Minichromosome maintenance protein 7 and geminin expression: Prognostic value in laryngeal squamous cell carcinoma in patients treated with radiotherapy and cetuximab

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ABSTRACT: *Background.* Minichromosome maintenance protein 7 (MCM7) is a downstream of human epidermal growth receptor (HER1) signaling. We examined MCM7, geminin, and HER1 expression in patients with laryngeal squamous cell carcinoma (SCC) treated with radiotherapy and cetuximab.

Methods. MCM7, geminin, and HER1 were evaluated by immunohistochemistry on 61 patients with laryngeal SCC. The follow-up (median, 32.1 months; range, 2–139 months) went from the beginning of therapy to tumor progression-free survival (PFS) and death (overall survival [OS]).

Results. MCM7, but not geminin, was associated only with HER1 expression, whereas no association was found 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, whereas MCM7 was an independent prognostic marker of OS.

Conclusion. MCM7-geminin tumor status may be prognostic for patients with laryngeal SCC treated with cetuximab and radiotherapy. © 2016 Wiley Periodicals, Inc. *Head Neck* **39:** 684–693, 2017

KEY WORDS: laryngeal squamous cell carcinoma, geminin, mini-chromosome maintenance protein 7 (MCM7), cetuximab, radiotherapy

INTRODUCTION

Minichromosome maintenance proteins (MCMs) belong to a family of 6 highly conserved and highly homologous proteins (MCM2–MCM7). MCM proteins 2 to 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]

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MCM proteins represent a reliable marker of cell cycle entry as their expression has been demonstrated in cells remaining in the cell cycle, whereas 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 has 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 clinicopathological 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 (SCC), 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 nonquiescent 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 a biomarker in laryngeal cancer still needs to be clarified.

Human epidermal growth factor receptor 1 (HER1) seems, at present, to be the most reliable molecular marker in head and neck SCC, as it is expressed in over 90% of head and neck squamous cell carcinomas (HNSCCs). HER1 overexpression in laryngeal SCC has been shown to correlate with worse clinical outcome,[11–16] decreased response to radiotherapy, and increased locoregional recurrence after definitive radiotherapy.[17]

Cetuximab is an immunoglobulin G1 monoclonal antibody that exclusively targets epidermal growth factor receptor (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 radiotherapy in locally advanced patients with HNSCC, 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 no 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] Because 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 laryngeal SCC 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 SCCs to reduce the biological and clinical heterogeneity associated with different sites of origin in the head and neck.

MATERIALS AND METHODS

Patients

Study design. From 1998 to 2012, 61 consecutive patients with untreated, newly diagnosed laryngeal SCC (age: mean, 65.4 years; median, 65 years; range, 40–86 years) suitable for an organ preservation protocol according to international guidelines (National Comprehensive Cancer Network version 2.2014), or, in case of T4 classification of disease, any N, refused other therapeutic options, and 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 laryngeal SCC, 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 head and neck 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 workup, including a complete head and neck examination, mirror, fiberoptic, and narrow band imaging-videoendoscopic examination, representative biopsy, chest CT, CT and MRI of the larynx and neck, and positron emission tomography/CT for advanced disease, as suggested by the National Comprehensive Cancer Network guidelines version 2.2014. After the workup, all cases were staged and discussed by the tumor board involving at least a medical oncologist, a radiation therapist, and a Head and Neck surgeon. Smoking and drinking status were assessed, 23 patients were ex-smokers (37.7%), 6 patients (9.8%) were never-smokers, 22 patients (36%) were smokers with a mean of 46 years of tobacco use, and, in 10 patients (16.4%), the tobacco use was not known. Current and previous smokers had a mean pack-year index of 61.4 (range, 6–250 pack-years). Thirty patients (49.2%) were current drinkers and 7 patients (11.5%) were previous drinkers. Fourteen patients (23%) 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 radiotherapy. We delivered 69.96 Gy at 2.12 Gy per fraction to the planning target volume (PTV) encompassing the gross tumor volume, 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 gross tumor volumes 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 intensity-modulated radiotherapy 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, narrow band imaging-videolaryngoscopy, imaging studies, and, if there was a high suspicion of residual laryngeal disease,

TABLE 1. Descriptive statistics of the main variables concerning patients and tumor parameters.

