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Lymph node metastasis in lower lip squamous cell carcinoma in relation to tumour size, histologic variables and $p27^{Kip1}$ protein expression

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Summary We studied a consecutive series of 95 patients undergoing radical surgical resection of lower lip squamous cell carcinoma (LLSCC) to assess the correlation between lymph node status and several prognostic variables, such as sex and age, tumour size, histologic grading, maximal microscopic tumour thickness, perineural infiltration and $p27^{Kip1}$ protein status, to see which of these might be predictive of the development of lymph node metastases. Statistical analysis demonstrated a significant association between node status and tumour size, histological grading, maximal thickness, perineural invasion and $p27^{Kip1}$ protein expression; additionally to node metastasis, low $p27^{Kip1}$ protein expression was significant correlated with high microscopic thickness. These results indicate that lower lip squamous cell carcinomas of >2 cm, with G3-G4 histological grading, maximal thickness of >6 mm, perineural invasion and low $p27^{Kip1}$ protein expression (LI $<19.7\%$) are at high risk for the development of lymph node metastases.

1. Introduction

Lower lip squamous cell carcinoma (LLSCC) is a fairly common tumour found mostly in middle-aged or elderly males.[1,2] The prognosis for such patients depends mainly on the clinical staging of the neoplasia, especially with regard to its size and to lymph node status.[3–6] In recent years, there have been numerous studies to identify new significant prognostic parameters and more suitable therapeutic approaches; interesting results have been obtained with the analysis of the cell cycle. The $p27^{Kip1}$ protein is a member of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors.[7] These molecules, which are potent negative regulators of the cell cycle, are potential tumour suppressor genes.[8,9] Experimental findings indicate that $p27^{Kip1}$ plays an important role in numerous fundamental

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cellular processes, including cell proliferation, cell differentiation, and apoptosis.[10] While alterations in the $p27^{Kip1}$ gene are rare,[11–13] low $p27^{Kip1}$ protein expression, commonly observed in human tumours, is correlated with enhanced specific proteasome-dependent degradation.[14] Findings in several studies indicate that low, or absent, $p27^{Kip1}$ protein expression is associated with tumour progression and a poor prognosis in several types of cancer.[15–21] As far as we know, there is no study concerning the expression of $p27^{Kip1}$ protein in lower lip squamous cell carcinoma.

The aim of our study was to assess the association between lymph node status and several prognostic variables, such as sex and age, tumour size, histologic grading, maximal tumour thickness, perineural infiltration and $p27^{Kip1}$ protein status, in a group of patients undergoing surgical treatment for LLSCC, and to show which of these might be predictive of the development of lymph node metastases.

2. Materials and methods

2.1. Clinical and pathologic findings

The study was performed on tissue samples deriving from a consecutive series of 110 patients undergoing radical surgical resection of LLSCC, at the Institute of Pathologic Anatomy and Histology of the University of Palermo between January 1988 and December 1997. Fifteen patients were excluded because of insufficient follow-up; only patients for whom adequate histologic material was available were included in the study.

Complete excision of the primary tumour was proved by means of the histologic examination of the resected margins. The tumours were divided by size into three categories, T_1, T_2 and T_3, according to the TNM system.[22] They were revaluated with regard to histological grading and classified as G1, G2, G3 and G4, following the method recommended by Anneroth and Hansen.[23] None of the patients examined presented distant metastases. All patients were treated with surgical resection of the primary tumour associated with a conservative or radical neck dissection in cases of suspect lymph nodes metastases as evidenced by palpation, ultrasound or computed tomography. The patients were followed up for at least 5 years after surgery; they had routine follow-up at 4 month intervals for the first year, at 6 month intervals for the second and third year and annually thereafter.

The 95 patients, made up of 92 men and three women, were divided into two groups: 74 cases (group I) without lymph node metastases and 21 subjects (group II), with lymph node metastases; the mean age of group I was of 67.1 years and of group II of 72.8 years. Table 1 shows the clinical features of patients.

