Correlation between levels of expression of minichromosome maintenance proteins, Ki-67 proliferation antigen and metallothionein I/II in laryngeal squamous cell cancer

KATARZYNA NOWINSKA[1], MAGDALENA CHMIELEWSKA[1,2], ALEKSANDRA PIOTROWSKA[1], BARTOSZ PULA[1], WOJCIECH PASTUSZEWSKI[3], TOMASZ KRECICKI[4], MARZENA PODHORSKA-OKOŁOW[1], MACIEJ ZABEL[1,5] and PIOTR DZIEGIEL[1,6]

[1]Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland; [2]Department of Physiotherapy, Wroclaw University School of Physical Education, Wroclaw; [3]Department of Otolaryngology, Municipal Hospital, Namsos, Norway; [4]Department of Otolaryngology, Head and Neck Surgery, Wroclaw Medical University, Wroclaw; [5]Department of Histology and Embryology, Poznań Medical University, Poznan; [6]Department of Vertebrate Biology and Conservation, Faculty of Biological Sciences, Wroclaw University, Wroclaw, Poland

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Abstract. MCM2, MCM3 and MCM7 are minichromosome maintenance proteins found in dividing cells and they play a role in DNA synthesis. Increased MCM expression level is observed in cells of different cancer types. Additionally, metallothioneins (MT-I/II) are involved in control of cell proliferation and differentiation and changes of their expression are observed in many types of cancer. Ki-67 is known cancer cell proliferation antigen currently used in prognostic evaluation. The study material consisted of 83 laryngeal squamous cell cancer (LSCC) cases and 10 benign hypertrophic lesions of larynx epithelium as a control group. For the present study, laryngeal cancer cell line HEp-2 and human keratinocytes were employed, and to evaluate expression of all the markers, immunohistochemical method (IHC), immunofluorescence (IF) and western blot analysis were used. Statistical analysis showed strong positive correlation between expression of MCM2, MCM3, MCM7 and Ki-67 antigen in LSCC. Additionally, moderate positive correlation was observed between MCM3 and MT-I/II expression. In cancer cells, the level of expression of MCM3, MCM2, MCM7 and Ki-67 markers was increasing with the grade of LSCC malignancy. IF and western blot analysis showed higher MCM2, MCM3, MCM7 expression in HEp-2 cells in comparison to their expression in keratinocytes. MCM proteins might be useful markers of cell proliferation in LSCC.

Correspondence to: Professor Piotr Dziegiel, Department of Histology and Embryology, Wroclaw Medical University, Chałbinskiego 6a, 50-365 Wrocław, Poland
E-mail: piotr.dziegiel@umed.wroc.pl

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Introduction

Head and neck cancers are the fifth most commonly occurring cancer type (1) and laryngeal squamous cell cancer (LSCC) accounts for ~60% of all cancers of this part of the body (2). LSCC development is a result of synergistic action of various risk factors. An important role in its ethology is played by: smoking, excessive drinking of alcohol (3-6), HPV infection (7), laryngeal-pharyngeal reflux (LPR) (8,9) and diet rich in preserved vegetables (10).

It was shown that proliferative potential increases due to the changes in cell cycle of cancer cells and that the increase of expression of genes involved in tumour cell proliferation is related to poorer prognosis (11,12). Proteins involved in regulation of DNA replication might be potentially used in routine diagnosis and prognosis of the course of cancer. Such proteins are *inter alia* minichromosome maintenance proteins (MCM). They are associated with the regulation of DNA synthesis and prevention of secondary replication in the same cell cycle (13-15). They are evolutionary conservative proteins and their expression is controlled by transcription factors of E2F family. Expression of MCM proteins is observed only in dividing cells and it is not present in resting, differentiating and senescencing cells (16,17). This group includes MCM2-9 proteins (18) characterised by the presence of specific MCM domain that contains motifs typical for ATPases (19,20). MCMs form a ring-shape complex composed of six subunits: MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7. In the presence of Cdc45 and GINS proteins (Sld5-Psf1-Psf2-Psf3), the complex has DNA double helix unwinding helicase activity and causes formation of replication forks (21,22). MCM2-7 are a part of pre-replication complex and they bind to replication initiation sites, therefore, enabling process of DNA synthesis (23). Additionally, multiple studies confirmed association between the increase of expression of non-histone, nucleus protein Ki-67 and cancer cell proliferation. Opposite

to the MCM proteins, Ki-67 expression is observed in all phases of the cell cycle (24,25). In laryngeal cancer, high Ki-67 expression might indicate biological aggressiveness of cancer, as well as its grade of malignancy (26).

Because of association with DNA synthesis, proteins from MCM family seem to be useful as markers of cellular proliferation. Many studies indicate that expression of these proteins increase in cancer (15,21,27-30). Moreover, MCMs might be better markers of proliferating cells than Ki-67, whose function has not yet been fully understood. On the contrary, function of MCMs is related only to cell proliferation and the proteins are found only in dividing cells. It is believed that besides engagement in processes related to cell division, Ki-67 protein may play a role in rRNA transcription (31,32). Additionally, Bullwinkel *et al* (32) showed that a slight amount of Ki-67 is present also in resting state cells, i.e. in G0 phase.

It has been shown that metallothioneins I/II (MT-I/II) are also involved in the control of cell proliferation and differentiation. The proteins might bind zinc and copper ions necessary for cell functioning, as well as ions of toxic metals such as cadmium, mercury and lead (33). MT-I/II might be donors of bound Zn ions for enzymes whose activity depend on the presence of such ions and therefore they might influence the activity of multiple factors controlling cell division cycle (34-36). Increased expression of MT-I/II was observed *inter alia* in hepatocytes during liver regeneration or in basal layer of stratified squamous epithelium (35,37). Changes in expression of the proteins are also observed in many cancers (38-40).

