

EMSI Gene Amplification Correlates with Poor Prognosis in Squamous Cell Carcinomas of the Head and Neck¹

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ABSTRACT

The relationship between *CCND1* and/or *EMSI* amplification and disease outcome was studied in a prospective series of 104 head and neck squamous cell carcinomas treated by surgical resection. The *CCND1* and *EMSI* copy number in tumor samples was estimated by differential PCR. The presence or absence of amplification was analyzed in relation to clinicopathological variables, tumor recurrence, and patient survival. *CCND1* amplification occurred in 32 cases (31%) and was associated with increased lymph node stage ($P = 0.005$) and advanced disease stage ($P = 0.003$). *EMSI* amplification was identified in 21 cases (20%) and was related with advanced T stages ($P = 0.001$), increased lymph node stage ($P = 0.02$), advanced disease stage ($P = 0.041$), poor histological differentiation ($P = 0.018$), recurrent disease ($P = 0.0004$), and reduced disease-specific survival ($P < 0.0001$). Coamplification of both genes occurred in 11 cases (11.5%). Multivariate analysis confirmed that in addition to regional lymph node status, *EMSI* amplification is an independent predictor of death from the tumor ($P = 0.0027$). *CCND1* amplification was not prognostic. These data indicate that *EMSI* amplification, but not *CCND1* amplification, predicts early recurrence and reduced survival in squamous cell carcinoma of the head and neck. The prognostic significance previously attributed to *CCND1* amplification may be attributable to its frequent coamplification with *EMSI*.

INTRODUCTION

The survival rate for patients with SCCHN³ (1) has remained unchanged in recent years despite the advances in diagnosis and treatment. Our ability to prognosticate for advanced

cases of SCCHN is particularly poor owing to variations in the biological behavior of the tumors and inadequacies of the present staging system. It is therefore essential to identify new markers (e.g., genetic markers) that may distinguish differences in tumor behavior.

Multiple genes and chromosomal regions are implicated in SCCHN tumorigenesis. Amplification of chromosomal region 11q13 is one of the genetic alterations most frequently observed in SCCHN (1–7). The amplified 11q13 region is estimated to be 3–5 megabases in size and includes four putative oncogenes: *CCND1* (*PRAD1*), *FGF3* (*INT2*), *FGF4* (*HST1*), and *EMSI*. *CCND1* and *EMSI* are the more prominent candidate oncogenes because they were found to be also overexpressed in all carcinomas with an 11q13 amplification (8). Therefore, the activation of these genes might confer the selective advantage to these tumors. The *CCND1* gene encodes the cell cycle regulating cyclin D1 protein involved in the G₁-to-S transition. *EMSI* encodes a cytoskeletal protein homologous to the avian F actin-binding protein (cortactin), which is thought to be involved in cell-to-cell interactions (8).

Amplification of the chromosome 11q13 region in SCCHN has been correlated with aggressive tumor growth (3, 9), the presence of lymph node metastases (7, 10–12), and poor prognosis (9). In addition, various studies have assessed the clinical and prognostic significance of *CCND1* amplification and/or overexpression, showing an association with recurrence and shortened overall survival (6, 13–17). However, little is known about the prognostic significance of *EMSI* amplification in SCCHN. Because *EMSI* amplification in tumors results in overexpression of cortactin and this is accompanied by a partial redistribution of cortactin from the cytoplasm into cell-matrix contact sites, it has been suggested that *EMSI* amplification can contribute to the invasive potential of tumor cells (8, 18).

We hypothesized that, although both *CCND1* and *EMSI* contribute to the growth advantage of SCCHN with 11q13 amplification, amplification of each gene individually may confer different properties to these tumors. The purpose of this study was therefore to assess the correlation between *CCND1* and/or *EMSI* amplification, the clinicopathological characteristics, and tumor behavior in SCCHN.

To evaluate *CCND1* and *EMSI* amplifications, we have used differential PCR, which is a rapid, simple, and sensitive method to determine semiquantitatively the number of gene copies. It only requires small amounts of DNA, which facilitates these studies in clinical medicine.

MATERIALS AND METHODS

Tumor Specimens. Tissue samples of 104 consecutive primary SCCHN were obtained from patients undergoing surgical resection of their tumor between January 1992 and July 1994. All patients included in our study had a single primary tumor, none had undergone treatment before surgery, and had

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³ The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; TH, tyrosine hydroxylase.

