Mini-chromosome maintenance protein 7 (MCM7) and geminin expression: prognostic value in laryngeal squamous-cell carcinoma in patients treated with radiotherapy and cetuximab.

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ABSTRACT

Background: MCM7 is a downstream of HER1 signaling. We examined MCM7, geminin and HER1 expression in laryngeal squamous-cell carcinoma (LSCC) patients treated with radiotherapy and cetuximab.

Methods: MCM7, geminin and HER1 were evaluated by immunohistochemistry on 61 LSCC patients. The follow-up (median: 32.1 months; range: 2-139) went from the beginning of therapy to tumor progression (PFS) and death (OS).

Results: MCM7, but not geminin, was associated only with HER1 expression, while no association was with other clinicopathological characteristics. Patients with MCM7 high - geminin high and MCM7 high - geminin low tumor status had a risk of progression 3.1 times and 17.7 times greater, respectively than patients with MCM7 low – geminin high tumor status. Tumor site, MCM7 and geminin were independent determinants of PFS, while MCM7 was an independent prognostic marker of OS.

Conclusions: MCM7-geminin tumor status may be prognostic for LSCC patients treated with cetuximab and radiotherapy.

INTRODUCTION

Minichromosome maintenance proteins (MCM) belong to a family of six highly conserved and highly homologous proteins (MCM2-7). MCM protein 2-7 form a functional hexameric complex, constituting an important part of the pre-recognition complex of proteins present at DNA replication origins during the G1 phase of the cell cycle [1].

MCM proteins show continuous expression patterns during the cell cycle, although they are bound to chromatin only in the late mitosis and early G1 phase [2]. In the course of the S-phase, MCM proteins become irreversibly detached from chromatin, assuring that DNA replication takes place only once in the cell cycle [3].

MCM proteins represent a reliable marker of cell cycle entry as their expression has been demonstrated in cells remaining in the cell cycle, while loss of MCM expression reflects the resting state of the cells [4]. Moreover, MCM proteins are also involved in transcription, chromatin remodeling and checkpoint responses [5].

The requirement for MCM proteins in cycling cells but their absence in quiescent cells have led to their potential clinical application as markers for cancer screening.

Dysregulation of MCM family members has been studied in several types of neoplasia in relation to important clinico-pathological characteristics and, as cell proliferation markers, they constitute diagnostic and prognostic tools of great clinical significance for patient management and survival. In particular, MCM7 has been associated with tumorigenesis in various human cancers, including oral squamous cell carcinoma, colorectal cancer, ovarian cancer, glioblastoma, and esophageal carcinoma [6].

Geminin is thought to be a regulator of the process that inhibits DNA re-replication in the same cell cycle. Geminin expression has been shown to be restricted to S, G2 and early M cell-cycle phase [7] and it functions as a protector of genome stability by preventing the untimely binding of MCM complex to chromatin during the S phase, the G2 phase and early mitosis [8]. Therefore, geminin is

involved in the regulation of the cell cycle and in keeping genomic integrity. Furthermore, geminin, as negative regulator of the MCM loading factor Cdt1 [9], may be a potential tumor suppressor gene [10].

Geminin expression might complement information obtained from evaluation of MCM labeling, giving an indication of cell-cycle rate. In fact, MCM proteins mark all non-quiescent cells, whereas geminin identifies the proportion of actively proliferating cells that have entered S-phase, but not exited mitosis [7].

The assessment of expression of both MCM7 and geminin may be a promising marker of cell proliferation, and it has been related to tumor progression and distant metastases [6], but its role as biomarker in laryngeal cancer is still need to be clarified.

Epidermal growth factor receptor (HER1) appears, at present, to be the most reliable molecular marker in head and neck squamous cell carcinogenesis, as it is expressed in over 90% of head and neck squamous cell carcinomas (SCCs). HER1 over-expression in laryngeal squamous-cell carcinoma (LSCC) has been shown to correlate with worse clinical outcome [11-16], decreased response to radiotherapy, and increased locoregional recurrence following definitive radiotherapy [17].

Cetuximab is an IgG1 monoclonal antibody that exclusively targets EGFR with high affinity, and inhibits endogenous ligand binding, thereby blocking receptor dimerization, tyrosine kinase phosphorylation, and signal transduction [18-20]. Furthermore, in-vitro and in-vivo studies have shown that there is synergy between cetuximab and radiotherapy with the combination resulting in a greater reduction in cellular proliferation than either treatment alone [21-24]. However, despite initial encouraging results of the pivotal trial of the anti-EGFr antibody cetuximab and radiation therapy in locally advanced HNSCC patients, demonstrating a significantly improved median time to progression, negative disappointing randomized phase III trials data [25,] indicate that much more

remains to be clarified with regard to EGFR biology and patient selection. In fact at present there is not reliable predictor of sensitivity to cetuximab including HER1 expression.

