REVIEW

Clinico-prognostic value of D-type cyclins and p27 in laryngeal cancer patients: a review

Il valore clinico-prognostico delle cicline D e di p27 nei pazienti affetti da carcinoma laringeo: una revisione

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Key words

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Summary

Despite recent improvements in surgical and radiation therapy, failures still occur in patients with laryngeal squamous cell carcinomas, which may have a very different clinical outcome even when their clinical and histopathological characteristics are similar. The apparent inadequacy of "traditional" prognostic factors in predicting the clinical evolution of laryngeal squamous cell carcinomas has led to attempts to develop additional markers capable of distinguishing patients with a good prognosis from those who are more likely to relapse. A number of studies have demonstrated a relationship between tumourigenesis and alterations in the expression of cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors, but the data regarding laryngeal squamous cell carcinomas are somewhat conflicting. Herein a review is made of the published literature concerning the clinico-prognostic role of cyclin D1, D3 and p27, and personal data are described concerning laryngeal squamous cell carcinoma patients who underwent surgical resection at the ENT Department of the University of Milan. The results of our multivariate analyses demonstrated that cyclin D1, p27 and cyclin D3 overexpression are statistically significant predictors of disease-free survival ($p = 0.0238$, $p = 0.0001$ and $p = 0.0217$, respectively); the statistical correlation with overall survival was significant in the case of p27 ($p = 0.0009$) and cyclin D3 ($p = 0.0189$), and borderline in the case of cyclin D1 ($p = 0.0622$). In relation to cyclin D1/p27 coexpression, the patients with a cyclin $D1^-/p27^+$ phenotype showed the best prognosis, those with a cyclin $D1^+/p27^+$ or cyclin $D1^-/p27^-$ phenotype, an intermediate prognosis, and those with a cyclin $D1^+/p27^-$ phenotype, the poorest prognosis ($p = 0.0001$ and $p = 0.0001$ for trend for disease-free survival; $p = 0.0015$ and $p = 0.0008$ for trend for overall survival). In the case of cyclin D1/cyclin D3 coexpression, the patients with cyclin $D1^+$/cyclin $D3^+$ tumours had the poorest overall survival, those with cyclin $D1^-$/cyclin $D3^+$ or cyclin $D1^+$/cyclin $D3^-$ tumours showed intermediate course, and those with cyclin $D1^-$/cyclin $D3^-$ tumours had the most favourable outcome ($p = 0.0002$). The findings of this review indicate that both types of cyclin D and p27 are involved in the genesis of laryngeal squamous cell carcinomas, and that immunohistochemical evalua-

Riassunto

I fattori clinico-patologici generalmente considerati sembrano non essere sufficienti a predire l'evoluzione clinico-prognostica dei pazienti con carcinoma squamocellulare della laringe, tanto che neoplasie con caratteristiche cliniche ed istopatologiche analoghe possono presentare andamenti clinici marcatamente differenti. Alla luce di queste considerazioni si sono sviluppati studi molecolari volti a ricercare ulteriori fattori prognostici da affiancare a quelli tradizionali. La letteratura dimostra un coinvolgimento nella tumorigenesi del carcinoma squamocellulare della laringe delle cicline, delle kinasi ciclino-dipendenti e dei loro inibitori. Gli Autori hanno condotto una review dei dati della letteratura sulla ciclina D1, D3 e su p27 riportando infine i risultati personali condotti su pazienti con carcinoma della laringe sottoposti ad intervento chirurgico presso la Clinica ORL dell'Università di Milano. I risultati dimostrano che la sovraespressione di ciclina D1 ($p = 0,0622$, $p = 0,0238$), p27 ($p = 0,0009$, $p = 0,0001$) e ciclina D3 ($p = 0,0189$, $p = 0,0217$) risultano essere fattori prognostici indipendenti, alla analisi multivariata, sia per la sopravvivenza globale che per quella libera da malattia. In merito alla coespressione di ciclina D1/p27 e di ciclina D1/D3 gli Autori selezionano un gruppo di pazienti a prognosi infausta sia per la sopravvivenza globale che per quella libera da malattia caratterizzati rispettivamente dai fenotipi $D1^+/p27^-$ ($p = 0,0015$, $p = 0,0001$) e $D1^+/D3^+$ ($p = 0,0002$). I dati riportati permettono di confermare un coinvolgimento delle cicline e di p27 nel processi di tumorigenesi del carcinoma squamocellulare della laringe, tanto da poter essere considerati markers clinico-prognostici aggiuntivi, da affiancare a quelli tradizionali, per selezionare sottogruppi di pazienti a prognosi sfavorevole che potrebbero essere sottoposti a trattamenti chirurgici più aggressivi, a protocolli complementari ed a follow-up più ravvicinati. Infine, anche se i dati della letteratura appaiono ancora preliminari, tali molecole sembrano essere il bersaglio di nuove terapie molecolari capaci di interferire su quei geni coinvolti nei complessi meccanismi del ciclo cellulare.

