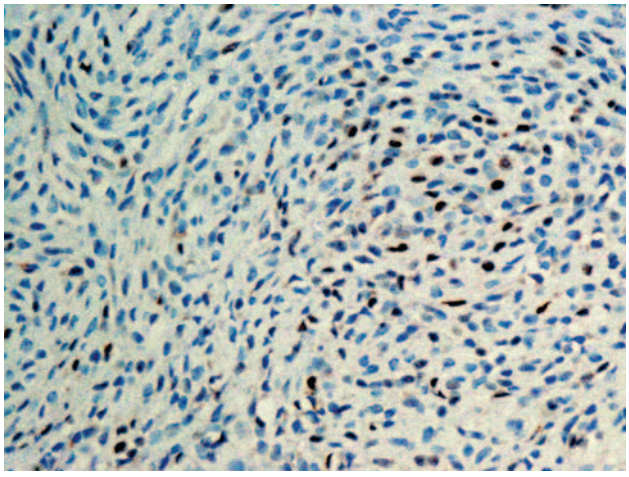

Figure 2. *High expression of MCM-3 (brown nuclei) in cells of canine soft tissue fibrosarcoma (×100, counterstained with hematoxylin).*

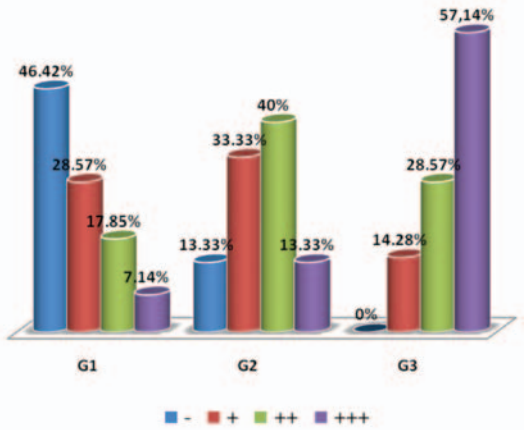


Figure 3. *Proportion of adenocarcinomas which manifest MCM-3 expression, as related to grade of malignancy (G).*

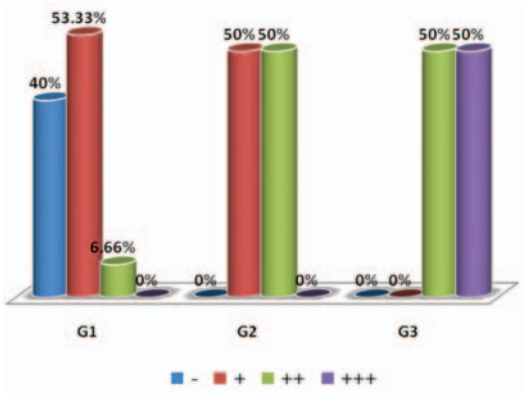


Figure 4. *Proportion of fibrosarcomas manifesting expression of MCM-3, as related to grade of malignancy (G).*

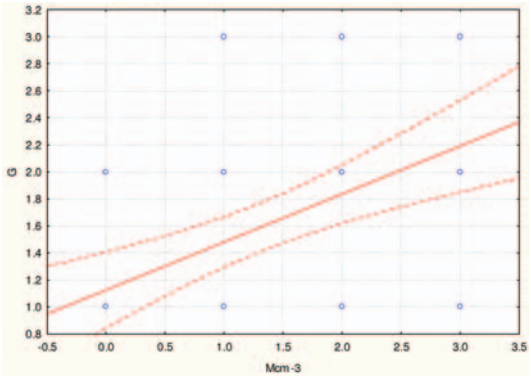


Figure 5. *Correlation between expression of MCM-3 and G in cells of canine mammary gland adenocarcinoma. Correlation coefficient r=0.52; p<0.05.*

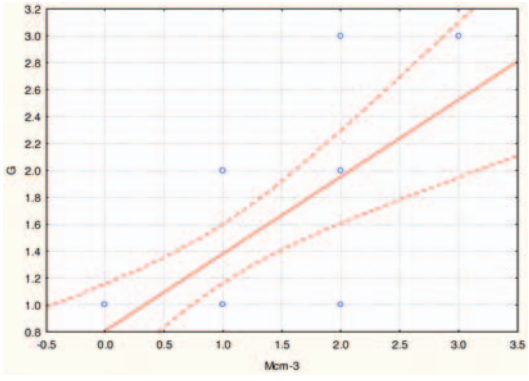


Figure 6. *Correlation between expression of MCM-3 and G in cells of canine fibrosarcoma. Correlation coefficient r=0.71; p<0.05.*

50% at ++. Moreover, in that subgroup there were no cases of no MCM-3 expression nor of +++. In the G3 subgroup of fibrosarcomas, half of the studied cases demonstrated MCM-3 expression at ++ and the other half at +++.