Recently, it has been proposed that HER1 enhances MCM7 phosphorylation and DNA replication through Lyn phosphorylation in human cancer cells thereby raising the possibility that MCM7 is a downstream target of HER1 signaling [26]. Since MCM7 is critical in DNA replication and involved in oncogenic signaling pathways, we set out to verify whether the expression of MCM7 and geminin in primary LSCC may potentially be used for screening and estimation of prognosis for patients who underwent radiotherapy in combination with cetuximab. Moreover, this study was site-specific with a series composed exclusively of laryngeal squamous cell carcinomas to reduce the biological and clinical heterogeneity associated with different sites of origin in head and neck.

MATERIALS AND METHODS

Patients

Study design

From 1998 to 2012, 61 consecutive untreated, newly diagnosed laryngeal squamous cell carcinoma (LSCC) patients (age: mean = 65.4 years; median = 65 years; range: 40-86 years) suitable for an organ preservation protocol according to International Guidelines (NCCN V2.2014), or, in case of T4 any N, refused other therapeutic options, treated definitively with cetuximab (C 225) concurrently with radiotherapy at Catholic University Head and Neck Cancer Center were reviewed (Institutional Review Tumor Board "SpiderNet"). Eligible patients had untreated, histologically confirmed, stage II, III or IV LSCC; Zubrod performance status 0 to 1; age > 18 years; any tobacco status; and adequate bone marrow, hepatic, heart, and renal functions. We excluded patients who received additional concurrent, induction, or adjuvant systemic therapy; weekly cisplatin; prior HN radiotherapy; or primary surgical resection; Histological grading and TNM classification was performed according to the recommendations of the International Union Against Cancer. All patients underwent a full diagnostic work up including a complete head and neck exam; mirror, fiberoptic, and narrow band imaging (NBI)-videoendoscopic examination; representative biopsy; chest CT; CT and MRI of larynx and neck; PET/CT for advanced disease, as suggested by NCCN guidelines (version 2.2014). After the work-up all cases were staged and discussed by the Tumor Board involving at least a medical oncologist, a radiation therapist, and an ENT surgeon. Smoking and drinking status were assessed, 23 patients were ex-smokers (37,7%), 6 (9,8%) patients were never-smokers, 22 (36%) patients were smokers with a mean of 46 years of tobacco use and in 10 (16,4%) cases the tobacco use was not known. Current and previous smokers had a mean pack/year index of 61,4 (range 6 - 250). Thirty (49,2%) patients were current drinkers and 7 (11,5%) were previous drinkers. Fourteen (23%) patients were both drinkers and smokers. Other patient and tumor characteristics are listed in table 1.

Treatment

All patients received dental care followed by intensity-modulated radiation therapy (IMRT). We delivered 69.96 Gy at 2.12 Gy per fraction to the planning target volume (PTV) encompassing the gross tumor volume (GTV), 59.4 Gy at 1.8 Gy per fraction to the PTV of the high-risk clinical target volume (CTV), and 54 Gy at 1.64 Gy per fraction to the PVT of the low-risk CTV. The GTVs and CTVs were each expanded 3 to 5 mm to generate their respective PTVs. Cetuximab was administered at an initial dose of 400 mg/m2 during the week before IMRT and then 250 mg/m2 per week during radiotherapy with a maximum of 7 additional doses. Toxicity was evaluated weekly during therapy using the Common Terminology Criteria for Adverse Events (version 3). Adverse events reported as definitely, probably, or possibly related were considered treatment-related events. During the treatment period the patients were examined weekly with transnasal fiber optic laryngoscopy to evaluate the respiratory laryngeal space and swallowing. Patients were assessed after the completion of treatment with physical examination, NBI-videolaryngoscopy, imaging studies, and, if there was a high suspicion of residual laryngeal disease, endoscopic laryngeal examination with biopsy plus a possible salvage surgery (i.e., partial or total laryngectomy was performed). Furthermore, if there was residual neck disease, comprehensive radical modified neck dissection was also performed. In the follow-up period, CT or MRI imaging was performed 8 to 9 weeks after treatment, at 6 months, and then annually, with physical examination every 3 months for 2 years, every 6 months through year 5, and then annually to assess tumor status and toxicity.

Immunohistochemistry

Tumor tissues were fixed in 10% buffered formalin and embedded in paraffin, according to standard procedures. Five-micrometer-thick sections cut from each case were deparaffinized in xylene, rehydrated, treated with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity, and subjected to heat-induced epitope retrieval in a microwave oven.

Immunohistochemistry was performed with a Ventana Benchmark XT autostainer according to the manufacturer's instructions (Ventana Medical System, Tucson, AZ). The following primary antibodies were used: mouse anti-MCM7 (clone 141.2; dilution 1:100; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and anti-HER1 (clone H11, dilution 1:150, Dako, Milano, Italy) monoclonal antibodies; rabbit anti-geminin polyclonal antibody (1:200; Santa Cruz Biotechnology). Normal skin and lymph node tissues were used as positive controls for MCM7, HER1 and geminin antibodies. Negative controls were performed using non-immunized rabbit and mouse serum, omitting the primary antibodies.

Quantification of immunohistochemical staining.