tions of biopsy samples may provide useful additional markers capable of identifying subgroups of patients with a poor prognosis who can be treated by means of more aggressive surgery, adjuvant radiotherapy and chemotherapy, as well as those requiring a closer and more prolonged follow-up. Finally, preliminary results suggest that the administration of new molecular therapies that exert their antitumoural activities by functionally subverting the pathways regulated by D-type cyclins and their cyclin-dependent kinase counterparts may represent a

Introduction

Laryngeal squamous cell carcinomas (LSCCs) account for approximately 2% of all of the cancers diagnosed annually in the Western world, and represent the most common tumours of the head and neck [1]. Despite recent developments in our understanding of cancer-regulating protein expression, improvements in surgical and radiation therapies, and the increased use of combined radio-chemotherapy, approximately 50% of the patients experience loco-regional recurrences, distant metastases or second primary tumours, which represent the most important causes of treatment failure [2-4]. Furthermore, as tumours with similar clinical and histo-pathological characteristics may have very different clinical outcomes, "traditional" prognostic factors alone (such as the primary tumour site, TNM staging and histological grading) seem to be inadequate in predicting the clinical history of LSCCs [5].

In order to improve the prediction of patient outcome, attempts have been made to develop new prognostic markers capable of distinguishing patients with a good prognosis from those who are more likely to relapse [6].

Tumour cells typically show acquired damage to the genes involved in controlling the cell cycle, particularly the G1/S restriction point [7], that is the best-known and most widely accepted point regulating the division of mammalian cells [8 9]. There is an overwhelming body of evidence indicating that the main event in the progression of the G1/S phase is phosphorylation of the retinoblastoma gene product (pRb), which overcomes the inhibition of the E2F family of transcription factors. The activity of E2F transcription factors permits the expression of the S phase-specific genes required for cell cycle progression [10 11], a critical checkpoint that is primarily regulated by a family of serine/threonine protein kinases consisting of a regulatory cyclin subunit and a catalytic cyclin-dependent kinase (CDK) subunit [12]. These kinases regulate the phosphorylation of pRB [13]. Unphosphorylated pRB inactivates transcription factors and prevents the G1/S transition, whereas phosphorylated pRB cannot bind transcription factors or promote cell cycle progression [14].

At least nine classes of cyclins and seven CDK catalytic subunits have been identified in mammalian cells [15]; two CDK subunits (CDK4 and CDK6) in combination with three D-type cyclins (D1, D2 and D3), and CDK2 in combination with cyclin E, are involved in G1/S progression and regulation [16].

Furthermore, molecular biological studies have recently revealed that CDKs are negatively regulated by a large group of CDK inhibitors, which can be divided into two families on the basis of their structural and functional properties: the INK4 family (which includes p15, p16, p18 and p19 and forms complexes with CDK4 and CDK6 and D-type cyclins) and the Cip/Kip family, which includes p21, p27 and p57, and its members are also known as universal CDK inhibitors because they inhibit the kinase activity of various CDK complexes [17]. These molecules form complexes with, and inactivate cyclins or CDKs, thus regulating cell cycle progression from G1 to S [7].

Several studies have demonstrated a relationship between tumourigenesis alterations in the expression of these cyclins, CDKs and CDK inhibitors that lead to the loss of cell cycle control especially during the G1/S phase [7 18 19].

p27

Progression from the G1 to the S phase of the cell cycle is regulated by the formation of cyclin/cyclin-dependent kinase complexes [7], but their kinase activity is inhibited by a number of specific proteins belonging to the INK4 and CIP/KIP families (p21, p27, p57) [20]. p27 can block this progression by binding cyclin E-cdk2 and cyclin A-cdk2 [21], and defective regulation of this major checkpoint may contribute to resistance against growth inhibitors, the deregulation cell proliferation, and oncogenic changes [7].

The p27 gene is located on chromosome 12p13 at the 12p12-12p13.1 junction [22], and is normally expressed in the nuclei of quiescent cells, whereas its activity is lost in actively proliferating cells since it responds to different signals and its level changes reciprocally as cells progress through G1, being high in quiescent cells and decreasing during the G0/S phase interval [23].

The intracellular levels of p27 increase in response to contact-dependent growth inhibition and a large number of extracellular anti-mitogenic signals and growth factors, such as transforming growth factor beta, cyclic AMP, lovastatin, rapamycin and tamoxifen [21 24-27].

It has been attributed with various functions. As the loss of p27 expression may lead to tumour development and progression, it has a potential function as a tumour suppressor gene [28-31] and may play a role in regulating drug resistance in solid tumours [32].

Although p27 mutations are rare in human tumours [33], recent studies indicate that its expression is less in a subset of tumours, and that this reduction is associated with an unfavourable prognosis. It is interesting to note that the lack of, or decreased expression of p27 has been found to be associated with metastases: it has been reported that the loss of p27 may give tumour cells the ability to grow in the presence of altered extracellular matrix properties and altered intercellular adhesion, both of which are conditions that may facilitate metastasis [32 34].