Characteristics	No. of patients (n = 61)
Age at diagnosis, y	
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	
Median	32.1 mo
Range	2–139 mo
Subsite (%)	
Supraglottic	25 (41)
Glottic	20 (32.8)
Transglottic	16 (26.2)
T classification (%)	
T2	33 (54)
T3–T4	28 (46)
N classification (%)	
Negative	44 (72.1)
Positive	17 (27.9)
Stage (%)	
1–2	26 (42.6)
3–4	35 (57.4)

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 minutes 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, in accord with 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, 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 nonimmunized 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 video camera. Five randomly selected fields, each containing at least 400 tumor cells, were counted independently by 2 pathologists and labeling index 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 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 computerized image analysis of all tissue sections were done by 3 pathologists without prior knowledge of the clinical and pathological parameters.

Statistical analysis

Survival data were available for all 61 patients. The Cox–Mantel method was used to evaluate the prognostic role of MCM7 and geminin as continuous variables. For survival analysis, MCM7 and geminin continuous variables were converted to binomial variables of high versus low expression around cutoff values chosen on the basis of the results of receiver-operator characteristic analyses conducted on the distribution of the labeling index values of MCM7 (area under the curve, 0.68; 95% confidence interval [CI], 0.56–0.81; $p = .02$) and geminin (area under the curve, 0.67; 95% CI, 0.53–0.81; $p = .02$). Tumors with MCM7 labeling index values >50% (43 of 61) or geminin labeling index values >15% (47 of 61) were considered positive. All medians and life tables were computed using the product-limit estimate by Kaplan–Meier and differences among 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 follow-up period (median, 32.1 months; range, 2–139 months; 95% CI, 25.7–52.9), the primary and secondary endpoints 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 2-sided. Statistical analyses were done by JMP 11 software (SAS Institute, Cary, NC).

RESULTS

All laryngeal SCC samples showed variable nuclear MCM7 and geminin immunolabeling. No substantial

TABLE 2. Minichromosome maintenance protein 7 and geminin labeling indices according to clinicopathological characteristics of 61 patients with laryngeal squamous cell carcinoma.

	MCM7			Geminin		
	No. of patients	(mean ± SE)	*p* value*	No. of patients	(mean ± SE)	*p* value
Age, y						
<60	17	63.9 ± 5.5†		17	18.5 ± 2.3	
≥60	44	67.5 ± 3.3	.64	44	19.9 ± 1.4	.57
Sex						
Female	6	59.3 ± 10.2		6	18.3 ± 3.8	
Male	55	67.6 ± 2.9	.29	55	19.6 ± 1.3	.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	.45	16	19.6 ± 3.1	.74
T classification						
2	33	68.9 ± 3.5		33	18.8 ± 1.8	
3–4	28	64.2 ± 4.5	.28	28	19.6 ± 1.5	.16
Stage						
1–2	26	67.0 ± 3.9		26	18.6 ± 2.2	
3–4	35	66.3 ± 4.0	.86	35	19.6 ± 1.3	.15
Nodal status						
Negative	44	65.0 ± 3.3		44	18.5 ± 1.5	
Positive	17	71.1 ± 5.5	.21	17	20.9 ± 1.9	.20
HER1						
≤14 ID	11	48.7 ± 6.2		11	21.3 ± 2.7	
>14 ID	50	71.2 ± 2.9	.003	50	18.8 ± 1.3	.38

Abbreviations: MCM7, minichromosome maintenance protein 7; HER1, human epidermal growth receptor 1; ID, integrated density.
* Wilcoxon test.
† Labeling index.

labeling for either protein was found in the normal peritumoral tissue, when present. The overall mean ± SE of MCM7 and geminin labeling indices 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 labeling index was observed.