The expression of MCM proteins has not been studied yet in relation to the expression of Ki-67 antigen and MT-I/II in LSCC. Therefore, their expression was evaluated in 83 cases of LSCC, taking into account clinical and pathological data of patients. Additionally, expression of MCM proteins was confirmed in laryngeal cancer cell line HEp-2 and human keratinocytes.

Materials and methods

Patients. The study material consisted of 83 blocks of paraffin-embedded LSCC samples (51 cases from Pathomorphology Division of J. Babiński Regional Hospital in Wroclaw and 32 cases from Department and Clinic of Otolaryngology, Head and Neck Surgery, Wroclaw Medical University), operated and treated in 1997-2003. Ten sample blocks of benign hypertrophic lesions of laryngeal epithelium were used as a control (vocal cord nodules and Reinke's oedema). Patients were observed for 156 months, and 46 of them died during this period (55%). During the treatment, the age of patients was in the range of 39-79 years (average age, 60 years). Of all patients, 12 were women and 71 were men. Grade of malignancy (G) and clinical stage of disease were determined based on TNM classification (41). Clinical and pathological characteristics of patients are shown in Table I.

Table I. Clinical and pathological characteristics of the studied LSCC patients.

Clinical/pathological parameter	N (%)
Gender	
Male	71 (85.46)
Female	12 (14.54)
Tumour size	
T1	2 (2.41)
T2	12 (14.46)
T3	36 (43.37)
T4	33 (39.76)
Lymph nodes	
N0	51 (61.45)
N1-N3	32 (38.55)
Clinical advancement	
I	2 (2.41)
II	8 (9.64)
III	34 (40.96)
IV	39 (46.99)
Grade of malignancy	
G1	23 (27.71)
G2	45 (54.22)
G3	15 (18.07)

Cell lines. Immunofluorescence (IF) experiments and western blot analyses were conducted with the use of reference adherent laryngeal carcinoma cells line, Larynx Epidermoid Carcinoma HEp-2 (collection of cell lines of Ludvik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland). The cells were cultured in MEM medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, streptomycin and L-glutamine. Pooled primary human foreskin keratinocytes (Gibco, Paisley, UK) cell line was used as a control. The cells were cultured on Keratinocyte-SFM medium (Gibco) with L-glutamine. SFM (Gibco) supplement containing human growth factors (EGF) and bovine pituitary extract (BPE) was added to the medium. After thawing, the cells were passaged three times before being used for research. Cultures were carried out in HERA cell (Heraeus, Hanau, Germany) incubator under constant conditions: temperature of 37˚C, CO_2 concentration of 5% and a 95% level of humidity.

Immunohistochemical reactions (IHC). Specimens of laryngeal carcinomas and benign lesions were fixed in 10% buffered-formalin, dehydrated and paraffin-embedded. In each case, histopathological evaluation and determination of LSCC grade was performed by haematoxylin and eosin (H&E) staining by two pathologists. In order to prepare H&E staining, paraffin blocks were cut into 7 μm thick section with the use of RM 2145 microtome (Leica Biosystem, Nussloch, Germany). For IHC reaction, paraffin blocks were cut in sections 4 μm thick, which were placed onto Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). Deparaffinisation, hydration and heat induced epitope demasking were carried out by boiling for 20 min in the temperature of 97˚C in Dako PT Link (Dako, Glostrup, Denmark) apparatus in high pH Target

Table II. The scale assessing the expression level MCM2, MCM3, MCM7 and the Ki-67 antigen in LSCC and benign lesions of the larynx.

Points	The percentage of cells with positive reaction (%)
0	0
1	1-10
2	11-25
3	26-50
4	>50

Table III. The scale assessing the level of MT-I/II expression in LSCC and benign lesions of the larynx (according to Remmele and Stegner (43).

Points	The percentage of cells with positive reaction (%)	Points	The intensity of the color reaction
0	0	0	No
1	1-10	1	Poor
2	11-50	2	Average
3	51-80	3	Strong
4	>80		

retrieval solution (Dako), according to 3-in-1 procedure. Endogenous peroxidase activity was blocked by incubation with peroxidase-blocking solution (Dako). For the evaluation of expression level of MCM2, MCM3, MCM7, Ki-67 antigen and MT-I/II in LSCC cells, IHC reactions were carried out in paraffin sections with the use of specific monoclonal mouse anti-human antibodies: anti-Ki-67 (dilution 1:100; clone MIB1, code no. F7268; Dako), anti-MT-I/II (dilution 1:100; clone E9, code no. M0639; Dako), anti-MCM2 (dilution 1:15; clone CRCT2.1, code no. NCL-MCM2; Leica Biosystems); anti-MCM3 (dilution 1:50; clone 101, code no. M7263; Dako); anti-MCM7 (dilution 1:50; clone DCS-141.1, code no. NCL-MCM7; Leica Biosystems). The antibodies were diluted in background-reducing reagent and the incubation with sections was carried out for 1 h in room temperature. All IHC reactions were carried out with the use of Autostainer Link 48 (Dako) apparatus and EnVision™ FLEX reagents (Dako) visualisation system. Sites, where a given antigen was located, were visualized in IHC reactions by brown DAB (3,3'-diaminobenzidine tetrahydrochloride) staining. Omitting the addition of primary antibody was used for negative control of IHC reaction, whereas LSCC cases characterised by high expression of studied markers served as a positive control.