Slides were observed with a Zeiss Axioskop 2 microscope and labeling counts for MCM7 and geminin antibodies were performed on a monitor, with the aid of a JVC color videocamera. Five randomly selected fields, each containing at least 400 tumor cells were counted independently by two pathologists and labeling index (LI) for each antibody was calculated as percentage of immunostained nuclei. The intensity of HER1 immunohistochemical staining was evaluated using image analysis based on Photoshop (AdobeSystems, San Jose, CA) together with 'The image-processing toolkit' (version 3.0, 1998, CRC Press, Boca Raton, FL) according to the method previously reported [27]. Briefly, Tumor tissue was manually selected in digitalized images and the integrated density (ID) of the immunostaining was calculated as the product of the mean density value of the immunoreactive regions by the percentage of the immunostained tumor tissue.

The computerised image analysis of all tissue sections were done by three pathologists without prior knowledge of the clinical and pathological parameters.

Statistical analysis

Survival data was available for all 61 patients. The Cox-Mantel method was used to evaluate the prognostic role of MCM7 and geminin as continous variables. For survival analysis, MCM7 and geminin continuous variables were converted to binomial variables of high versus low expression

around cut-off values chosen on the basis of the results of receiver-operator curve (ROC) analyses conducted on the distribution of the LI values of MCM7 (Area under curve = 0.68; CI 95% = 0.56-0.81; p = 0.02) and geminin (Area under curve = 0.67; 0.53-0.81; p = 0.02).. Tumors with MCM7 LI values >50% (43 out of 61) or geminin LI values > 15% (47 out of 61) were considered positive. All medians and life tables were computed using the product-limit estimate by Kaplan and Meier and and differences among the the Kaplan-Meier estimates were calculated using the log-rank test. Reported survival percentages of event-free patients at 5 year follow-up were based on the Kaplan-Meier estimator. Univariate and multivariate analyses were performed by the Cox proportional hazards model. In the the follow-up period (median: 32.1 months; range: 2-139; 95% CI: 25.7-52.9), the primary and secondary end points went from the beginning of therapy to clinical or pathological recurrence (progression-free survival: PFS) and to death (overall survival: OS), respectively. All p values were two-sided. Statistical analyses were done by JMP 11 software (SAS Institute Inc., Cary, NC).

RESULTS

All LSCC samples showed variable nuclear MCM7 and geminin immunolabeling. No substantial labeling for either protein was found in the normal peritumoral tissue, when present. The overall mean ± SE of MCM7 and geminin labeling indices (LI) were 68.6% ± 2.7 (median: 65%; range, 15-100) and 19.4% ± 1.2 (median: 15%; range, 5-60), respectively. No significant correlation between MCM7 and geminin LI was observed.

MCM7 and geminin LI were not associated with age, sex, tumor site, T, stage and nodal status of patients while the mean value of MCM7 LI was significantly higher in tumors expressing high levels of HER1 (Table 2).

During the follow-up period (median: 32.1 months; range: 2-139; 95% CI: 25.7-52.9) 30 out of 61 (49.2%) patients had tumor progression and 10 out of 61 (16.4%) died.

All the enrolled patients completed the treatment without interruptions.

MCM7 and geminin LI were first analyzed by Cox regression analysis as continuous variables. MCM7 LI values were directly associated with the risk of progression and death. The hazard risk ratios were 5.5 (95% C.I.= 1.3-26.4; p = 0.043) and 11.31 (95% C.I.= 0.9-232.2; p = 0.056) for progression and death, respectively. Geminin LI values were inversely associated only with the risk of progression. The hazard risk ratios were 53.7 (95% C.I.= 2.2-2042.8; p = 0.012). The plots of the estimates of survival as a function of MCM7 and geminin levels showed that the increase of MCM7 LI was associated with a reduction of the progression-free and overall survival fraction of patients at 5-year follow-up while the increase of geminin LI was associated with an increased number of progression-free patients at 5-year follow-up (Fig 1).

Kaplan-Meier analyses of survival curves according to MCM7 status showed a significant relationship between positive MCM7 LI and short progression-free (log-rank test: p = 0.0005; Fig. 2A) and overall (log-rank test: p = 0.045 : Fig. 2B) survival. The median PFS was 45 and 26 months for MCM7 LI <50% and MCM7 LI $\geq$ 50%, respectively. Kaplan-Meier analysis of survival

curves revealed that, at 5 year follow-up, the percentage of progression-free surviving patients with MCM7 negative tumors was 86.9% + 8.7 SE while that of progression-free surviving patients with MCM7 positive tumors was 24.7% + 10.4 SE. Relative to the overall survival, at 5 year follow-up, the percentage of patients still alive was 92.3% + 7.4 SE and 53.4% + 13.7 SE for subjects with MCM7 negative and positive tumors, respectively.

Kaplan-Meier analyses of survival curves according to geminin status showed a significant relationship between positive geminin LI and long progression-free survival (log-rank test: $p = 0.0001$; Fig. 2C), while no significant association was found between geminin LI and overall survival. The median PFS was 33 and 16 months for geminin LI $\geq$ 15% and geminin LI < 15%, respectively. Kaplan-Meier analyses of survival curves revealed that at 5 year follow-up the percentage of progression-free surviving patients with geminin positive tumors was 54.1% + 9.3 SE while that of progression-free surviving patients with geminin negative tumors was 0.