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

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

All the enrolled patients completed the treatment without interruptions.

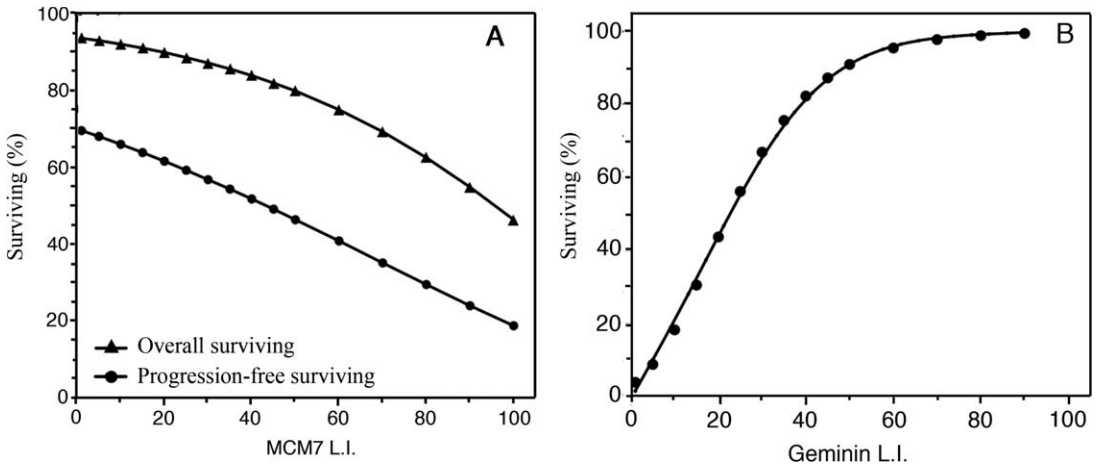

FIGURE 1. Plots of the estimates of progression-free and overall survival as a function of minichromosome maintenance protein 7 (MCM7) labeling index (L.I.) (A) and geminin labeling index (B) values. The proportional hazards model was evaluated at each covariate value and the proportion of patients without event at 5-year follow-up was estimated from the computed survival functions.

MCM7 and geminin labeling index were first analyzed by Cox regression analysis as continuous variables. MCM7 labeling index values were directly associated with the risk of progression and death. The hazard risk ratios were 5.5 (95% CI, 1.3–26.4; $p = .043$) and 11.31 (95% CI, 0.9–232.2; $p = .056$) for progression and death, respectively. Geminin labeling index values were inversely associated only with the risk of progression. The hazard risk ratios were 53.7 (95% CI, 2.2–2042.8; $p = .012$). The plots of the estimates of survival as a function of MCM7 and geminin levels showed that the increase of MCM7 labeling index was associated with a reduction of the PFS and OS fraction of patients at 5-year follow-up, whereas the increase of geminin labeling index was associated with an increased number of patients with PFS at 5-year follow-up (see Figure 1).

Kaplan–Meier analyses of survival curves, according to MCM7 status, showed a significant relationship between positive MCM7 labeling index and short PFS (log-rank test, $p = .0005$; Figure 2A) and OS (log-rank test, $p = .045$; Figure 2B). The median PFS was 106 and 18 months for MCM7 labeling index <50% and MCM7 labeling index ≥50%, respectively. Kaplan–Meier analysis of survival curves revealed that, at 5-year follow-up, the percentage of patients with PFS with MCM7-negative tumors was 86.9% + 8.7 SE, whereas that of patients with PFS with MCM7-positive tumors was 24.7% + 10.4 SE. Relative to the OS, 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 MCM7-positive tumors, respectively.