Table 1 Characteristics of the patient population and their tumors

	No. of patients
Mean age at resection (median)	
Total population: 58.7 (60) yr	
Alive/died of other causes: 60.3 (60.5) yr	
Died of index cancer: 57 (59) yr	
Sex	
Male	103
Female	1
Site	
Oral cavity	5
Oropharynx	24
Supraglottic larynx	23
Glottic larynx	15
Hypopharynx	37
pT stage	
T ₁	3
T ₂	25
T ₃	33
T ₄	43
pN stage	
N ₀	35
N ₁	21
N ₂	32
N ₃	16
Disease stage	
I	2
II	11
III	30
IV	61
Histopathological grade	
Well-differentiated	51
Moderately differentiated	43
Poorly differentiated	10
Outcome	
Alive at last follow-up	35
Died of index cancer	52
Died of other causes	17
Mean length of follow-up (median)	
Total population: 24.4 (18) mo	
Alive at last follow-up: 41.4 (40) mo	
Died of other causes: 17.3 (14) mo	
Died of index cancer: 15.3 (14) mo	

microscopically clear surgical margins. None of the patients were thought to have had distant metastases at the time of surgery. All but one subject were smokers, and 94 of them were also habitual alcohol drinkers. The exceptionally low proportion of women in the sample (only one) is usual for this kind of tumor in our media. A total of 56 patients received postoperative radiotherapy. As a general rule, this was administered to the patients with histologically N₂ or N₃ neck lesions, and also to the patients with N₀-N₁ neck lesions with locally advanced stage (T₄). The clinicopathological data from the patients are shown in Table 1. The stage of disease was determined after the surgical resection of the tumor (with lymph node dissection where appropriate) according to the TNM system of the Union Internationale Contre le Cancer (Ed. 4).

DNA Isolation and PCR Analysis. Tissue specimens were frozen immediately in liquid nitrogen and stored at -70°C until DNA extraction. High-molecular-weight DNA was isolated by standard phenol-chloroform extraction as previously described (19).

The PCR mixtures contained 0.2–0.5 µg of target DNA, 10

mm Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM each of deoxynucleotide triphosphate, 1 µM of each primer, and 1 unit of Taq polymerase (Boehringer Mannheim, Mannheim, Germany) in a total volume of 50 µl with a 50-µl mineral oil overlay. The PCR cycles included 1 min at each temperature (94°C, 56°C, and 72°C), for a total of 30 cycles, and a final cycle at 72°C for 7 min. Two different sets of primers, one for the target gene (*CCND1* or *EMSI*) and the other for the control gene, were present simultaneously in the reaction vessel, as previously described (20, 21). The *TH* gene, which is located on the same chromosome as the target genes, was used as the control gene. Primer sequences were designed from the genomic sequences (obtained from GenBank).⁴ Primers for the *CCND1* gene were 5'-CGTACCCCG-ATGC-CAACC and 5'-ATGGACGGCAGGACCTCC, and they amplified a fragment of 121bp. Primers for the *EMSI* gene were 5'-TCCCCTGATGCCAGGTC and 5'-TCC-CAATCCAGAG-ACCCG, and they amplified a sequence of 111 bp. Primers for the *TH* gene were 5'-GCCCCAGCTGCATCCTAC and 5'-CTTG-GCAGACACCTGGGG, and they amplified a sequence of 188 bp. The primers were purchased from MWG-Biotech (Mannheim, Germany).

Samples of DNA from normal tissues (tonsils) obtained from noncancer patients were used as negative controls. As positive controls, a mixture of DNA from normal tissue and increasing amounts of a previously PCR-amplified sequence of the target gene (*CCND1* or *EMSI*), mimicking different degrees of amplification, was used as template in the PCR reaction, as previously described (19).

Electrophoresis and Quantitation of PCR Products.

After PCR, 10 µl of each sample were electrophoresed on gels containing 3% NuSieve agarose (FMC, Rockland, ME) and 1% molecular biology grade agarose (Promega, Madison, WI) for 1.5 h at 65 V in 40 mM Tris-Acetate and 2 mM EDTA buffer. The gels were stained with ethidium bromide, and the images of the UV-illuminated gels were captured using a digital camera and stored in an IBM-compatible PC system. The bands were thereafter quantitated by computerized densitometric analysis techniques (Kodak Digital Science 10, Eastman Software, Billerica, MA), and the *CCND1* or *EMSI:TH* ratios were determined. The results of densitometry were corroborated by visual inspection of the gels.