Evaluation of immunohistochemical reaction. Two pathologists carried out the evaluation independently. The estimation of nuclear expression of MCM2, MCM3, MCM7 and Ki-67, antigens was performed at x200 magnification with the use of BX41 (Olympus, Tokyo, Japan) light microscope coupled with visual circuit and Cell^{D} (Olympus) software for computer image analysis. The intensity of MCM2, MCM3, MCM7 and Ki-67 expression was determined with the use of five-point evaluation scale (score 0-4), taking into account the percent of cancer cells exhibiting reaction relative to all cancer cells in a given specimen (Table II) (38,42). For the evaluation of MT-I/II cytoplasmic expression level, semiquantitative IRS (method according to Remmele and Stegner - immunoreactive score) was used (43). The intensity of colour reaction (score 0-3) and percentage amount of IRS-positive cancer cells (score 0-4) was estimated. The final result was the product of scores obtained for the evaluation of both parameters and values from 0 to 12 were considered (Table III).

Western blot analysis. After 72 h of culturing, the cells were trypsinised, centrifuged and PBS-washed. The number of cells was estimated using Burker's chamber. For each western blot analysis, 3.5-4x10^6 of laryngeal cancer cells and keratinocytes in exponential growth phase were taken. After washing with cold PBS, the cells were resuspended in 1 ml of isotonic buffer for cell nuclei isolation (5 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl pH 7.5, 250 mM sucrose). Cells were mechanically broken on ice in isotonic buffer, in glass Potter's homogeniser. In order to separate nuclei pellet from cytoplasm, homogenate was centrifuged for 10 min in 4˚C, 2000 x g. Nuclei precipitate was solubilised on ice using nuclei lysis buffer [50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% Igepal-NP-40 (Sigma, St. Louis, MO, USA), protease and phosphatase inhibitors (Sigma), 0.5 mM PMSF]. The suspension was centrifuged for protein separation. Supernatant was stored in -20˚C. For the determination of concentration of obtained protein, BCA method was used (Thermo Fisher Scientific-Pierce, Rockford, IL, USA). Cellular extracts were mixed with GLB sample buffer (250 mM Tris pH 6.8, 40% glycerol, 20% β-mercaptoethanol, 100 mM DTT, 0.33 mg/ml bromophenol blue, 8% SDS) and then denatured for 10 min in 95˚C. Equal amounts of proteins from cancer cells and normal keratinocytes were loaded into a gel (15 μg/lane) and separated by electrophoresis in 7.5% polyacrylamide gel with SDS in Mini-Protean 3 apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Next, the proteins were electrophoretically transferred onto PVDF-Immobilon P (Millipore, Billerica, MA, USA) membrane according to Towbin *et al* (44) and the membrane was blocked with milk solution in TBS with 0.1% Tween-20 (TBST) for non-specific binding sites.

The expression of MCM2, MCM3 and MCM7 was determined using specific mouse monoclonal anti-human antibodies: anti-MCM2 (dilution 1:100; clone CRCT2.1, code no. sc-56321; Santa Cruz Biotechnology, Dallas, TX, USA), anti-MCM3 (dilution 1:400; clone 36-Q-7, code no. sc-81848; Santa Cruz Biotechnology); anti-MCM7 (dilution 1:200; clone 141.2, code no. sc-9966; Santa Cruz Biotechnology). Incubation was carried out overnight at 4˚C with mild shaking. The membrane was washed three times using 0.2% TBST buffer. Afterwards, it was incubated in the solution of donkey anti-mouse antibody conjugated with horseradish peroxidase (1:3000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The detection was performed with the use of chemiluminescence substrate from Immun-Star HRP Chemiluminescent kit (Bio-Rad Laboratories). The data were collected at various exposition times ranging from 2 sec to 30 min in Chemi-Doc