Kaplan–Meier analyses of survival curves according to geminin status showed a significant relationship between positive geminin labeling index and long PFS (log-rank test, $p = .0001$; Figure 2C), whereas no significant association was found between geminin labeling index and OS. The median PFS was 61 and 16 months for geminin labeling index ≥15% and geminin labeling index <15%, respectively. Kaplan–Meier analyses of survival curves revealed that at 5-year follow-up the percentage of patients with PFS with geminin-positive tumors was 54.1% + 9.3 SE, whereas that of patients with PFS 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 the tumor adds to better understanding of the prognostic significance of these 2 proteins. Based on the combined expression of MCM7 and geminin, 3 subpopulations of patients were categorized: MCM7 ≤50% and geminin ≥15% ($n = 17$); MCM7 >50% and geminin ≥15% ($n = 31$); and MCM7 >50% and geminin <15% ($n = 13$). Two illustrative cases are shown in Figure 3: one belonging to the subgroup with tumor status MCM7 ≤50% and geminin ≥15% (Figure 3A and 3B) and the other to the subgroup with tumor status MCM7 >50% and geminin <15% (Figure 3C and 3D).

Kaplan–Meier analyses of survival curves showed a significant relationship between MCM7-geminin status and PFS (log-rank test, $p = .0001$; Figure 4A). There was no significant association with OS. Kaplan–Meier analyses of survival curves revealed that at 5-year follow-up the percentage of PFS according to MCM7-geminin status was: 82.5% ± 17.0 SE for MCM7 ≤50% and geminin ≥15%; 30.4% ± 12.8 SE for MCM7 >50% and geminin ≥15%; and 0 for MCM7 >50% and geminin <15%.