2.2. Histology

All the tissue samples were fixed in 10% buffered formalin, dehydrated in ethanol and paraffin-embedded according to the routine technique. Sections 5 μm thick were cut and stained with H&E, PAS, Alcian blue and Masson's trichrome (Fig. 1). Assessment of histologic grading and inspection for the possible presence of perineural invasion, reported as present or absent, were performed on each sample. The measurement of microscopic thickness was performed with a 2.5 magnifying lens by an image analysis system (Leica QM500, Cambridge, UK) consisting of an optical Leitz (Wetzlar, Germany) microscope (mod. Dialux 20) fitted with a single chip color CCD video camera (JVC TK 1280E, Yokohama, Japan) with a resolution of 752×582 (horizontal×vertical) TV lines, a color monitor and an image processing unit installed in a Pentium II (Intel Corporation, Santa Clara, CA, USA) 350 MHz processor-based personal computer. The resulting software processing was expressed in mm. The cases were divided into three groups according to infiltration depth: <3 mm, between 3 and 6 mm and >6 mm. We determined the maximal thickness

Table 1 Clinico-pathological characteristics of patients

Parameter	No. of patients
Sex	
Male	92
Female	3
Age (year)	
<60	18
60–70	22
>70	55
Size	
T1	71
T2	18
T3	6
Node status	
Node–	74
Node +	21
Histologic grade	
G1	9
G2	46
G3	30
G4	10

of each tumour measuring vertically from the surface of the tumour; surface keratin and exudate were excluded from the measurement. For exophytic, papillary lesions, we measured the thickness from the tip of a papilla to an imaginary line connecting the bases of two adjacent pegs. All thickness measurements were performed separately by two different operators; since variation between the results was less than 5%, first observer data were used. With regard to grading and perineural invasion, discordances in valuations were discussed in order to reach a consensus between the two pathologists.

2.3. Immunohistochemistry

$p27^{Kip1}$ protein expression was detected immunohistochemically using the monoclonal antibody K25020 (Transduction Laboratories, Lexington, KY, USA) generated from mouse $p27^{(Kip1)}$ protein. Sections were cut at 5 μm thickness and placed in an oven at 60 °C for ⩾2 h. Tissue sections were deparaffinized and rehydrated. Microwave heating of tissue sections was used for antigen retrieval. Sections immersed in 10 mM of citrate buffer, at pH6.0, were boiled for four 5-min cycles at 750 W. Jars were refilled with the buffer between each cycle. All slides were then placed in phosphate-buffered saline (PBS) and were incubated with 10% whole horse serum PBS for 20 min at room temperature. Sections were then incubated overnight at 4 °C with the monoclonal K25020 antibody at a concentration of 1:1.200. Negative control studies were carried out by replacing the primary antibody with PBS. Biotinylated alpha mouse IgG obtained from horse serum (ABC Kit, Vector Laboratories, Burlingame, CA, USA) was applied as a secondary antibody at 1:400 in PBS for 30 min at room temperature. Immunostaining was performed using the avidin-biotin peroxidase complex technique (ABC Kit, Vector Laboratories) applied for 30 min. Finally, 3,3-diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark) in distilled water was used as the chromogen for 10 min, and sections were counterstained using Mayer's hematoxylin. Internal positive controls were provided by normal epithelium adjacent to the tumours and infiltrating lymphocytes usually present within the specimens.

The grade of $p27^{Kip1}$ protein expression in each specimen was evaluated according to the percentage of positively stained cells among the total number of counted cancer cells. Although there was variability in the total number of cells counted in each specimen, the percentage of positively stained cells was estimated by counting 200 cells under a ×40 magnification in each of five randomly selected areas, giving a total of 1000 cells; all positive cells were counted regardless of intensity of staining and used as labeling index (LI). The cases were evaluated separately by two different operators; since variation was less than 5%, first observer data were used. Based on the median value of $p27^{Kip1}$ protein expression in this series, $p27^{Kip1}$ protein status was classified as low ($p27^{Kip1}$ protein nuclear staining in less than 19.7% of tumour cells), or high ($p27^{Kip1}$ protein nuclear staining in 19.7% or more tumour cells).

2.4. Statistical methods

Fisher's exact test and the χ^2 test were used. A *P*-value less than 0.05 was considered statistically significant. Correlation between pairs of continuous variables was evaluated with Spearman's rank test. These analyses were performed with SPSS software (release 8.0; Chicago, IL, USA, 1997).

3. Results

On examining the distribution of tumours without lymph node metastases (group I) and with lymph node metastases (group II) no significant differences were found for sex and age. However, statistical analysis demonstrated a significant association between node status and tumour size, histologic grading, maximal thickness, perineural invasion and $p27^{Kip1}$ protein expression. Table 2 shows the results obtained.