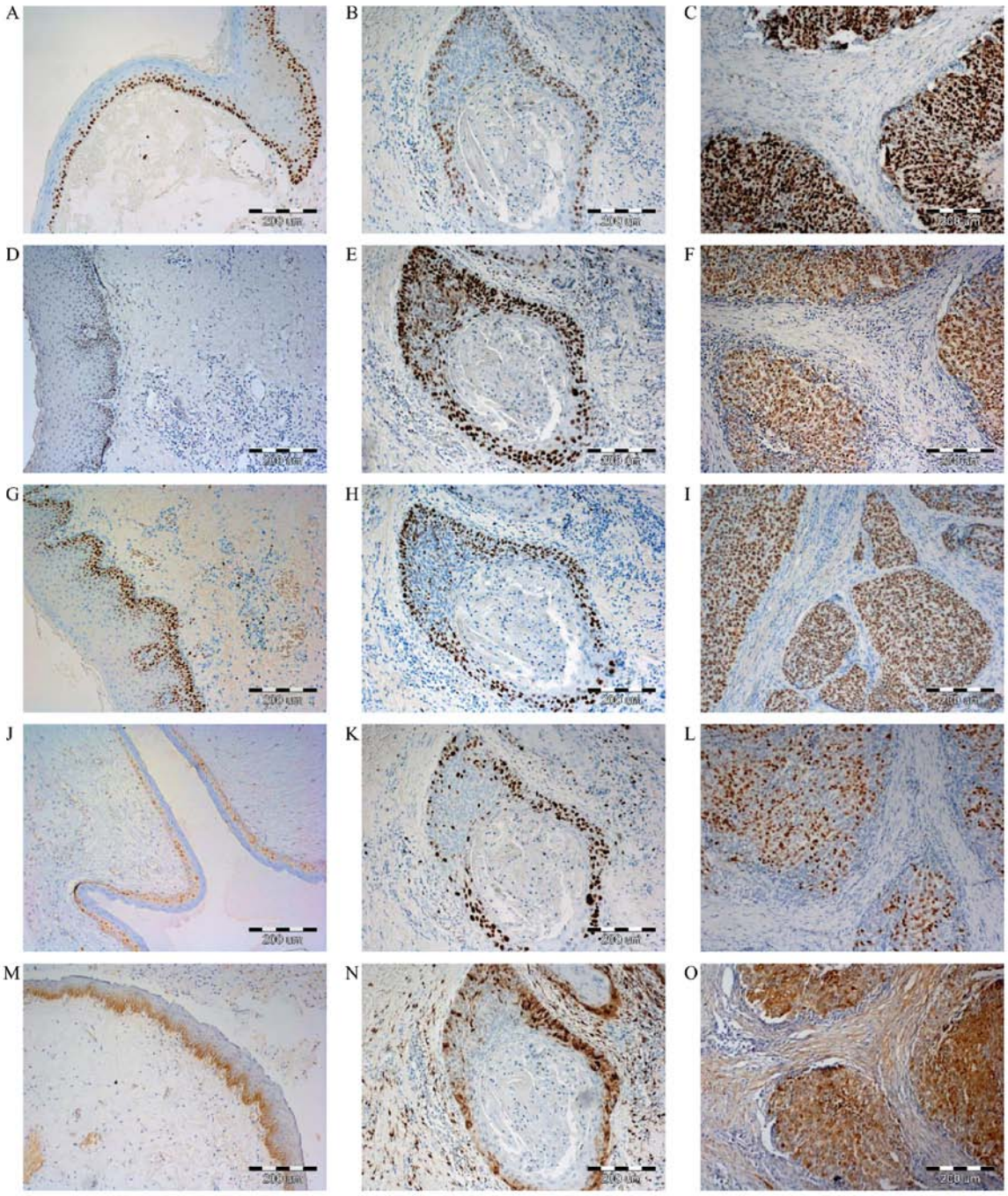

Figure 1. Positive immunohistochemical reaction (brown nuclei) indicating MCM2, MCM3, MCM7, MT-I/II (brown cytoplasm) and Ki-67 (brown nuclei) antigen expression carried out on laryngeal benign lesions and in different histological grade of LSCC (G) (A-C). MCM2 expression - benign lesion (A) and LSCC (B in G1, C in G3). MCM3 expression in benign lesion (D) and LSCC (E in G1, F in G3). MCM7 expression in benign lesion (G) and LSCC (H in G1, I in G3). Ki-67 antigen expression in benign lesion (J) and LSCC (K in G1, L in G3). MT-I/II expression in benign lesion (M) and LSCC (N in G1, O in G3).

XRS Molecular Imager (Bio-Rad Laboratories) apparatus. Antibodies were washed from the membrane to perform densitometric studies and the detection of β-actin was conducted with the use of mouse monoclonal primary antibody (dilution 1:300; clone AC-40, code no. A4700; Sigma, St. Louis, MO, USA) in SNAP i.d (Millipore) apparatus, as a control of amount of loaded protein. Quantity One (Bio-Rad Laboratories) software was used for measurements of the protein band optical density. In each lane, the amount of protein in MCM2, MCM3 and MCM7 bands was normalised to β-actin.

Immunofluorescence (IF). In order to perform immunofluorescence reactions on cells of laryngeal cancer cell line HEp-2 and normal human keratinocytes, 24-h micro-cultures were set up on slides with 8-wells covered with Teflon. The cells were trypsinised, centrifuged and resuspended in 5 ml of medium. For microculture inoculum, 50 μl of $5x10^4$ cells/ml suspension was instilled into wells on the slide. Slide-microcultures were placed on Petri dishes in an incubator at 37˚C for 24 h. The medium was removed after the incubation and wells were washed with PBS. The cells were

Table IV. Intensity of MCM2, MCM3, MCM7, Ki-67 and MT I/II expression in benign larynx lesions and analyzed LSCC cases.

	Patient group				
		Grade of malignancy LSCC			
Expression	Benign laryngeal (n=10)	G1 (n=23)	G2 (n=45)	G3 (n=15)	P-value (Kruskal-Wallis test)
MCM2	1.2±0.42	1.96±1.02	2.84±1.02	3.33±0.82	**0.036**
MCM3	1.1±0.32	2.39±1.03	2.87±1.10	3.07±0.96	**<0.0001**
MCM7	1.2±0.42	2.26±0.96	2.89±1.01	3.07±1.03	**0.0002**
Ki-67	1.2±0.42	1.91±0.85	2.38±1.01	3.27±0.96	**0.0156**
MT I/II	2.4±1.43	6.13±3.14	7.44±3.05	7.47±4.31	**0.0004**

Statistically significant values are indicated in bold.

Table V. Spearman's rank correlation between the expression levels of MCM proteins, Ki-67 antigen and MT-I/II in LSCC.

	MT-I/II	Ki-67	MCM7	MCM3
MCM2	r=0.20, P=0.6004	r=0.60, P<**0.0001**	r=0.47, P<**0.0001**	r=0.35, P=**0.0011**
MCM3	r=0.35, P=**0.0013**	r=0.52, P<**0.0001**	r=0.70, P<**0.0001**	
MCM7	r=0.10, P=0.3218	r=0.54, P<**0.0001**		
Ki-67	r=0.16, P=0.1402			

Statistically significant values are indicated in bold.

fixed in wells with the use of 4% formaldehyde in PBS for 12 min at room temperature. Cell membrane permeabilisation was performed with 0.2% Triton in PBS for 10 min at room temperature. Specific primary mouse monoclonal antibodies were used for the reaction: anti-MCM2 (dilution 1:15; clone CRCT2.1, NCL-MCM2; Leica Biosystems), anti-MCM3 (dilution 1:20; clone 101, M7263; Dako), anti-MCM7 (dilution 1:50; clone DCS-141.1, NCL-MCM7; Leica Biosystems). Overnight incubation with primary antibodies was carried out at 4˚C. Next, preparations were incubated for 1 h with donkey anti-mouse secondary FITC-conjugated antibody (Jackson ImmunoResearch Laboratories) diluted 1:50 in the reagent with background-reducing component. Preparations were mounted using Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA), which contains DAPI for cellular DNA visualisation. Observations were made at x200 magnification with the use of BX51 microscope (Olympus) coupled with Cell^F software (Olympus).