Considering that geminin expression might complement information obtained from MCM7 labeling, we further examined whether combining MCM7 and geminin status of tumor adds better understanding of the prognostic significance of these two proteins. Based on the combined expression of MCM7 and geminin, three subpopulations of patients were categorized : MCM7 $\leq$ 50% - geminin $\geq$ 15% (n = 17); MCM7 > 50% - geminin $\geq$ 15% (n = 31) and MCM7 > 50% - geminin < 15% (n = 13). Two illustrative cases are shown in Figure 3: one belonging to the subgroup with tumor status MCM7 $\leq$ 50% - geminin $\geq$ 15% (A, B) and the other to the subgroup with tumor status MCM7 > 50% - geminin < 15% (C, D).

Kaplan-Meier analyses of survival curves showed a significant relationship between MCM7-geminin status and progression-free survival (Log-Rank test: $p = 0.0001$; Fig. 4A) There was no significant association with overall survival. Kaplan-Meier analyses of survival curves revealed that at 5 year follow-up the percentage of progression-free survival according to MCM7-geminin status was: 69.5% $\pm$ 17.0 SE for MCM7 $\leq$ 50% - geminin $\geq$ 15%, 30.4% $\pm$ 12.8 SE for MCM7 > 50% -

geminin ≥ 15%, and 0 for MCM7 > 50% - geminin < 15%.

Cox's proportional hazard analysis revealed that, relative to patients with MCM7 ≤ 50% - geminin ≥ 15% tumor status, patients with MCM7 > 50% - geminin ≥ 15% and MCM7 > 50% - geminin < 15% tumor status had a risk of progression 3.1 times (95% C.I. = 1.3-7.2; Likelihood ratio tests: p = 0.014) and 17.7 times (95% C.I. = 4.7-88.6; Likelihood ratio tests: p = <0.0001) greater, respectively. This finding suggests that geminin co-expression can discriminate patients at highest risk of progression, among those with MCM7 positive tumors (>50%).

We then examined whether MCM7-geminin status retains a prognostic value for the duration of progression-free survival in the subpopulation of cetuximab treated patients with tumors expressing highest levels of HER1. The Survival curves showed a significant relationship between MCM7-geminin status and progression-free survival also in this patient sub-population (Fig. 4B). Kaplan-Meier analyses revealed that at 5 year follow-up the proportion of progression-free surviving patients according to MCM7-geminin status was: 60.0% ± 17 SE for MCM7 ≤ 50% - geminin ≥ 15%, 28.7% ± 12.3 SE for MCM7 > 50% - geminin ≥ 15%, and 0 for MCM7 > 50% - geminin < 15% (Log-Rank test: p = 0.0002).

Cox proportional hazard analysis revealed that, relative to patients with MCM7 ≤ 50% - geminin ≥ 15% tumor status, patients with MCM7 > 50% - geminin ≥ 15% and MCM7 > 50% - geminin < 15% tumor status had a risk of progression 3.5 times (95% C.I. = 1.3-8.3; Likelihood ratio tests: p = 0.011) and 17.1 times (95% C.I. = 3.8-124.6; Likelihood ratio tests: p = 0.0001) greater, respectively.

As revealed by univariate analysis cases with transglottic tumor site, high HER1, MCM7 tumor expression and low geminin LI were associated with an increased risk of progression while only high MCM7 expression was associated with increased risk of death (Table 3). In multivariate analysis, the tumor site, MCM7 and geminin LI of tumor retained an indipendent prognostic significance relative to the progression-free survival while the age of patient and the MCM7 LI each

behaved as an independent prognostic covariate relative to the overall survival (Table 4). Moreover, MCM7-geminin status of tumor, when considered, in multivariate analysis, instead of MCM7 and geminin covariates separately, showed an independent prognostic significance relative to PFS. Patients with MCM7 > 50% - geminin < 15% tumor status had a risk of progression 7.36 higher than those with MCM7 > 50% - geminin $\geq$ 15% tumor status (95% C.I. = 2.5-21.8; Likelihood ratio tests: p = <0.0004). Relative to the overall survival, MCM7-geminin status was not considered as geminin did not show a significant prognostic role.

DISCUSSION

MCM7 LI values were directly associated with the risk of progression and death in LSCC patients. These findings are consistent with previous studies that have indicated MCM7 as a sensitive prognostic marker of various human cancers, including oral squamous cell carcinoma, colorectal cancer, ovarian cancer, glioblastoma, and esophageal carcinoma [6]. Moreover, relative to normal epithelium, increased expression of MCM2 has been previously reported in LSCC [28].