Spearman's correlation analysis showed statistically significant positive correlation between expression of MCM-3 and the G grade both in mammary adenocarcinomas (r=0.52; $p<0.05$) and in soft tissue fibrosarcomas (r=0.71; $p<0.05$) (Figures 5, 6).

## Discussion

In the present study, the expression of MCM-3 as related to G grade of malignancy was analysed in 71 cases of the most frequently manifested malignant tumours in dogs, namely in mammary adenocarcinomas and in soft tissue fibrosarcomas.

The results show that a growing grade of tumour malignancy (both of fibrosarcomas and adenocarcinomas) is accompanied by an increasing level of MCM-3 expression: from 7% G1 adenocarcinomas to 53% G3 tumours manifesting strong (+++) MCM-3 expression; in soft tissue fibrosarcomas, from total absence of the pronounced MCM-3 expression in G1 tumours to 50% of G3 tumours with strong (+++) MCM-3 expression. In a similar way, Rodins *et al*. (17) documented a positive correlation between expression of MCM-2 and malignancy grade in 56 cases of malignant tumours of human kidney (clear cell carcinoma, chromophil carcinoma, oncocytoma and transitional cell carcinoma). Moreover, the authors detected a significant correlation between MCM-2 expression level and expression of Ki-67 proliferation-associated antigen, although in all the studied tumours antibodies directed to MCM-2 labelled around ten-fold higher numbers of cells than Ki-67 antibodies did. It should be added that several authors showed that in non-neoplastic tissues, expression of MCM family proteins reached higher or similar levels as compared to expression of Ki-67, the recognized proliferation marker (18, 19). In turn, Meng *et al*. (20) studied 92 cases of prostate gland and detected a significantly increased level of MCM-2 expression in tumours as compared to the normal tissue. In these studies, patients with high expression of MCM-2 also manifested shorter disease-free survival times. Similarly to other types of malignant epithelial cells, such as non-small cell lung carcinoma or oral cavity squamocellular carcinoma, increased expression of MCM-2 correlated with shorter survival of the patients (21, 22).

The presented study shows a positive correlation between expression of MCM and malignancy grade in fibrosarcomas, which was also noted in malignant mesenchymal tumours in humans. Halfenstein *et al*. (23) examined 17 cases of chondrosarcoma and 14 cases of enchondroma and found that expression of MCM-6 clearly correlated with tumour malignancy grade. In addition, expression of MCM-6 was markedly higher in G1 chondrosarcomas than in enchondromas, which, according to the authors, might facilitate differential diagnosis of the tumours. Similarly, Sington *et al*. (24) analysed 51 myxofibrosarcomas in humans and documented positive correlation between intensity of MCM-2 expression and tumour malignancy grade and mitotic indices. Moreover, the authors noted that increased expression of MCM-2 was accompanied by increasingly shorter time to relapse.

In the cases of cervical intraepithelial neoplasia, previously termed a pre-invasive carcinoma, Ki-67 could be detected in around 10% superficial epithelial cells while MCM-5 was present in almost all the cells (25). In a similar way, in many other dysplastic conditions in humans, such as Bowen's disease or colon adenomas, MCM undergo a markedly higher expression than that of Ki-67 (26-28). These results allow us to suggest that MCM proteins may serve as a proliferation marker of not only neoplastically transformed cells but also of cells in the course of neoplastic transformation process. The cells remaining in the cell cycle due to disturbed proliferation control stay at the threshold between a normal and a neoplastically transformed cell. Their early detection might permit implementation of an appropriate treatment to prevent development of a neoplastic disease. In conclusion, MCM proteins may provide a sensitive cell proliferation marker in line with the routinely used antigens such as Ki-67 or PCNA.

## References

1 Weiss SW and Goldblum JR (ed.). Soft Tissue Tumors. Mosby, St. Louis, pp. 21-44, 2001.

2 Nowak M and Madej JA: Prevalence of neoplasms in domestic animals in Lower Silesia during 2000-2004. Medycyna Wet *62*: 900-904, 2006 (in Polish).

3 Baez JL, Hendrick MJ, Szofer FS, Goldkamp D and Sorenmo KU: Liposarcomas in dogs: 56 cases (1989-2000). J Am Vet Med Assoc *224*: 887-891, 2004.

4 Nowak M, Madej JA and Dziegiel P: Expression of E-cadherin, β-catenin and Ki-67 antigen and their reciprocal correlations in fibrosarcomas of soft tissues in dogs. In Vivo *21*: 751-756, 2007.

5 Nowak M, Madej JA and Dziegiel P: Expression of E-cadherin, β-catenin and Ki-67 antigen and their reciprocal relationships in mammary adenocarcinomas in bitches. Folia Histochem Cytobiol *45*: 233-238, 2007.

6 Sapierzynski R and Sapierzynska E: Mesenchymal neoplasms of the skin and subcutaneous tissue in dogs and cats. Part III. High Malignancy sarcomas. Zycie Wet *79*: 488-492, 2004 (in Polish).