Statistical analysis. Statistical analyses were performed using Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA) and Statistica 8.0 (Dell Software, Aliso Viejo, CA, USA) software. In order to study the relationship between the antigens, Spearman's rank correlation was used. Mann-Whitney U test, a non-parametric equivalent of Student's t-test, as well as Kruskal-Wallis test, a non-parametric equivalent of variation analysis, were used for the evaluation of relationship between the intensity of gene expression and LSCC grade. Fisher's exact test was used to determine the correlation between the intensity of marker expression and clinical and pathological factors. To verify the correlation between protein expression levels and the patient survival, Kaplan-Meier method and Cox proportional hazards regression analysis were used. Statistically significance was set at P-value <0.05.

Results

Expression of MCM2, MCM3, MCM7, MT-I/II and Ki-67 antigen in immunostained LSCC sections. Nuclear expression of MCM2, MCM3, MCM7 and Ki-67, as well as cytoplasmic-nuclear MT-I/II expression in LSCC was observed (Fig. 1) in histopathological specimens prepared by IHC. Strong MCM2 protein expression was reported in 47 (56.6%) cases of LSCC. Similarly high MCM3 expression was observed in 50 (60.2%) cases, while high MCM7 expression was recorded in 48 (57.8%) cases. Intensity of Ki-67 expression in laryngeal cancer cells was weaker. High expression was reported in 39 (46.9%) cases of LSCC. Higher expression of all tested MCM proteins, as well as Ki-67 and MT-I/II in LSCC was visible in comparison to analysed benign lesions (Fig. 1). Statistical analysis revealed that this difference is statistically significant in the case of each of proteins being evaluated (Table IV).

Spearman's rank correlation coefficient showed strong positive correlation between the level of MCM2, MCM3 and MCM7 expression and Ki-67 antigen (r=0.60, r=0.52, r=0.54; P<0.05) in LSCC (Table V). The strongest correlation was observed between MCM2 and Ki-67. Additionally, moderate positive correlation between expression of MCM3 and MT-I/II was found (r=0.35, P<0.05). No statistically significant corre-

Table VI. Correlations between levels of MCM2, MCM3, MCM7, Ki-67 and MT I/II expression and grade of malignancy of LSCC.

Expression	Grade of malignancy LSCC			P-value (Mann-Whitney U test)
	G1 (n=23)	G2 (n=45)	G3 (n=15)	
MCM2	1.96±1.02 SD	2.84±1.02 SD	3.33±0.82 SD	**G1 vs. G2, 0.0018** G1 vs. G3, 0.1102 **G2 vs. G3, 0.0003**
MCM3	2.39±1.03 SD	2.87±1.10 SD	3.07±0.96 SD	G1 vs. G2, 0.0819 **G1 vs. G3, 0.0529** G2 vs. G3, 0.6039
MCM7	2.26±0.96 SD	2.89±1.01 SD	3.07±1.03 SD	**G1 vs. G2, 0.0367** **G1 vs. G3, 0.0134** G2 vs. G3, 0.3687
Ki-67	1.91±0.85 SD	2.38±1.01 SD	3.27±0.96 SD	**G1 vs. G2, 0.0638** G1 vs. G3, 0.0003 **G2 vs. G3, 0.0042**
MT-I/II	6.13±3.14 SD	7.44±3.05 SD	7.47±4.31 SD	G1 vs. G2, 0.1624 G1 vs. G3, 0.3628 G2 vs. G3, 0.9931

Statistically significant values are indicated in bold.