MCM7 and geminin LI were not associated with any clinicopathologic parameter and did not correlate to each other. The mean value of MCM7 LI, but not geminin, was significantly higher in tumors expressing high levels of HER1. This finding could be explained by the recent observation that MCM7 binds splicing factor SF3B3 and is essential for splicing of epidermal growth factor receptor and other critical growth factor receptor RNAs [29]. Consequently, the MCM7 RNA splicing activity may be exerted through the expression of HER1 and other critical growth factor receptors. This suggests that MCM7 has a significant role in producing the pro-growth phenotype that is associated with MCM7 overexpression in LSCC. Moreover, it has been recently reported that HER1 phosphorylates the p56 isoform of Lyn, which, in turn, phosphorylates MCM7. Activated MCM7 then is recruited to the origin of replication complex and initiation of DNA replication ensues [26]. Therefore, MCM7 and HER1 could reinforce the activities of each other in supporting the progression of LSCC phenotype. This possibility can be further supported by the finding that the activity of Lyn in DNA synthesis and cell proliferation is potentiated in cancer cells whose survival depends on EGFR signaling. Furthermore, in this subset of cancer cells, but not in cancer cells less growth-dependent on HER1 signaling, a synergistic efficacy of Lyn targeting with EGFR TKIs treatment has been documented [26]. In view of these observations, the finding that MCM7 over-expression is associated with an adverse clinical outcome in patients with HER1 expressing LSCC, provides clinical implications for the treatment of LSCC with deregulated MCM7 status, using EGFR and Lyn kinase inhibitors.

Geminin LI behaves as a significant predictor of better prognosis in LSCC patients relative to the progression-free survival. Our data are in agreement with previous reports in patients with oral squamous cell carcinoma [30] and high-grade astrocytic brain tumors [31] where high geminin expression is a significant predictor of better prognosis. However, in patients with breast [32], renal [33] and colorectal cancer [34] geminin over-expression has been associated with poor prognosis and the frequent overexpression in tumor cells together with the ability to stimulate cell cycle progression and proliferation have supported its role of a classical oncogene.

Our results in LSCC patients are inconsistent with the oncogenic role attributed to geminin. However, the favorable prognostic role of geminin can be explained considering that the elevated levels of geminin could indicate the chemo-radio sensitivity of LSCC.

This possibility was supported by the observation that high levels of geminin expression promote G1 to S progression and accumulation of cancer cells in the S-G2-M phase of the cell cycle [35] where they are most chemo-radio sensitive. On the other hand, a significant reduction of geminin expression was observed after chemo-radio treatment in primary rectal cancers containing high levels of geminin [35], further suggesting that cells expressing high levels of geminin might be more susceptible to chemo–radio treatment. Then, evaluation of geminin expression might serve as a prognostic marker of response to chemo–radio therapy in LSCC patients. This possibility is also in agreement with what was observed in patients with oral squamous cell carcinoma [30] and high-grade astrocytic brain tumors [31] who have undergone chemo-radio treatment.

Geminin expression, as a prognostic marker of response to chemo–radio therapy, complements the prognostic informations obtained from MCM7 expression in tumor cells. In fact, patients with LSCC containing high levels of MCM7 had a significantly reduced risk of progression when their cancer cells also expressed high levels of geminin. Furthermore, the MCM7-geminin status of the tumor behaves as a prognostic marker independent from the expression levels of HER1 and, then, it seems to improve the prognostic role of the HER1 expression, identifying a subgroup of patients

with different local response to radiotherapy and biotherapy. All the patients in the present study were treated by the combination of cetuximab with radiotherapy, for whom HER1 expression does not seem to act as a reliable predictor of the response, and does not help in treatment selection. MCM7-geminin status, which is a downstream marker of EGFR pathway, may on the other hand reveal very useful for this aim and be employed in the clinical practice for the selection of patients to submit to concurrent treatment with c225 or other anti-EGFR drugs instead of the other standard drug for concomitant RTCT in the head and neck which is cisplatin.

In conclusion, to the best our knowledge, this is the first study demonstrating that MCM7-geminin status is a reliable independent prognostic marker of the clinical outcome in LSCC patients. A well-designed, prospective, randomized multi-institutional study with a higher number of patients and a longer follow-up is needed to confirm our preliminary results. In fact, if the MCM7–geminin prognostic role was validated on independent cohorts, their gene expression patterns may provide valuable information that can be used to assist in treatment decisions for HNSCC.

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FIGURE LEGENDS

Figure 1. Plots of the estimates of progression-free and overall survival as a function of MCM7 LI (A) and geminin LI (B) values. The proportional hazards model was evaluated at each covariate value and the proportion of patients without event at 5-year follow-up was estimated from the computed survival functions.

Figure 2. Kaplan-Meier analysis of survival curves as a function of MCM7 (A, B) and geminin (C) status of tumor in laryngeal squamous carcinoma patients. Cut-off values of the variables were chosen according to the results of ROC curve analyses. The median progression-free survival were 26 and 45 months for MCM7 LI > 50% and < 50%, respectively and 33 and 16 months for geminin LI > 15% and < 15%, respectively. The median overall survival for MCM7 LI > 50% was 27 months while that for MCM7 LI < 50% was undefinied.

Figure 3. Immunohistochemical analysis of two illustrative cases of laryngeal squamous carcinoma: one expressing low MCM7 (A) and high geminin (B) LI and the other showing high MCM7 (C) and low geminin (D) LI. Bar = 50x.

Figure 4. Kaplan-Meier analysis of progression-free survival curves as a function of MCM7-geminin status of tumor in the overall patient population (A) and in the subpopulation of patients with tumors expressing high levels of HER1 (B).