Statistical Analysis. Statistical analysis was performed using χ^2 , with Yates' correction where appropriate, and Fisher's exact tests. Survival curves were calculated using the Kaplan-Meier product limit estimate (22). Deaths from causes other than the index tumor or its metastases were not considered treatment failures, and these patients were censored in all analysis involving the length of survival. Differences between survival times were analyzed by the log-rank method (23). Multivariate Cox proportional hazards models (24) were used to examine the relative impact of either variables demonstrated to be statistically significant in univariate analysis or those variables likely to have an effect on outcome (e.g., tumor site and histopathological grade). In these models, tumor sites were dichotomized as oral cavity and pharynx versus larynx. Similarly, T-stage

⁴ Internet address: <http://www.ncbi.nlm.nih.gov>.

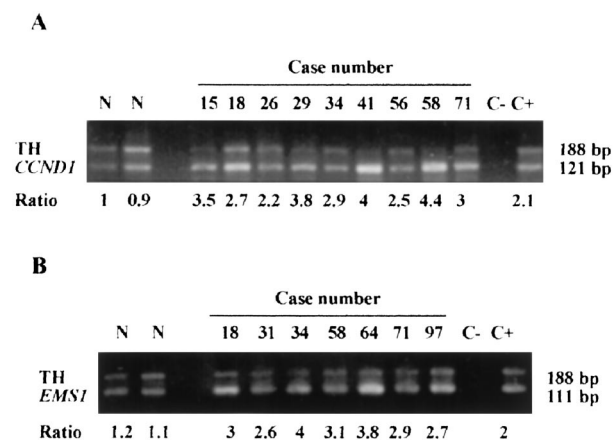


Fig. 1 Differential PCR analysis of *CCND1* (A) and *EMS1* (B) gene amplifications from various tumor samples in which gene amplification was found. The PCR products were separated by agarose gel electrophoresis and quantified by image analysis densitometry. *N*, controls with normal tissue DNA; *C+*, the positive controls (2-fold amplification); *C-*, the negative controls.

classification was dichotomized as T_1 , T_2 , or T_3 versus T_4 . Lymph node metastasis classification was dichotomized as N_0 or N_1 versus N_2 or N_3 . Stage was dichotomized as stage I, stage II, or stage III versus stage IV. Finally, histopathological grade was dichotomized as well- and moderately differentiated versus poorly differentiated. $P_s \leq 0.05$ were considered to be statistically significant. Patients were observed for at least 36 months.

RESULTS

Differential PCR. A total of 104 samples from patients with SCCHN were studied. Each sample was investigated for *CCND1* and *EMS1* amplifications by differential PCR using the gene for TH as the control. Samples of DNA obtained from normal tissues showed *CCND1:TH* ratios of 1.2 ± 0.21 (mean \pm SD) and *EMS1:TH* ratios of 0.9 ± 0.1 (mean \pm SDs obtained from 10 different samples). The positive controls showed that there was an increase in the PCR product from the target gene, compared with the PCR product from the control gene, as the target gene copy number used as the template was increased (data not shown). Experimentally, gene amplification was defined as at least a 2-fold increase in the *CCND1:TH* or *EMS1:TH* ratios, relative to the ratios obtained with normal tissue DNA. Because of the semiquantitative nature of the differential PCR, the ratios obtained were not converted to a score of amplification; the results were expressed as amplification or no amplification of the *CCND1* or *EMS1* genes. PCR was carried out at least twice in the positive cases.

CCND1 and *EMS1* amplifications were found in 32 (31%) and 21 (20%) of the 104 tumor samples, respectively. Coamplification of both genes was demonstrated in 11 cases (11.5%). None of the cases considered nonamplified presented a *CCND1:TH* ratio higher than 1.9 (mean \pm SD, 1.3 ± 0.2), or an *EMS1:TH* ratio higher than 1.8 (mean \pm SD, 1.2 ± 0.2). Fig. 1 shows the results of the differential PCR in some of the amplified cases.