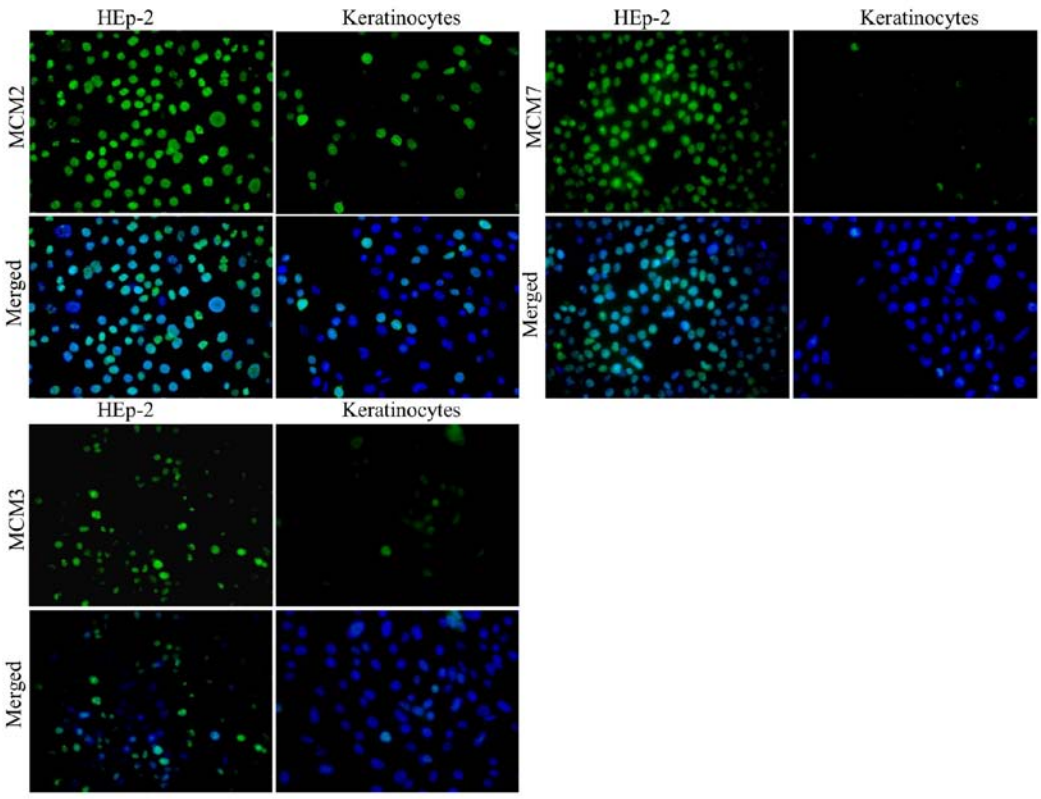

Figure 2. Nuclear expression of MCM2, MCM3 and MCM7 observed in cells from larynx epidermoid carcinoma HEp-2 (HEp-2) cell line in comparison to normal human keratinocytes from pooled primary human foreskin keratinocytes, magnification, x400.

lation was found between expression of MT-I/II and two other MCM proteins (Table V).

The greater the level of expression of MCM2, MCM3, MCM7 and Ki-67 marker was in cancer cells, the higher was

Table VII. Associations between the expression intensity of MCM2, MCM3, MCM7, Ki-67 and MT-I/II and clinicopathological characteristics of analyzed LSCC patients.

		MCM2 expression LSCC N, (%)			MCM3 expression LSCC N, (%)			MCM7 expression LSCC N, (%)			Ki-67 expression LSCC N, (%)			MT-I/II expression LSCC N, (%)		
Characteristics	(No (%)	0-2	3-4	P-value	0-2	3-4	P-value	0-2	3-4	P-value	0-2	3-4	P-value	0-4	6-12	P-value
Age (years)																
≤60	40 (48.2)	20 (24.1)	20 (24.1)	0.2734	18 (21.6)	22 (26.6)	0.3772	18 (21.6)	22 (26.6)	0.6609	24 (28.9)	16 (19.2)	0.2731	10 (12)	30 (36.2)	0.8077
>60	43 (51.8)	16 (19.2)	27 (32.6)		15 (18.1)	28 (33.7)		17 (20.6)	26 (31.2)		20 (24.1)	23 (27.8)		12 (14.4)	31 (37.4)	
Tumour size																
T1-T2	14 (16.8)	7 (8.4)	7 (8.4)	**0.0179**	4 (4.8)	10 (12)	0.3890	5 (6)	9 (10.8)	0.7684	6 (7.2)	8 (9.6)	0.7771	2 (2.4)	12 (14.4)	0.3345
T3-T4	69 (83.2)	29 (34.9)	40 (48.1)		29 (35)	40 (48.2)		30 (36.2)	39 (47.1)		33 (39.8)	36 (43.4)		20 (24.1)	49 (59.1)	
Lymph nodes																
N0	51 (61.4)	29 (34.9)	22 (26.5)	**0.0028**	23 (27.7)	28 (33.7)	0.2534	25 (30.2)	26 (31.2)	0.1702	35 (42.2)	16 (19.2)	**0.0006**	12 (14.4)	39 (47)	0.4455
N1, N2, N3	32 (38.6)	7 (8.4)	25 (30.2)		10 (12)	22 (26.6)		10 (12)	22 (26.6)		9 (10.8)	23 (27.8)		10 (12)	22 (26.6)	

Expression intensity: low, 0-2; high, 3-4. Significant differences for the Fisher's exact test are indicated in bold.

the grade (G) of the analysed LSCC. Additionally, statistically significant difference was shown between G1 and G3 in the level of expression of MCM3, MCM7 and Ki-67 proteins, and between G1 and G2 in the case of MCM2 and MCM7, and between G2 and G3 in the case of MCM2 and Ki-67 (Table VI and Fig. 1). The difference in MCMs, Ki-67 and MT-I/II expression was observed in cases of LSCC with and without lymph node metastasis. The difference was statistically significant in the case of MCM2 and Ki-67 (Table VII).

No significant difference was found in the survival of patients with high (score 3-4 for MCM 2, 3, 7 and Ki-67; score 6-12 for MT-I/II) and low (score 0-2 for MCM 2, 3, 7 and Ki-67; score 0-4 for MT-I/II) expression of studied proteins, neither was the survival dependent on the age of the patients. However, significant relationship was shown between patient survival and lymph node metastasis, as well as the size of the tumour (Table VIII).

Immunofluorescence reaction. In order to visualise the localisation and intensity of expression of MCM2, MCM3 and MCM7 in HEp-2 cells and normal keratinocytes, *in vitro* studies were performed. IF reactions were conducted with the use of specific antibodies on slide-microcultures. Next, fluorescence of nuclei in HEp-2 cells and normal keratinocyte cell lines was observed under the microscope (Fig. 2). Importantly, larger number of cells was expressed in the studied MCM proteins in laryngeal cancer cells than in normal keratinocytes.

Western blot analysis. MCM2, MCM3 and MCM7 proteins were detected in nuclei isolates of HEp-2 cell line and keratinocytes by means of western blot technique and with the use of specific antibodies. Densitometrically measured level of expression of MCM2, MCM3 and MCM7 was higher in HEp-2 cells in comparison with their expression in keratinocytes (Fig. 3).

Discussion

In most of the cancers, an increased level of expression of genes encoding proteins that regulate cancer cell proliferation is observed. The intensity of their expression might indicate fast tumour growth and it is also frequently associated with a worst prognosis for patient (12-13,17). Recognised cell proliferation markers, such as PCNA (proliferating cell nuclear antigen) and Ki-67, are useful indicators of the intensity of this process in various types of cancers. However, some studies have shown that they can indicate also other than dividing cells (45). The above facts led to a search for such cell proliferation markers, which would be expressed in dividing cells only. MCM protein family members fulfil this criterion, because they are associated with the regulation of DNA synthesis (13).