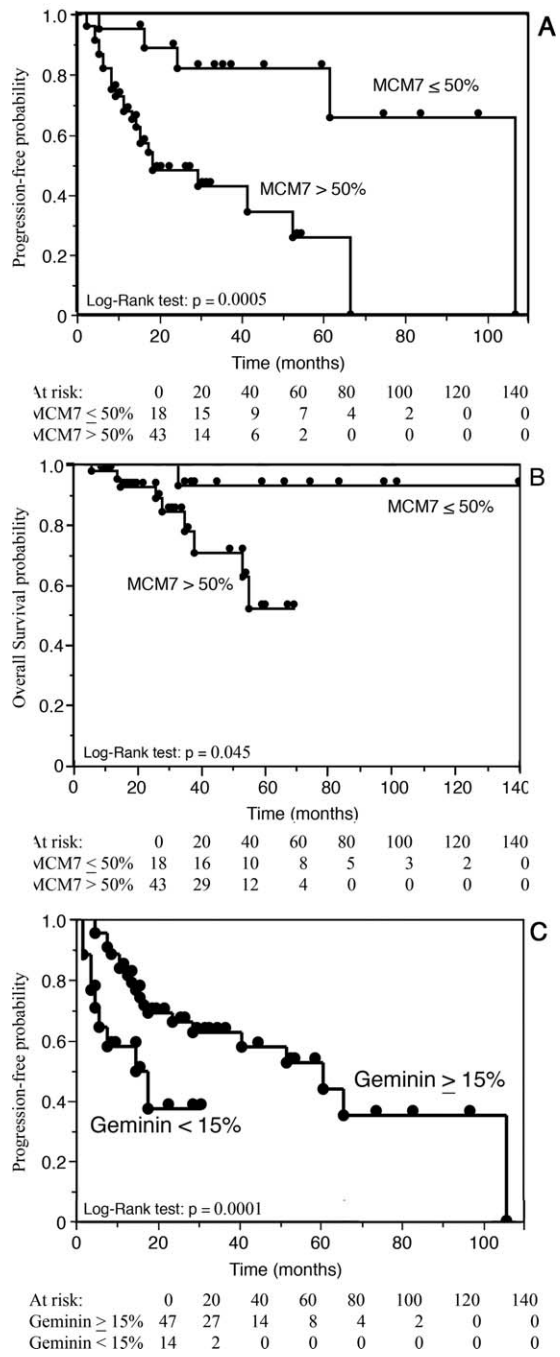

FIGURE 2. Kaplan–Meier analysis of survival curves as a function of minichromosome maintenance protein 7 (MCM7) (A, B) and geminin (C) status of tumor in patients with laryngeal squamous carcinoma. Cutoff values of the variables were chosen according to the results of receiver operating characteristic curve analyses. The median progression-free survival were 18 and 106 months for MCM7 labeling index >50% and ≤50%, respectively, and 61 and 16 months for geminin labeling index ≥15% and <15%, respectively. The median overall survival for MCM7 labeling index was undefined.