3.1. Lymph node status

Group I (74 patients) presented no metastases, either at the moment of surgery, or subsequently, whereas in group II, eight of the 21 patients (38%) had histologically confirmed lymph node metastases at the time of primary surgery. The other 13 cases (62%) in this group developed metastases following surgery.

3.2. Tumour size

In group I (without metastases), 90% (67/74) of the tumours were T_1, 7% (5/74) were T_2 and 3% (2/74) were T_3. In group II (with metastases), 19% (4/21) were T_1, 62% (13/21) were T_2 and 19% (4/21) were T_3. These differences were statistically significant ($P<0.001$).

3.3. Histologic grading

In group I, 11% (8/74) of the tumours were classified as G1, 53% (39/74) as G2, 31% (23/74) as G3

Table 2 Comparison of tumour size, histologic grading, maximal thickness, perineural invasion and $p27^{Kip1}$ protein expression in non metastatic (group I) and metastatic (group II) squamous cell carcinoma of the lower lip

Parameters	Group I (node−) (%)	n	Group II (node +) (%)	n	P value
Size					<0.001
T1	(90)	67	(19)	4	
T2	(7)	5	(62)	13	
T3	(3)	2	(19)	4	
Histologic grading					=0.016
G1	(11)	8	(5)	1	
G2	(53)	39	(33)	7	
G3	(31)	23	(33)	7	
G4	(5)	4	(29)	6	
Maximal thickness					<0.001
<3 mm	(51)	38	(0)	0	
3–6 mm	(42)	31	(10)	2	
>6 mm	(7)	5	(90)	19	
Perineural invasion					=0.008
Absent	(99)	73	(81)	17	
Present	(1)	1	(19)	4	
$p27^{Kip1}$ protein expression					<0.001
LI≥19.7%	(87)	64	(24)	5	
LI<19.7%	(13)	10	(76)	16	

and 5% (4/74) as G4. In group II, 5% (1/21) of the cases were G1, 33% (7/21) were G2, 33% (7/21) were G3 and 29% (6/21) were G4. The differences between the two groups proved to be significant (P=0.016).

3.4. Maximal thickness

The mean depth of neoplastic invasion in group I subjects was of 4.2 mm (range 1.2–12.1); 51% of these tumours (38/74) reached a depth of <3 mm, 42% (31/74) of between 3 and 6 mm, and 7% (5/74) of >6 mm. The group II cases showed a mean invasion depth of 11.2 mm (range 4.1–16.7); none of these was <3 mm, 10% (2/21) were between 3 and 6 mm, and 90% (19/21) were of >6 mm. The differences between the two groups proved to be significant (P<0.001).

3.5. Perineural invasion

Perineural invasion was found in five cases only, one case in group I and four cases in group II; this result was statistically significant (P=0.008).

3.6. $p27^{Kip1}$ protein expression

The median percentage of tumour cells with $p27^{Kip1}$ protein expression was 19.7% (range 0–90.1%). In all cases, the signal was confined mainly to the nuclei of the tumour cells (Fig. 1). Low $p27^{Kip1}$ protein expression (LI <19.7%) was found in 26 cases (27%); in 16 cases (17%), $p27^{Kip1}$ was expressed in less than 5% of neoplastic cells and in 40 cases (42%), it was expressed in more than 50% of the neoplastic cells. A significant association was found between low $p27^{Kip1}$ protein expression and lymph node metastasis. In group I (without metastases), 87% (64/74) of the tumours were high $p27^{Kip1}$ protein expression and 13% (10/74) were low $p27^{Kip1}$ protein expression; in group II (with metastases), 76% (16/21) were low $p27^{Kip1}$ protein expression and 24% (5/21) were high $p27^{Kip1}$ protein expression. These differences were statistically significant (P<0.001).

On examining the distribution of tumours with high and those with low $p27^{Kip1}$ protein expression, no significant differences were found for sex, age, tumour size, histological grading, and the presence of perineural invasion. Statistical analysis, however, demonstrated a significant association between low $p27^{Kip1}$ protein expression and high microscopic thickness, additionally to node metastasis (Table 3). $p27^{Kip1}$ protein expression was low in two of the 38 (5%) tumours with a depth of <3 mm, in five of the 33 (15%) between 3 and 6 mm, and in 19 of the 24 (79%) of >6 mm. Similar results were obtained using continuous variables in the analyses: a striking decrease in $p27^{Kip1}$ protein expression and a reciprocal increase in microscopic thickness were observed (Rho=−0.895; P<0.001); the bivariate scattergram (Fig. 2) indicated a graphical negative trend between the two variables.