The role of Ki-67 and MT-I/II in cancer cell proliferation and their use as prognostic factors are described in many publications (42, 44-46). As the involvement of Ki-67 antigen and MT-I/II in cancer cell proliferation is well documented, it seems important to investigate whether their expression correlates with the expression of MCM proteins. We have shown that expression of all studied MCM proteins was positively correlated with the level of expression of Ki-67 antigen in LSCC cells. The correlation between MCM proteins and

Table VIII. Univariate Cox proportional hazards analysis in 83 patients with LSCC.

	Overall survival		
Clinicopathological parameter	HR	95% CI	P-value
Age (≤60 vs. >60 years)	0.8357	0.4814-1.457	0.5302
pT (pT1-T2 vs. pT3-T4)	2.018	1.019-3.994	**0.0440**
pN (pN0 vs. pN^+)	1.935	1.069-3.902	**0.0291**
Tumour size (T1-T2 vs. T3-T4)	0.4957	0.2504-0.9812	**0.0440**
Grade of malignancy (G1, G2 vs. G3)	0.8071	0.3845-1.694	0.5710
Lymph node involvement (N0 vs. N^+)	0.5168	0.2856-0.9350	**0.0291**
Ki-67 (<25 vs. ≥25%)	1.124	0.6427-1.967	0.6815
MCM 2 (<25 vs. ≥25%)	0.8557	0.4911-1.491	0.5821
MCM 3 (<25 vs. ≥25%)	1.514	0.8523-2.688	0.1572
MCM 7 (<25 vs. ≥25%)	0.8370	0.4783-1.465	0.5331
MT I/II (0-4 vs. 5-12)	1.168	0.6171-2.210	0.6336

HR, hazard ratio; CI, confidence interval. Statistically significant values are indicated in bold.

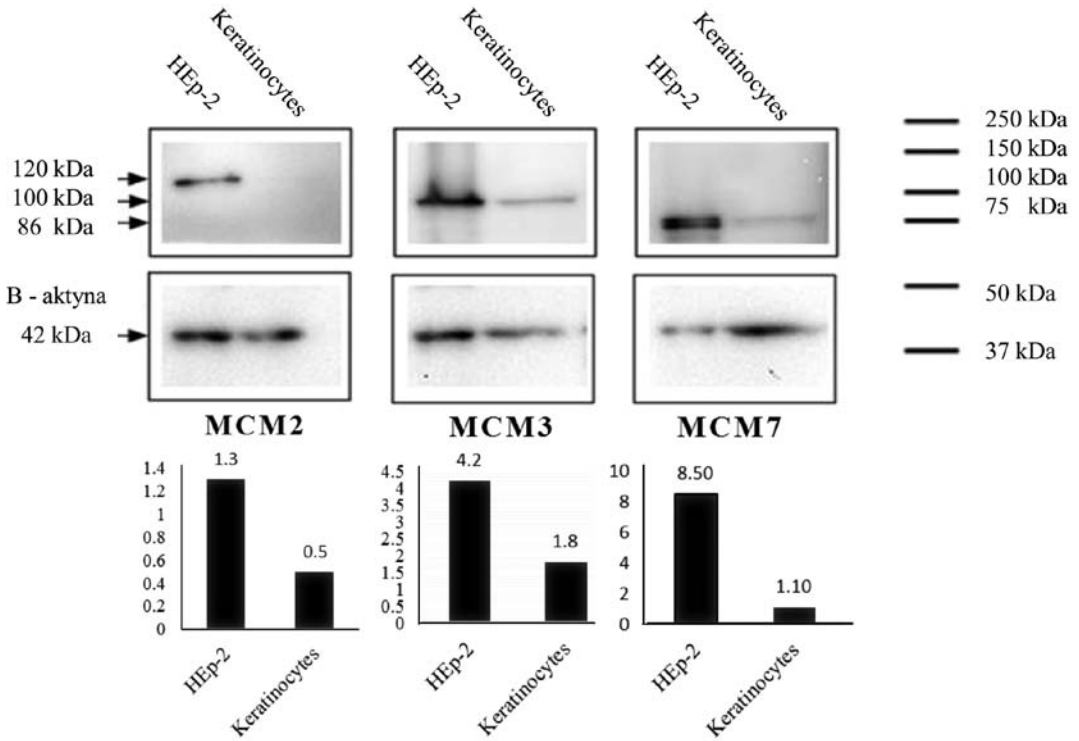

Figure 3. MCM proteins expression detected by using western blot technique in the cell nucleus of HEp-2 cells and pooled primary human foreskin keratinocytes. MCM2, 125 kDa; MCM3, 100 kDa; MCM7, 86 kDa. Comparison of densitometrical analysis of MCM proteins expression in HEp-2 cell line and in normal keratinocytes is indicated.

Ki-67 was strong and values of expression in the subsequent grades (G) were higher for MCM in comparison to Ki-67. It might support the proposal by Ha *et al* (11) that MCM proteins might by more specific than Ki-67 antigen in evaluation of the intensity of cancer cell proliferation and might be used as replacement markers for Ki-67 antigen. Similar results were obtained by authors studying correlation between expression of MCM proteins and Ki-67 antigen in various types of carcinomas (27,29-30,45,47-50). Chatrath *et al* (50) presented the result of experiments carried out on a small group of LSCC. They investigated the expression of MCM2, MCM5 and antigen Ki-67 in 20 patients with dysplasia of laryngeal mucus membrane and in only 10 patients with LSCC. They found an increase of expression of both MCM proteins and Ki-67 in LSCC in comparison to control tissues. Moreover, they observed that in neoplastic lesions of larynx,

the number of cells showing the expression of MCM2 is higher than cells expressing antigen Ki-67 in the same tissue localisation. However, the presented results are based on a small test group and for the analysis only samples fixed in formalin and embedded in paraffin were used. Apart from the results obtained with the use of archival material, in the present study we show also those conducted *in vitro* on laryngeal cancer cells. Corresponding study of these authors conducted on similar number of LSCC patients gave almost identical results. Additionally, they studied coexpression of MCM2 protein and cell cycle markers. They found that MCM2 protein is expressed in the same cells as cyclins A and D1, and also in some cells expressing cyclin B1 and phosphorylated histone H3. This fact suggests that MCM2 proteins are present during all phases of cell cycle, but their strongest expression is observed in phase G1 and S (51).