Table 1. Descriptive statistics of the main variables concerning patients and tumour parameters.

Characteristic	**61 patients**
Age at diagnosis	
Median	65
Range	40-86
Sex-no. (%)	
Male	55 (90.2%)
Female	6 (9.8%)
Smoking Status	
Smokers	22 (36,1%)
Non Smokers	29 (47.5%)
Unknown status	10 (16.4%)
Drinking Status	
Drinkers	30 (49.2%)
Non Drinkers	24 (39.3%)
Previous Drinkers	7 (11.5%)
Follow-up period in months	
Median	32.1 months
Range	2-139 months
Subsite	
Supraglottic	25 (41%)
Glottic	20 (32,8%)
Transglottic	16 (26.2%)
T	
T2	33 (54%)
T3-4	28 (46%)
N	
Negative	44 (72.1%)

Positive	17 (27.9%)
Stage	
1-2	26 (42.6%)
3-4	35 (57.4%)

Table 2. MCM7 and Geminin labeling indices according to clinico-pathological characteristics of 61 laryngeal squamous carcinoma patients.

		MCM7	p[1]		GEMININ	p
AGE	N	(mean ± SE)		N	(mean ± SE)	
< 60	17	63.9[2] ± 5.5		17	18.5 ± 2.3	
≥ 60	44	67.5 ± 3.3	0.64	44	19.9 ± 1.4	0.57
SEX						
female	6	59.3 ± 10.2		6	18.3 ± 3.8	
male	55	67.6 ± 2.9	0.29	55	19.6 ± 1.3	0.86
TUMOR SITE						
supraglottic	25	67.5 ± 4.8		25	19.5 ± 1.8	
glottic	20	63.0 ± 4.7		20	19.2 ± 1.5	
transglottic	16	70.3 ± 4.9	0.45	16	19.6 ± 3.1	0.74
T						
2	33	68.9 ± 3.5		33	18.8 ± 1.8	
3-4	28	64.2 ± 4.5	0.28	28	19.6 ± 1.5	0.16
STAGE						
1-2	26	67.0 ± 3.9		26	18.6 ± 2.2	
3-4	35	66.3 ± 4.0	0.86	35	19.6 ± 1.3	0.15
NODAL STATUS						
negative	44	65.0 ± 3.3		44	18.5 ± 1.5	
positive	17	71.1 ± 5.5	0.21	17	20.9 ± 1.9	0.20
HER1						
≤ 14 I.D.[3]	11	48.7 ± 6.2		11	21.3 ± 2.7	
> 14 I.D.	50	71.2 ± 2.9	0.003	50	18.8 ± 1.3	0.38

[1)] Wilcoxon test; [2)]Labeling index; [3)]Integrated density.

Table 3. Univariate analysis of prognostic covariates in laryngeal squamous cancer patients.

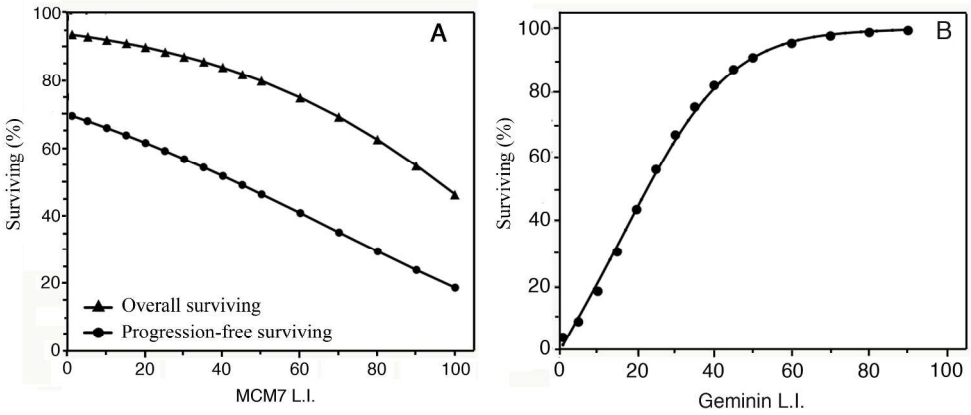
	RELAPSE-FREE SURVIVAL			OVERALL SURVIVAL		
	N	R.R. (C.I. 95%)	p[1]	N	R.R. (C.I. 95%)	p
AGE						
≥ 60	44	1[2]		44	1	
< 60	17	1.15 (0.5-2.6)	0.74	17	4.10 (1.2-16.1)	0.028
SEX						
female	6	1		6	1	
male	55	4.15 (0.9-74.1)	0.08	55	1.15 (0.2-21.3)	0.89
T classification						
2	32	1		32	1	
3-4	29	0.98 (0.5-2.1)	0.96	29	1.03 (0.3-4.2)	0.97
STAGE						
1-2	26	1		26	1	
3-4	35	0.74 (0.6-2.8)	0.43	35	1.22 (0.3-5.8)	0.77
TUMOR SITE						
supraglottic / glottic	45	1		45	1	
transglottic	16	3.72 (1.6-8.3)	0.0023	16	2.48 (0.5-9.3)	0.23
NODAL STATUS						
negative	44	1		44	1	
positive	17	0.87 (0.3-1.9)	0.75	17	2.81 (0.7-10.7)	0.14
HER1						
≤ 14 I.D.	11	1		11	1	
> 14 I.D.	50	4.67 (1.4-29.0)	0.009	50	2.70 (0.5-49.5)	0.29
MCM7						
≤ 50%	18	1		18	1	
> 50%	43	5.15 (1.9-18.2)	0.0007	43	7.01 (1.3-130.1)	0.021
GEMININ						
≥ 15%	30	1		30	1	
< 15%	31	4.60 (1.9-10.6)	0.0012	31	1.23 (0.1-15.7)	0.85

[1)] Likelihood ratio tests; [2)]Reference risk.