Table 3 Low $p27^{Kip1}$ expression (LI <19.7%) and maximal tumour thickness and lymph node status in 95 patients with LLSCC

Parameters	Total	low $p27^{Kip1}$ expression no.	(%)
Maximal thickness			
<3 mm	38	2	(5)
3–6 mm	33	5	(15)
>6 mm	24	19	(79)
Node			
Node −	74	10	(14)
Node +	21	16	(76)

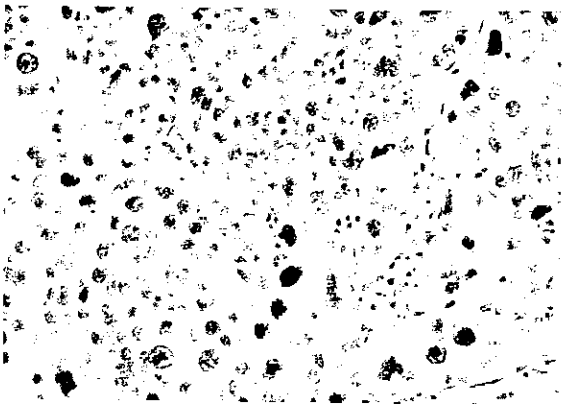

Figure 1 Section of lower lip squamous cell carcinoma stained with antibody to $p27^{Kipl}$ protein (LI=9.5%).

4. Discussion

The clinical features of our patients were similar to those reported by other authors.[1–4] Most tumours occurred in males between 50 and 70 years of age. The first correlation is between primary tumour size and development of lymph node metastases; a revision of literature shows that on the whole, larger tumours have a poorer prognosis;[1,3] Beltrami et al.[6] concluded that the diameter of the tumour is small in live patients with a long follow-up, and, in fact, a diameter of 2 cm or less is to be considered as a favorable prognostic factor. Moore et al.[5] stated that the development of metastases from carcinomas of the mouth is less likely if the tumour is of 2 cm or less. In our own cases, 90% of the non-metastatic tumours were T_1 ($\leqslant$2 cm), compared to 19% of those with metastases.

It is interesting to note that with regard to histologic variables, in the group II patients, there was generally higher grading and deeper neoplastic infiltration, while in group I, with no metastases, only 5% were G4, and the two subgroups G3–G4 together reached 36%, whereas in the group II subjects, 29% were G4, and the two subgroups G3–G4 together reached 62%. Furthermore, in group I, only 7% of the tumours showed thickness of >6 mm, while this feature reached 90% in group II. The assessment of maximal tumour thickness has been applied up till now to tumours of the skin and of the cervix,[24,25] but rarely to carcinomas of the oral cavity. Frierson et al.[4] have found a correlation between microscopic thickness and prognosis in a series of lower lip squamous cell carcinomas and conclude that the cut-off level for prognostic assessment is of 6 mm: three quarters of the patients with at least 6 mm of invasion developed metastases. The same Authors have also found that G3–G4 tumours were more common in patients with metastases. Moore et al.[5] reported a correlation between grading and lymph node status; well-differentiated tumours do not generally lead