MT-I/II, similarly to Ki-67, are associated with cell proliferation process. Therefore, we also studied their relationship with MCMs. Interestingly, the moderate positive correlation between expression of MT-I/II and MCM3 was observed with regard to the lack of association between metallothionein expression in LSCC and two other proteins, MCM2 and MCM7. All proteins belonging to MCM family have zinc-binding site, however this motif is different in MCM3 chain than in all other proteins belonging to this family and it enables chelation of zinc. It was proved that zinc influences MCM2-7 complex assembly and ATPase activity (52,53). On the contrary, metallothioneins are proteins that can bind zinc and regulate its balance in the organism (54). Their association with the balance and availability of zinc ions might explain the fact of correlation between MT-I/II and MCM3. Similar studies and analysis have rarely been conducted. The relationship between MT-I/II and MCM2 was reported in adrenal cortex adenocarcinomas, non-small cells lung cancer and invasive breast cancer (39-40,53). However, to date the literature does not include studies on the association between expression of MT-I/II and MCM markers in laryngeal squamous cell cancer. Our results, as well as those of other authors, show that MCM proteins might be important markers of cancer cell proliferation. Additionally, the presence of MCM in cancer and dysplastic cells suggests their clinical utility in diagnosis of pre-invasive and invasive cancers (50).

Apart of the importance of MCMs in cancer cell proliferation process, it is a key issue to study their prognostic value. We observed the increase of studied MCM proteins expression in LSCC in comparison to their expression in benign hypertrophic lesions of stratified squamous epithelium of larynx. Moreover, we found statistically significant differences between expressions of MCMs in studied carcinomas of various grades of malignancy. The level of MCMs expression increased with the increase of malignancy grade of the studied tumours. Based on western blot analysis and IF experiments it was also found that MCMs are present in higher number of laryngeal cancer cells than in normal cells in an *in vitro* model. Additionally, the results obtained with the use of IHC show that MCM2 might be of significance for the evaluation of the risk of metastasis to lymph nodes. Its expression is significantly higher in patients with LSCC who were diagnosed with lymph node metastasis. Our presented study results are consistent with the results on MCM expression obtained by other authors. The increase of MCM proteins expression in cancer cells was observed in various cancers (45,50,52,55-61). However, there is still a lack of analysis of MCM protein expression and the prognostic significance in LSCC. The importance of MCM2 protein as a prognostic marker in LSCC was described in two studies (27,51), whereas the significance of MCM3 and MCM7 in LSCC has not been reported so far. Expression of MCM7 was in turn evaluated in other cancers of head and neck area, e.g. in squamous cell oral cancer. It was shown that expression of this protein is significantly higher in oral cancer in comparison to normal and dysplastic lesions. The authors also found that MCM7 is positively correlated with the grade of malignancy of above-mentioned carcinomas (30).

We observed also significant difference between expression of Ki-67 in laryngeal cancers and benign lesions, as well as the possibility of use of this antigen for the evaluation of LSCC aggressiveness. Our results are consistent with those of Rodrigues *et al* (26) and Sarafoleanu *et al* (62). The intensity of Ki-67 expression in non-metastatic tumours (N0) was significantly lower than in metastatic cancers (N1-N3) to regional lymph nodes. In agreement with Pastuszewski *et al* (38) studied the expression of Ki-67 in the cells of squamous cell laryngeal cancer. They found that it is significantly higher in laryngeal cancer in comparison with benign hypertrophic lesions. Higher Ki-67 expression was also associated with poorer prognosis for patients. As Pastuszewski *et al* (38), we also found remarkably higher MT-I/II expression in squamous cell laryngeal cancers than in hyperplastic lesions. The increase of MT-I/II expression was observed also in other cancers (63-65). In some cancers, the expression of MT-I/II correlates with the size of the tumour and the stage of the disease, as well as with resistance to chemotherapy and radiotherapy and poor prognosis for patients (35).

We were unable to show association of MCM, Ki-67 and MT-I/II proteins with the patient survival. The difference in survival of patients with high and low level of the studied proteins was not statistically significant. Possibly due to the rather small test group. However, it was well selected, as evidenced by statistically significant dependency between the patient survival and pT and pN parameters. Bukholm *et al* (65) who observed the lack of association between MCM2 and the patient survival, obtained similar results. In turn, Tamura *et al* (30) showed that survival of patients with higher MCM7 expression was shorter than patients with its lower expression in cancers of head and neck region.

In summary, nuclear expression of MCM proteins was observed in nuclei and it was higher in LSCC cells than in control tissue, benign hypertrophic lesions. Our studies also confirmed that the proteins might be useful as markers of proliferating cancer cells due to strong correlation of their expression with the intensity expression of antigen Ki-67 and also MT-I/II in case of MCM3 protein. The results of the present study also suggest the association of increased expression of MCM proteins with higher aggressiveness of squamous cell laryngeal cancer (grade of malignancy, G). Moreover, the analysis of the results indicates that MCM2 protein might be of importance for the evaluation of risk of lymph nodes metastasis. Further studies on the use of MCM proteins as markers of cancer cell proliferation might enable evaluation of their usefulness in diagnostic and prognostic assessment of LSCC.

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