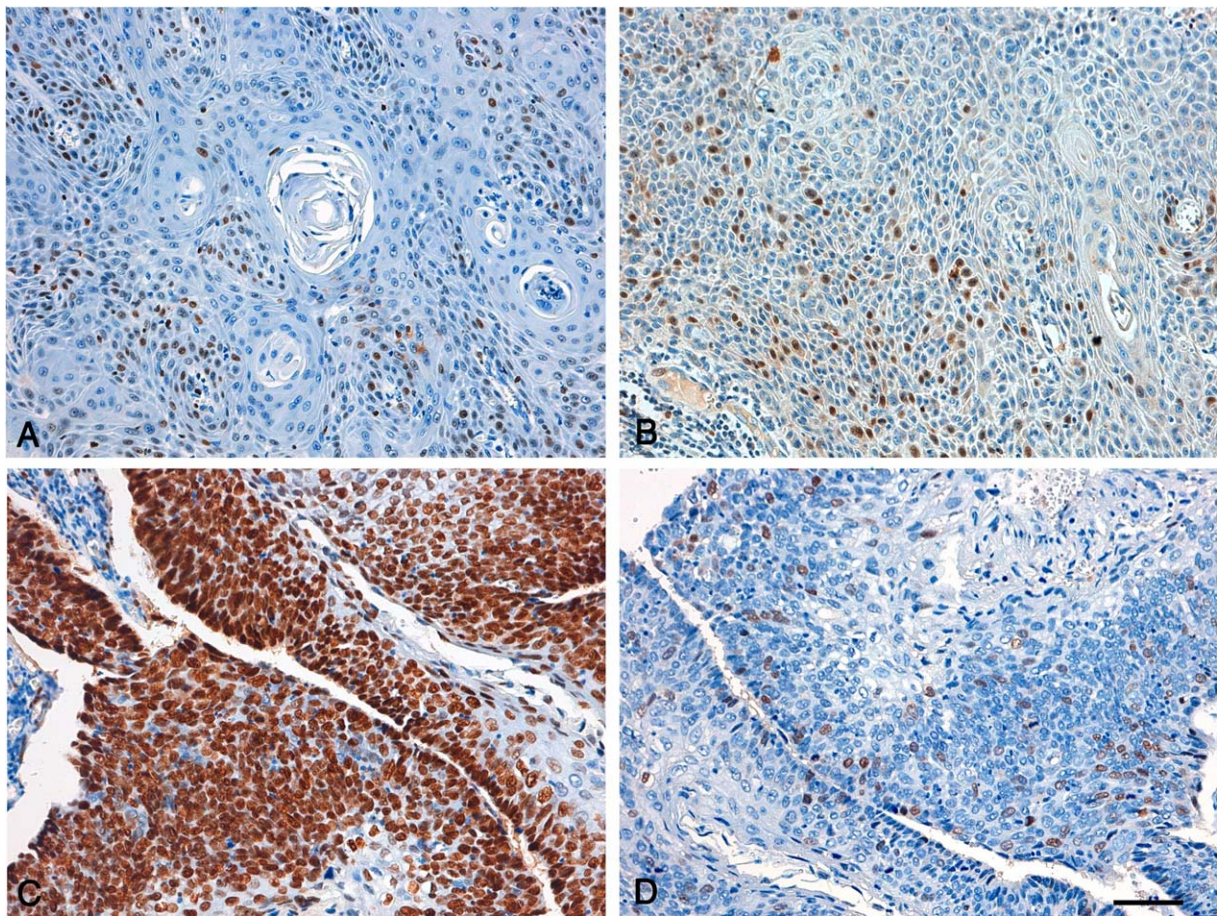

FIGURE 3. Immunohistochemical analysis of 2 illustrative cases of laryngeal squamous carcinoma: one expressing low minichromosome maintenance protein 7 (MCM7) (A) and high geminin (B) labeling index, and the other showing high MCM7 (C) and low geminin (D) labeling index. (Bar original magnification ×50).

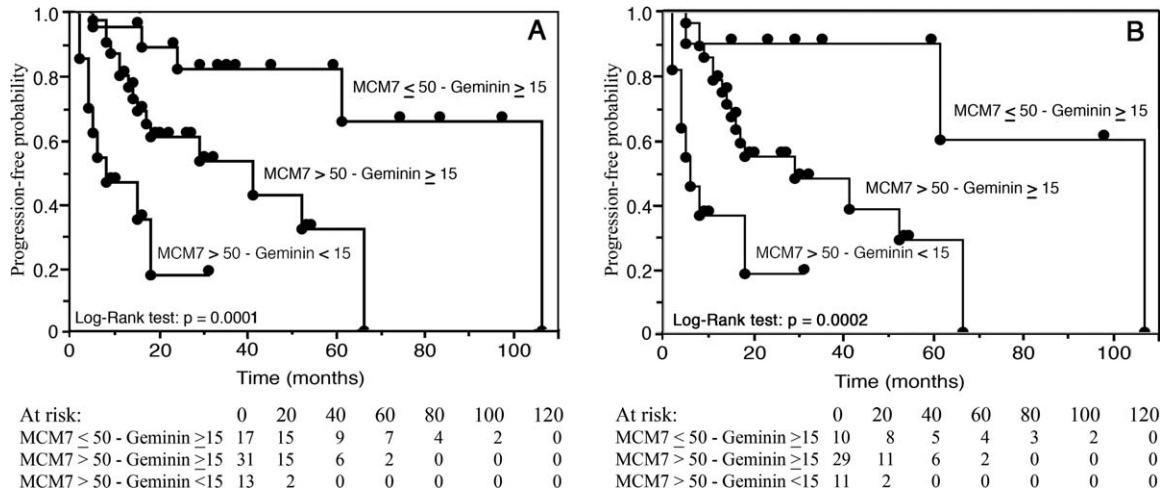

FIGURE 4. Kaplan–Meier analysis of progression-free survival curves as a function of minichromosome maintenance protein 7 (MCM7)-geminin status of tumors in the overall patient population (A) and in the subpopulation of patients with tumors expressing high levels of human epidermal growth factor receptor 1 (B).

TABLE 3. Univariate analysis of prognostic covariates in patients with laryngeal squamous cell carcinoma.