Table 4. Mulivariate analysis of prognostic covariates in laryngeal squamous cancer patients.

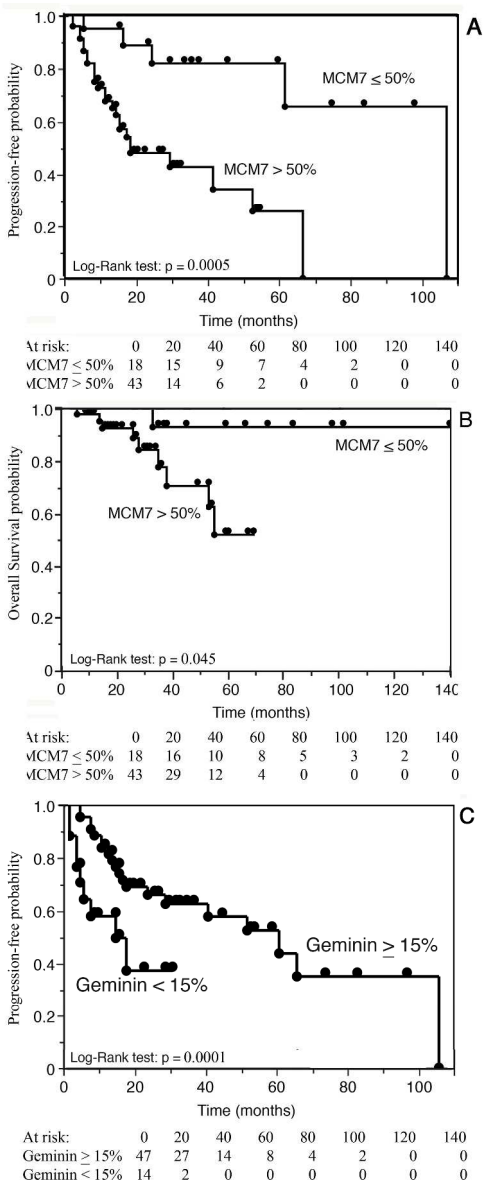
	RELAPSE-FREE SURVIVAL			OVERALL SURVIVAL		
	N	R.R. (C.I. 95%)	p[1]	N	R.R. (C.I. 95%)	p
AGE						
≥ 60	44	1[2]		44	1	
< 60	17	1.47 (0.5-3.9)	0.45	17	57.37 (4.0-1927.5)	0.001
SEX						
female	6	1		6	1	
male	55	2.02 (0.3-38.6)	0.48	55	0.10 (0.02-4.2)	0.20
T classification						
2	32	1		32	1	
3-4	29	2.95 (0.5-11.7)	0.29	29	1.34 (0.06-17.3)	0.82
STAGE						
1-2	26	1		26	1	
3-4	35	0.33 (0.5-2.0)	0.23	35	0.47 (0.01-13.5)	0.67
TUMOR SITE						
supraglottic / glottic	45	1		45	1	
transglottic	16	2.96 (1.1-7.8)	0.028	16	11.42 (0.8-219.8)	0.07
NODAL STATUS						
negative	44	1		44	1	
positive	17	1.34 (0.4-4.6)	0.65	17	2.29 (0.2-28.6)	0.51
HER1						
≤ 14 I.D.	11	1		11	1	
> 14 I.D.	50	1.78 (0.6-6.3)	0.31	50	1.34 (0.05-72.5)	0.86
MCM7						
≤ 50%	18	1		18	1	
> 50%	43	3.90 (1.2-16.3)	0.02	43	6.17 (0.9-67.8)	0.04
GEMININ						
≥ 15%	30	1		30	1	
< 15%	31	7.36 (2.5-21.8)	0.004	31	4.44 (0.1-224.7)	0.40

[1)] Likelihood ratio tests; [2)]Reference risk.



Plots of the estimates of progression-free and overall survival as a function of MCM7 LI (A) and geminin LI (B) values. The proportional hazards model was evaluated at each covariate value and the proportion of patients without event at 5-year follow-up was estimated from the computed survival functions.

1188x517mm (72 x 72 DPI)



Kaplan-Meier analysis of survival curves as a function of MCM7 (A, B) and geminin (C) status of tumor in laryngeal squamous carcinoma patients. Cut-off values of the variables were chosen according to the results of ROC curve analyses. The median progression-free survival were 26 and 45 months for MCM7 LI > 50% and < 50%, respectively and 33 and 16 months for geminin LI > 15% and < 15%, respectively. The median overall survival for MCM7 LI > 50% was 27 months while that for MCM7 LI < 50% was undefinied.