Association of Amplification of *CCND1* or *EMS1* with Clinicopathological Parameters. Table 2 presents the correlation of *CCND1* and *EMS1* amplifications with clinical stage, pathological grading, primary site, relapse, and disease outcome. Amplified cases were found at each primary head and neck anatomical site, with no statistically significant differences. However, in the cases with *CCND1* amplification, there was a tendency toward higher frequency of amplification in those cases with hypopharyngeal localization. Sixteen (76%) of the 21 cases with *EMS1* amplification were observed in T_4 -stage tumors, with the association being statistically significant ($P = 0.001$). *CCND1* amplification was also more frequent in T_4 -stage tumors, although in this case, the differences were not statistically significant ($P = 0.12$). Both *CCND1* and *EMS1* amplifications were associated with increased regional lymph node disease (Table 2). Because *CCND1* and *EMS1* amplifications correlate with advanced T and N stages, none of the patients with either stage I or II disease had *CCND1* or *EMS1* amplification, resulting in a statistically significant association between advanced disease stage and amplification of these genes. No relationship was observed between *CCND1* amplification and histopathological differentiation. However, *EMS1* amplification was significantly associated with poorly differentiated tumors ($P = 0.018$).

Amplification of *CCND1* and/or *EMS1* and Disease Course. Seventeen patients that died from causes not related to the index tumor were excluded from the recurrence analysis (four of them had *CCND1* amplification, and three had *EMS1* amplification). Of the remaining 28 *CCND1*-amplified cases, 21 (75%) had tumor recurrence (local/regional recurrence or distant metastases), compared with 35 (57%) in the nonamplified group, that did not reach statistical significance (Table 2). The differences in recurrence rates were statistically significant when the cases with and without *EMS1* amplification were compared (Table 2): 18 (100%) of the 18 *EMS1*-amplified cases versus 37 (54%) of the 69 nonamplified cases presented recurrence. Then, it seems that *EMS1* amplification has a stronger relationship with tumor recurrence than *CCND1* amplification. It is confirmed if we compare the recurrence rates when the coamplified cases are considered separately (Table 3); in this fashion, recurrences were found in 10 (59%) of 17 cases with *CCND1* amplification, 7 (100%) of 7 cases with *EMS1* amplification, 11 (100%) of 11 coamplified cases, and 27 (52%) of 52 nonamplified cases.

The results of univariate Kaplan-Meier analyses are shown in Fig. 2. The analyses demonstrated that *EMS1* amplification had a statistically significant association with shorter disease-specific survival (log-rank $P < 0.0001$) in this patient population. The data in Fig. 2 also suggest that there was an association, although not statistically significant (log-rank $P = 0.073$), of *CCND1* amplification with disease course. This is likely to be the result of its frequent coamplification with *EMS1*. Other parameters that had statistically significant association with a reduced disease-specific survival were: tumor size T_4 (log-rank $P = 0.047$), locoregional lymph node metastases of class N_2 or greater (log-rank $P = 0.0002$), stage IV disease (log-rank $P = 0.0018$), and pharyngeal localization of the tumor (log-rank $P = 0.031$). The influence of postoperative radiotherapy on survival cannot be addressed because it was administered mainly to the

Table 2 *CCND1* and *EMSI* amplification by clinicopathological findings, site and relapse

	Total cases	<i>CCND1</i> -amplified cases (%)	<i>P</i> ^a	<i>EMSI</i> -amplified cases (%)	<i>P</i> ^a
Mean age (yr)	58.7	57.4	0.38 ^b	58.8	0.98 ^b
pT stage					
T ₁ -T ₂	28	6 (21)	0.12	1 (3.5)	0.001
T ₃	33	8 (24)		4 (12)	
T ₄	43	18 (42)		16 (37)	
pN stage					
N ₀	35	5 (14)	0.0054	5 (14)	0.02
N ₁	21	8 (38)		1 (5)	
N ₂	32	9 (28)		8 (25)	
N ₃	16	10 (62)		7 (43)	
Pathologic grade					
Well-differentiated	51	15 (29)	0.94	6 (12)	0.018
Moderately differentiated	43	14 (32)		10 (23)	
Poorly differentiated	10	3 (30)		5 (50)	
Disease stage					
I-II	13	0	0.036	0	0.041
III	30	11 (37)		4 (13)	
IV	61	21 (34)		17 (28)	
Site					
Oral cavity	5	1 (20)	0.087	1 (20)	0.62
Oropharynx	24	4 (17)		6 (25)	
Supraglottic larynx	23	9 (39)		2 (9)	
Glottic larynx	15	2 (13)		3 (20)	
Hypopharynx	37	16 (43)		9 (24)	
Recurrence ^c					
No recurrence	32	7 (22)	0.16	0	0.0004
Loco-regional recurrence	43	18 (42)		16 (37)	
Distant metastases	12	3 (25)		2 (17)	
Disease status					
Alive without disease	35	8 (23)	0.19	1 (3)	0.0019
Dead of index cancer	52	20 (38)		17 (33)	
Died of other causes	17	4 (24)		3 (18)	

^a χ^2 test.