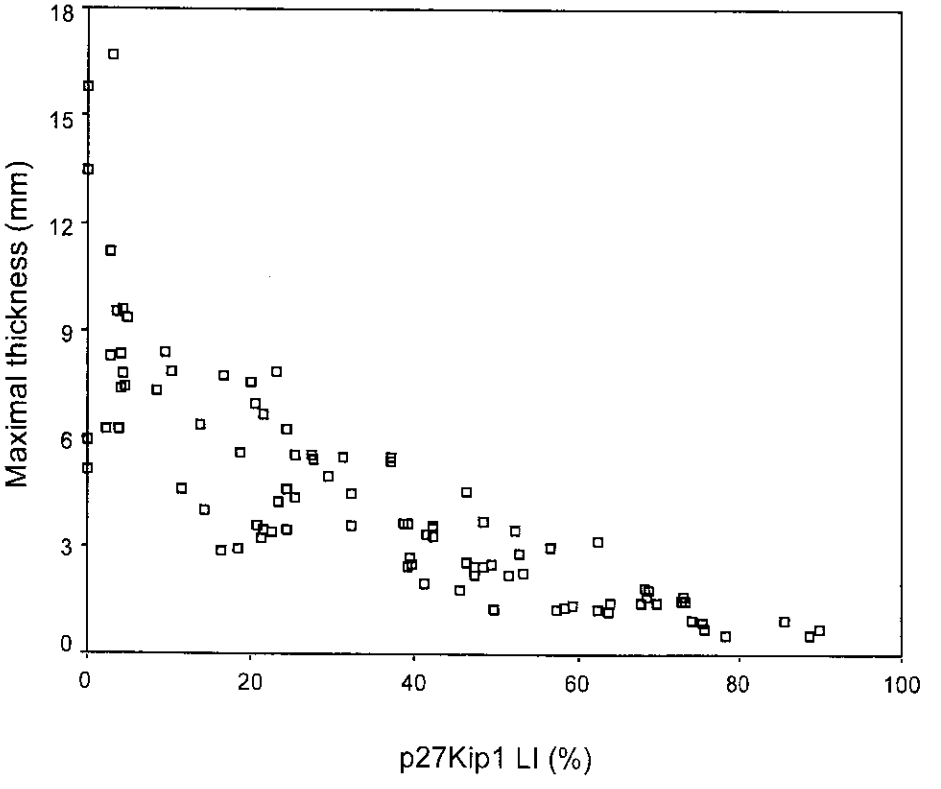

Figure 2 Bivariate scatter plot of $p27^{Kipl}$ LI and maximal tumour thickness.

to lymph node metastases. Beltrami et al.[6] reported a poor prognosis for patients with poorly differentiated tumours. In a study involving a series of 114 squamous cell carcinomas of the head and neck, Klijanienko et al.[26] found a high correlation between poor differentiation and high N stage.

In our series a significant correlation was also found between perineural infiltration and prognosis; four of five cases with perineural invasion occurred in group II. Frierson et al.[4] stated that perineural infiltration is an extra histologic parameter for the assumption of more aggressive tumoural behavior.

Though absent or low expression of $p27^{Kip1}$ protein has been shown to indicate poor prognosis for many tumours, the precise biological role of $p27^{Kip1}$ in human tumours is still unclear. In our own study, the low $p27^{Kip1}$ protein expression was strongly associated with lymph node metastases; only 13% of the cases in group I showed low $p27^{Kip1}$ protein expression, versus 76% in group II. We also found a clear correlation between low $p27^{Kip1}$ protein expression and high microscopic thickness. These findings are in line with previous studies on oral squamous cell carcinoma[16–18] and other types of cancer; it has been shown that patients with colorectal,[14] breast,[19] prostate,[20] or non-small cell cancer[21] with low or absent $p27^{Kip1}$ protein expression had a poor prognosis. Loda et al.[14] studied the prognostic value of $p27^{Kip1}$ protein expression in 149 primary human colorectal carcinomas, and found that $p27^{Kip1}$ had independent value; they also found that carcinomas with low or absent $p27^{Kip1}$ protein displayed enhanced proteolytic activity specific for $p27^{Kip1}$ and suggested that low $p27^{Kip1}$ expression may result from increased proteosome mediated degradation rather than altered gene expression. Similar results have also been reported in non-small cell lung cancer.[21] Several studies have suggested that $p27^{Kip1}$ may have additional functions such as cell-cell adhesion.[27–29] Thomas et al. have observed a reduction of $p27^{Kip1}$ expression in metastasis of colorectal carcinoma compared to primitive tumours.[30] These findings may indicate that loss of $p27^{Kip1}$ expression confers to tumour epithelial cells the ability to survive without anchorage and provide the tumour cells the opportunity to invade any tissue and to develop metastases. Moreover, although data reported on the relationship between $p27^{Kip1}$ expression and the susceptibility of cells to apoptosis are contradictory, several experimental studies indicate that the $p27^{Kip1}$ protein plays an indirect role in the induction of apoptosis.[31–33]

In conclusion, our own study has shown that lower lip squamous cell carcinomas of >2 cm, with G3–G4 histological grading, maximal thickness of >6 mm, perineural invasion and low $p27^{Kip1}$ protein expression are at high risk for the development of lymph node metastases. These variables can be used in routine diagnosis and even on biopsy material prior to surgery and are suitable for use in the selection of a high-risk sub-group of patients who might benefit from a more aggressive therapeutic approach. Further investigations on a larger number of LLSCC should be studied to see if the findings here presented can be confirmed.

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