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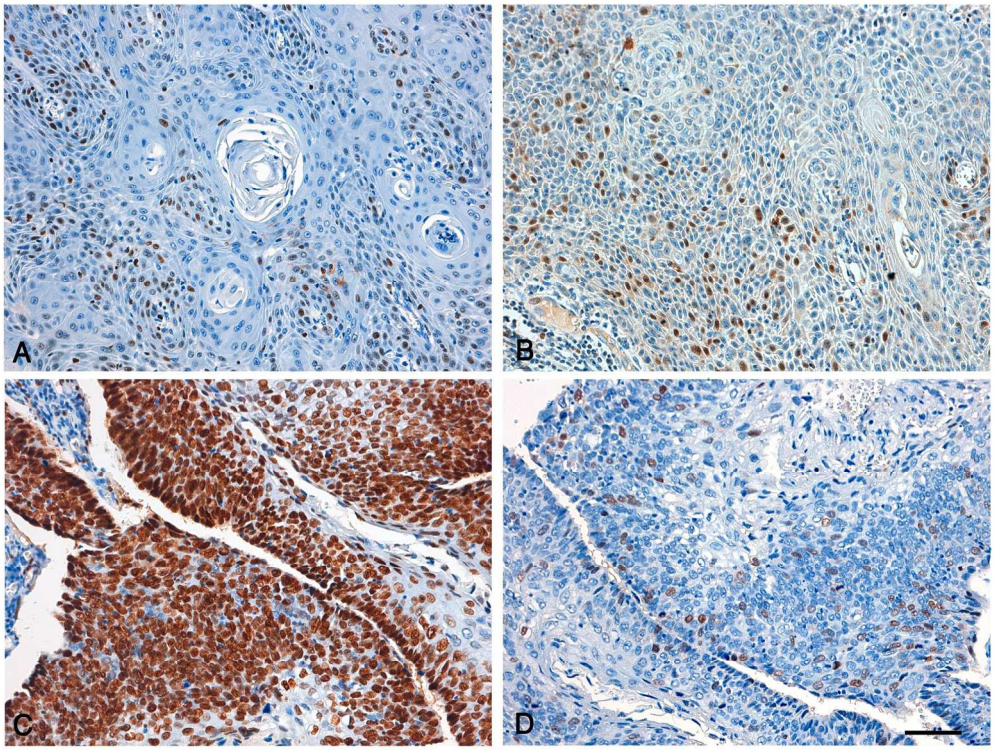

Immunohistochemical analysis of two illustrative cases of laryngeal squamous carcinoma: one expressing low MCM7 (A) and high geminin (B) LI and the other showing high MCM7 (C) and low geminin (D) LI. Bar = 50x.

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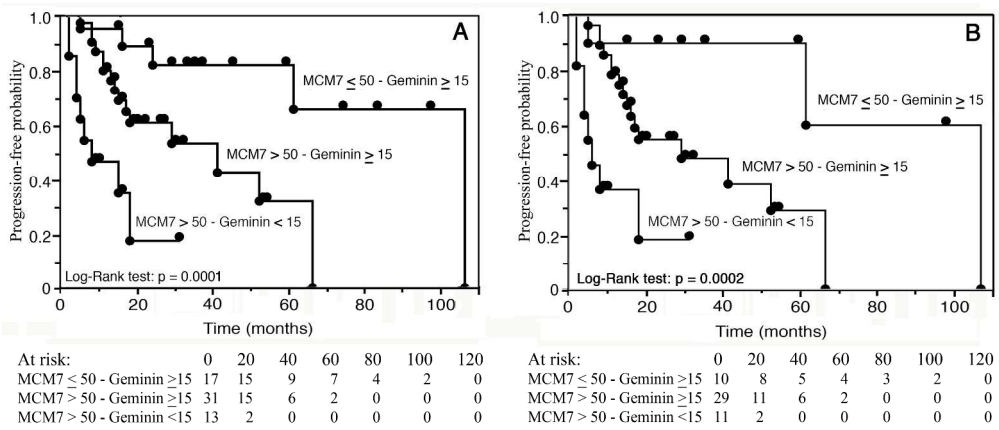

At risk:	0	20	40	60	80	100	120
MCM7 ≤ 50 - Geminin ≥15	17	15	9	7	4	2	0
MCM7 > 50 - Geminin ≥15	31	15	6	2	0	0	0
MCM7 > 50 - Geminin <15	13	2	0	0	0	0	0

At risk:	0	20	40	60	80	100	120
MCM7 ≤ 50 - Geminin ≥15	10	8	5	4	3	2	0
MCM7 > 50 - Geminin ≥15	29	11	6	2	0	0	0
MCM7 > 50 - Geminin <15	11	2	0	0	0	0	0

Kaplan-Meier analysis of progression-free survival curves as a function of MCM7- geminin status of tumor in the overall patient population (A) and in the subpopulation of patients with tumors expressing high levels of HER1 (B).

1449x630mm (72 x 72 DPI)