^b *t* test.

^c Seventeen patients who died from causes not related to the index tumor were excluded from the recurrence analysis.

Table 3 Gene amplification and relapse in 87 patients with SCCHN with adequate follow-up

Gene amplification	No. of cases	Loco-regional recurrence or distant metastases (%)	<i>P</i> ^a
None	52	27 (52)	
<i>CCND1</i>	17	10 (59)	0.83
<i>EMSI</i>	7	7 (100)	0.044
<i>CCND1</i> and <i>EMSI</i>	11	11 (100)	0.009

^a χ^2 test.

patients in stage IV disease (46 of 56 patients that received this treatment). Because *CCND1* and *EMSI* amplifications were associated with advanced disease stage, most of the amplified cases received this treatment (25 of 32 with *CCND1* amplification and 17 of 21 with *EMSI* amplification). Then, it seems unlikely that this can influence the differences in survival between the amplified and the nonamplified cases.

The results of the multivariate Cox proportional hazards model showed two parameters that were statistically significant independent predictors of a reduced disease-specific survival (Table 4): cervical lymph node metastases of class N₂ or greater and *EMSI* amplification. Amplification of the *EMSI* gene ap-

peared to confer an adjusted relative risk of 2.58 for dying of the index cancer (95% confidence interval, 1.3–8.99). Amplification of the *CCND1* gene did not enter in either of the multivariate Cox models.

DISCUSSION

Amplification of the chromosome 11q13 region has been observed in a variety of human malignancies. The highest frequencies of amplification have been found in adenocarcinomas of the breast and in squamous cell carcinomas of the esophagus and the head and neck region (8). Although various genes are consistently present on the amplified 11q13 region, only *CCND1* and *EMSI* are likely to be the key genes within this region because in addition to being frequently coamplified, they were found to be overexpressed in all tumors with an 11q13 amplification (8). *CCND1* encodes the cyclin D1, a G₁ cyclin involved in the G₁-to-S transition that functions as a positive cell cycle regulator (25). The *EMSI* gene encodes cortactin, a cytoskeletal protein involved in regulating the interactions between components in the adherens junctions, and as such, its overexpression might affect the organization and functioning of cytoskeleton and cell-adhesion structures and hence, contribute to the invasive behavior of tumor cells (18, 26).

In SCCHN, amplification of 11q13 has been reported in

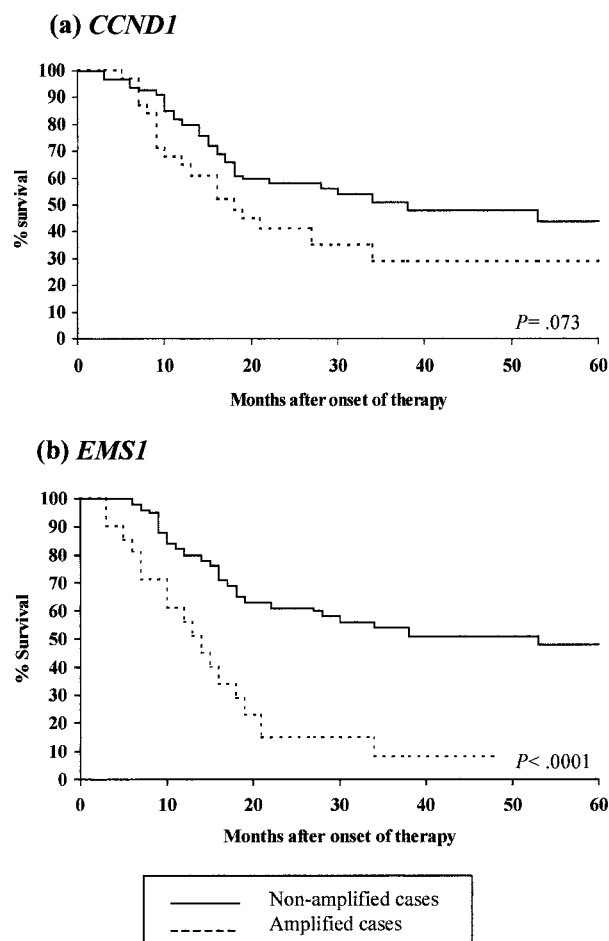


Fig. 2 Kaplan-Meier disease-free survival curves of the patients with (---) and without (—) *CCND1* (A) or *EMS1* (B) gene amplification. Differences between survival times were analyzed by the log-rank method.