7 Tye BK: MCM proteins in DNA replication. Annu Rev Biochem *68*: 649-861, 1999.

8 Dutta A and Bell SP: Initiation of DNA replication in eukaryotic cells. Annu Rev Cell Dev Biol *13*: 293-332, 1997.

9 Labib K, Tercero JA and Diffley JF: Uninterrupted MCM2–7 function required for DNA replication fork progression. Science *288*: 1643-1647, 2000.

10 Lei M and Tye BK: Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci *114*: 1447-1454, 2001.
11 Tye BK and Sawyer S: The hexameric eukaryotic MCM helicase: building symmetry from nonidentical parts. J Biol Chem *275*: 34833-34836, 2000.
12 Forsburg SL: Eukaryotic MCM proteins: beyond replication initiation. Microbiol Mol Biol Rev *68*: 109-131, 2004.
13 Musahl C, Holthoff HP, Lesch R and Knippers R: Stability of the replicative Mcm3 protein in proliferating and differentiating human cells. Exp Cell Res *241*: 260-264, 1998.
14 Todorov IT, Werness BA, Wang HQ, Buddharajul N, Todorova PD, Slocum HK, Brooks JS and Huberman JA: HsMcm2/BM28: a novel proliferation marker for human tumours and normal tissues. Lab Invest *78*: 73-78, 1998.
15 Madine MA, Swietlik M, Pelizon C, Romanowski P, Mills AD and Laskey RA: The roles of the MCM, Orc and Cdc6 proteins in determining the replication competence of chromatin in quiescent cells. J Struct Biol *129*: 198-210, 2000.
16 Lei M: The MCM complex: its role in DNA replication and implications for cancer therapy. Curr Cancer Drug Targets *5*: 365-380, 2005.
17 Rodins K, Cheale M, Coleman N and Fox SB: Minichromosome maintenance protein 2 expression in normal kidney and renal cell carcinomas: relationship to tumor dormancy and potential clinical utility. Clin Canc Res *8*: 1075-1081, 2002.
18 Stoeber K, Tlsty TD, Happerfield L, Thomas GA, Romanov S, Bobrow L, Williams ED and Williams GH: DNA replication licensing and human cell proliferation. J Cell Sci *114*: 2027-2041, 2001.
19 Todorov IT, Wemess BA, Wang HQ, Buddharaju LN, Todorova PD, Slocum HK, Brooks JS and Huberman JA: HsMCM2/BM28: a novel proliferation marker for human tumors and normal tissues. Lab Invest *78*: 73-78, 1998.
20 Meng MV, Grossfeld GD, Williams GH, Dilworth S, Stoeber K, Mulley TW, Weinberg V, Carroll PR and Tisty TD: Minichromosome maintenance protein 2 expression in prostate: characterization and association with outcome after therapy for cancer. Clin Canc Res *7*: 2712-2718, 2001.
21 Ramnath N, Hernandez FJ, Tan DF, Huberman JA, Natarajan N, Beck AF, Hyland A, Todorov IT, Brooks JS and Bepler G: MCM2 is an independent predictor of survival in patients with non-small cell lung cancer. J Clin Oncol *22*: 4259-4266, 2001.
22 Szelachowska J, Dziegiel P, Jelen-Krzeszewska J, Jelen M, Matkowski R, Pomiecko A, Spytkowska B, Jagas M, Gisterek I and Kornafel J: Mcm-2 protein expression predicts prognosis better than Ki-67 antigen in oral cavity squamocellular carcinoma. Anticancer Res *26*: 2473-2478, 2006.
23 Holfenstein A, Frahm SO, Krams M, Drescher W, Parwaresch R and Hassenpflug J: Minichromosome maintenance protein (MCM6) in low-grade chondrosarcoma. Am J Clin Pathol *122*: 912-918, 2004.
24 Sington JD, Freeman A, Morris LS, Vowler SL, Arch BN, Fisher C and Coleman N: Minichromosome maintenance protein in myxofibrosarcoma. Modern Path *17*: 235-240, 2004.
25 Williams GH, Romanowski P, Morris L, Madine M, Mills AD, Stoeber K, Marr J, Laskey RA and Coleman N: Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. PNAS *95*: 14932-14937, 1998.
26 Alison MR, Hunt T and Forbes SJ: Minichromosome maintenance (MCM) proteins may be pre-cancer markers. Gut *50*: 290-291, 2002.
27 Freeman A, Morris LS, Mills AD, Stoeber K, Laskey RA, Williams GH and Coleman N: Minichromosome maintenance proteins as biological markers of dysplasia and malignancy. Clin Cancer Res *5*: 2121-2132, 1999.
28 Going JJ, Keith WN, Neilson L, Stoeber K, Stuart RC and Williams GH: Aberrant expression of minichromosome maintenance proteins 2 and 5, and Ki-67 in dysplastic squamous oesophageal epithelium and Barrett's mucosa. Gut *50*: 373-377, 2002.

*Received June 13, 2008*
*Revised December 11, 2008*
*Accepted December 15, 2008*