	Relapse-free survival			OS		
	No. of patients	RR (95% CI)	*p* value*	No. of patients	RR (95% CI)	*p* value
Age, y						
≥60	44	1†		44	1	
<60	17	1.15 (0.5–2.6)	.74	17	4.10 (1.2–16.1)	.028
Sex						
Female	6	1		6	1	
Male	55	4.15 (0.9–74.1)	.08	55	1.15 (0.2–21.3)	.89
T classification						
2	32	1		32	1	
3–4	29	0.98 (0.5–2.1)	.96	29	1.03 (0.3–4.2)	.97
Stage						
1–2	26	1		26	1	
3–4	35	0.74 (0.6–2.8)	.43	35	1.22 (0.3–5.8)	.77
Tumor site						
Supraglottic/glottic	45	1		45	1	
Transglottic	16	3.72 (1.6–8.3)	.0023	16	2.48 (0.5–9.3)	.23
Nodal status						
Negative	44	1		44	1	
Positive	17	0.87 (0.3–1.9)	.75	17	2.81 (0.7–10.7)	.14
HER1						
≤14 ID	11	1		11	1	
>14 ID	50	4.67 (1.4–29.0)	.009	50	2.70 (0.5–49.5)	.29
MCM7						
≤50%	18	1		18	1	
>50%	43	5.15 (1.9–18.2)	.0007	43	7.01 (1.3–130.1)	.021
Geminin						
≥15%	30	1		30	1	
<15%	31	4.60 (1.9–10.6)	.0012	31	1.23 (0.1–15.7)	.85

Abbreviations: OS, overall survival; RR, risk ratio; CI, confidence interval; HER1, human epidermal growth receptor 1; MCM7, minichromosome maintenance protein 7.
* Likelihood ratio tests.
† Reference risk.

The Cox proportional hazard analysis revealed that, relative to patients with MCM7 ≤50% and geminin ≥15% tumor status, patients with MCM7 >50% and geminin ≥15% and MCM7 >50% and geminin <15% tumor status had a risk of progression 3.1 times (95% CI, 1.3–7.2; likelihood ratio tests, $p = .014$) and 17.7 times greater (95% CI, 4.7–88.6; likelihood ratio tests, $p < .0001$), respectively. This finding suggests that geminin coexpression 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 PFS 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 PFS also in this patient subpopulation (Figure 4B). Kaplan–Meier analyses revealed that at 5-year follow-up the proportion of patients with PFS according to MCM7-geminin status was: 91% ± 8 SE for MCM7 ≤50% and geminin ≥15%; 28.7% ± 12.3 SE for MCM7 >50% and geminin ≥15%; and 0 for MCM7 >50% and geminin <15% (log-rank test, $p = .0002$).

The Cox proportional hazard analysis revealed that, relative to patients with MCM7 ≤50% and geminin ≥15% tumor status, patients with MCM7 >50% and geminin ≥15% and MCM7 >50% and geminin <15% tumor status had a risk of progression 3.5 times (95% CI, 1.3–8.3; likelihood ratio tests, $p = .011$) and 17.1 times (95% CI, 3.8–124.6; likelihood ratio tests, $p = .0001$) greater, respectively.

As revealed by univariate analysis cases with transglottic tumor site, high HER1, MCM7 tumor expression, and low geminin labeling index were associated with an increased risk of progression, whereas only high MCM7 expression was associated with increased risk of death (Table 3). In multivariate analysis, the tumor site, MCM7, and geminin labeling index of tumor retained an independent prognostic significance relative to the PFS, whereas the age of the patient and the MCM7 labeling index each behaved as an independent prognostic covariate relative to the OS (Table 4). Moreover, MCM7-geminin status of tumors, when considered in multivariate analysis instead of MCM7 and geminin covariates separately, showed an independent prognostic significance relative to PFS. Patients with MCM7 >50% and geminin <15% tumor status had a risk of progression 7.36 higher than those with MCM7 >50% and geminin ≥15% tumor status (95% CI, 2.5–21.8; likelihood ratio tests, $p < .0004$). Relative to the OS, MCM7-geminin status was not considered, as geminin did not show a significant prognostic role.

DISCUSSION

MCM7 labeling index values were directly associated with the risk of progression and death in patients with

TABLE 4. Multivariate analysis of prognostic covariates in patients with laryngeal squamous cell carcinoma.