20–52% of patients (1–3, 5, 7, 9–11), which compares well with 34% in the present study. Several works have focused on *CCND1* amplification and showed similar results (4, 6, 13, 17). In contrast, only a few studies have also analyzed *EMS1* amplification (3, 8, 9, 12). In these series, as in our study, *CCND1* and *EMS1* have not been uniformly amplified in all cases. These differences in amplification of 11q13 markers were also described in breast carcinomas (27), and they are in agreement with the structure of the 11q13 amplicon proposed by Schuurings (8): the amplified 11q13 region harbors at least three, or even more, separate cores of amplification. The first core extends from *BCL1* to *FGF4*, with the *CCND1* gene as the best candidate key gene. The second core extends from *FGF3* to the *EMS1* gene, with the *EMS1* gene as the best candidate key gene. Another two cores of amplification have been described in the centromeric and the telomeric borders, respectively, of the 11q13 region; as yet, no gene has been identified in these subregions.

The results of the present study indicate that the amplification of *EMS1* is of prognostic value for SCCHN independent

Table 4 Forward stepwise Cox analysis of disease-specific survival in 104 patients with squamous cell carcinoma of the head and neck

Variable	<i>P</i> s for each step		
	0	1	2
Site	0.031	0.081	0.16
pT stage	0.047	0.24	0.90
pN stage	0.0002	0.0035	0.0043 ^a
Disease stage	0.0018	0.012	0.55
Pathologic grade	0.20	0.43	0.54
<i>EMS1</i> amplification	0.0000	0.0001 ^a	0.0027
<i>CCND1</i> amplification	0.073	0.17	0.59

^a *P*s indicate the variable entered in the model at each step.

of other known risk factors. In fact, all of the patients with *EMS1* amplification experienced disease recurrence. In contrast, *CCND1* amplification has not found to be of prognostic significance in multivariate analysis. In other reports, 11q13 amplification (without reference to specific genes) was observed in patients with advanced disease, a poorly differentiated histology of the tumor, and deeply invasive growth (3). In concordance with the presumed association with progressed disease, the amplified cases develop more frequent recurrences and have an increased risk of tumor-associated death (6, 7, 9). Opposite reports (5, 11) indicate the controversy on this point. This may be attributable to the use of different 11q13 markers, measuring only one of the amplification cores to analyze 11q13 amplification. On the other hand, the amplification and/or overexpression of *CCND1* has been reported to be a poor prognostic sign in various studies focused on this gene (6, 13–17). Again, there are also reports on the contrary (4, 28). In addition, the association of *CCND1* amplification and/or overexpression and poor prognosis is not absolute, suggesting that amplification and/or overexpression of *CCND1* contributes only partially to the process of tumor progression. Our findings indicate that the amplification of *CCND1* loses its prognostic significance when the cases in which *CCND1* is coamplified with the *EMS1* gene are eliminated. Thus, the prognostic significance attributed to the *CCND1* amplification may be attributable to its frequent coamplification with the *EMS1* gene. This agrees with the proposed role of these genes in tumor development (8): *CCND1* overexpression might confer to the tumor cells the ability to proliferate under reduced growth factor conditions because of its effects in the G₁-to-S transition of the cell cycle; *EMS1* overexpression might mediate the increased invasive and metastatic behavior of tumor cells attributable to its effects in the organization and the functioning of cytoskeleton and cell adhesion structures.

Differential PCR has previously been shown as a simple, rapid, and sensitive method to detect genetic amplification (17, 19–21). Our results corroborate that of others, showing that this method is suitable to detect gene amplification. It is reproducible, and its main advantage is that small quantities of DNA are needed, making possible its clinical use with samples obtained from small biopsies or fine needle aspirations. Using this technique, we have found an 11q13 amplification rate in agreement with previous studies that used conventional techniques.

We conclude that amplification of the *EMS1* gene is an independent prognostic marker for survival, and it specifies a

subgroup of operable SCCHN patients that is at increased risk and might benefit from more intensive treatment and follow-up. Whether this is the result of greater tumor aggressiveness or of decreased responsiveness to adjuvant radiotherapy remains to be elucidated. Presently, clinical and histopathological parameters are used as guides for the application of adjunctive therapy, such as radiotherapy or even resection of the surgical field. Our data suggest that *EMSI* amplification can augment the predictive power of those clinical and histopathological markers. Routine implementation of this molecular test will require confirmation of our findings in larger prospective studies.

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