	Relapse-free survival			OS		
	No. of patients	RR (95% CI)	*p* value*	No. of patients	RR (95% CI)	*p* value
Age, y						
≥60	44	1†		44	1	
<60	17	1.47 (0.5–3.9)	.45	17	57.37 (4.0–1927.5)	.001
Sex						
Female	6	1		6	1	
Male	55	2.02 (0.3–38.6)	.48	55	0.10 (0.02–4.2)	.20
T classification						
2	32	1		32	1	
3–4	29	2.95 (0.5–11.7)	.29	29	1.34 (0.06–17.3)	.82
Stage						
1–2	26	1		26	1	
3–4	35	0.33 (0.5–2.0)	.23	35	0.47 (0.01–13.5)	.67
Tumor site						
Supraglottic/glottic	45	1		45	1	
Transglottic	16	2.96 (1.1–7.8)	.028	16	11.42 (0.8–219.8)	.07
Nodal status						
Negative	44	1		44	1	
Positive	17	1.34 (0.4–4.6)	.65	17	2.29 (0.2–28.6)	.51
HER1						
≤14 ID	11	1		11	1	
>14 ID	50	1.78 (0.6–6.3)	.31	50	1.34 (0.05–72.5)	.86
MCM7						
≤50%	18	1		18	1	
>50%	43	3.90 (1.2–16.3)	.02	43	6.17 (0.9–67.8)	.04
Geminin						
≥15%	30	1		30	1	
<15%	31	7.36 (2.5–21.8)	.004	31	4.44 (0.1–224.7)	.40

Abbreviations: OS, overall survival; RR, risk ratio; CI, confidence interval; HER1, human epidermal growth receptor 1; MCM7, minichromosome maintenance protein 7.
* Likelihood ratio tests.
† Reference risk.

laryngeal SCC. These findings are consistent with previous studies that have indicated MCM7 as a sensitive prognostic marker of various human cancers, including oral SCC, colorectal cancer, ovarian cancer, glioblastoma, and esophageal carcinoma.[6] Moreover, relative to normal epithelium, increased expression of MCM2 has been previously reported in laryngeal SCC.[28]

MCM7 and geminin labeling index were not associated with any clinicopathologic parameter and did not correlate to each other. The mean value of MCM7 labeling index, 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 EGFR 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 progrowth phenotype that is associated with MCM7 overexpression in laryngeal SCC. 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 laryngeal SCC 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 tyrosine kinase inhibitors treatment has been documented.[26] In view of these observations, the finding that MCM7 overexpression is associated with an adverse clinical outcome in patients with HER1 expressing laryngeal SCC, provides clinical implications for the treatment of laryngeal SCC with deregulated MCM7 status, using EGFR and Lyn kinase inhibitors.

Geminin labeling index behaves as a significant predictor of better prognosis in patients with laryngeal SCC relative to the PFS. Our data are in agreement with previous reports in patients with oral SCC[30] and high-grade astrocytic brain tumors,[31] in which high geminin expression is a significant predictor of better prognosis. However, in patients with breast,[32] renal,[33] and colorectal cancer,[34] geminin overexpression 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 patients with laryngeal SCC 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 chemoradiosensitivity of laryngeal SCC.

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] in which they are most chemoradiosensitive. On the other hand, a significant reduction of geminin expression was observed after chemoradiosensitive 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 chemoradiosensitive treatment. Then, evaluation of geminin expression might serve as a prognostic marker of response to chemoradiosensitive therapy in patients with laryngeal SCC. This possibility is also in agreement with what was observed in patients with oral SCC[30] and high-grade astrocytic brain tumors[31] who have undergone chemoradiosensitive treatment.

Geminin expression, as a prognostic marker of response to chemoradiosensitive therapy, complements the prognostic information obtained from MCM7 expression in tumor cells. In fact, patients with laryngeal SCC 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 the EGFR pathway, may reveal to be very useful for this purpose and be used 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 radiotherapy/chemotherapy in the head and neck, which is cisplatin.

In conclusion, to the best of our knowledge, this is the first study demonstrating that MCM7-geminin status is a reliable independent prognostic marker of the clinical outcome in patients with laryngeal SCC. 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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