Furthermore, several authors have shown a correlation between p27 levels and poor prognosis in various human neoplasms [35-53].

Most authors have demonstrated the clinical relevance of p27 expression in head and neck squamous cell carcinoma (SCC). In patients with oral SCC, reduced p27 expression correlates with metastases [54], a poor prognosis [55] and an unfavourable treatment response [56]. Mineta et al., studied a group of 94 patients with SCC of the tongue, and showed that low p27 expression was statistically associated with nodal involvement and an advanced stage; at multivariate analysis, it was found to be a predictor of reduced survival [57].

In patients with muco-epidermoid cancer of the salivary glands, Okabe et al. observed that low p27 expression levels were a risk factor for worse disease-free survival [58].

This was confirmed by Choi et al., who reported a significant correlation between low p27 levels and clinico-pathological parameters, and found that multivariate analysis indicated that p27 expression was the most significant predictor of overall survival, with patients expressing low p27 showing a poor prognosis [59].

Finally, an association between p27 and prognosis has been observed in naso-pharyngeal carcinoma [60] and hypo-pharyngeal cancer [61].

Only a limited number of authors have investigated the clinico-prognostic role of p27 in LSCCs. Fan et al. used immunohistochemistry to study 109 patients, and found that the absence of p27 expression was statistically associated with an advanced clinical stage, lymph node involvement, and distant metastases; p27 also proved to be the most powerful prognostic marker both at univariate and multivariate analysis [62]. These findings were confirmed by Qin et al. and Tamura et al., who reported a significant correlation between low p27 levels and a poor prognosis in terms of overall and disease-free survival [63 64]. Korkmaz et al. recently found that p27 overexpression was a significant predictor of recurrence in a group of 68 patients who underwent external beam radiation for T1 and T2 laryngeal carcinomas [65].

D-Type cyclins

D-type cyclins are proteins involved in the cell cycle regulation that are essential for G1 phase progression, which act during the late G1 phase by complexing with cyclin-dependent kinases (CDK) [19]. The regulatory function of the cyclins cdk (CDK4 and CDK6) complex are due to the phoshorylation of the proteins involved in cell cycle control, such as pRb, and there is evidence that both cyclins and pRb belong to the same regulatory pathway [18] that releases the repression of E2F-dependent transcription and allows the expression of the genes required for progression to the S-phase of the cell cycle [66].

Cyclin D1 also indirectly promotes cell proliferation by sequestering p21 and p27, thus leading to the activation of CDK2 [67 68]. This protein degradation is mediated by phosphorylation-triggered, ubiquitin-dependent proteolysis [69].

The overexpression of cyclin D1 contracts the G1 phase, decreases cell size, and reduces the serum requirements for growth and the transition from the G1 to the S phase [70 71]. It has been demonstrated that cyclin D3 overexpression is capable of predisposing cells to malignant transformation, and that it is significantly associated with a worse prognosis.

DNA amplification is the most frequent abnormality affecting the CCND1 gene and, in the majority of the cases, correlates with the overexpression of cyclin D1 protein [72], a frequent event in a large number of primary human neoplasms and cell lines [73-88].

A correlation between cyclin D1 overexpression and prognosis has been reported for colorectal cancer [89-91], cell cancers of the urinary bladder [92], ovarian cancers [93], breast cancer [94], non-small cell lung cancer [95 96], and oesophageal cancer [97-100].

Cyclin D1 overexpression or CCND1 gene amplification have been detected in 35-64% of head and neck SCC [101-105], with significant differences between tumours at different anatomic sites in the head and neck region: it has been reported that cancers of the hypopharynx show gene amplification more frequently than those of the larynx and the oral cavity [106-109].

Moreover, tumour overexpression of cyclin D1 has been associated with the development of multiple primary upper aerodigestive tract carcinomas [110],

which represents one of the most important and unsolved problems associated with head and neck cancer, may occur in up to 15% of patients, and particularly affects malignant neoplasms of the hypo-pharynx and oesophagus [111-113].

Several authors have found that the cyclin D1 status is associated with the recurrence of head and neck SCC [102-105], and survival [103 107 114-116].

Only a limited number of studies on LSCCs have investigated the clinical importance of cyclin D1 overexpression, and the data are somewhat conflicting. In a retrospective study of 102 patients, Dong et al. found that it was significantly associated with tumour site and size, lymph node metastases, an advanced clinical stage, and poor disease-free and overall survival. Furthermore, multivariate analysis showed that this was an independent predictor of disease-free survival [117]. Similar results were obtained by Krecicki et al. [118] and Bellacosa et al. [119], who found a significant correlation between cyclin D1 gene amplification and shorter overall survival. Finally, Wang et al. carried out immunohistochemical studies on the overexpression of cyclin D1 in 92 patients with laryngeal carcinoma, and found significantly higher levels of cyclin D1 in those experiencing local recurrences [120].

On the contrary, Ioachim et al. found no significant differences in the risk of recurrence or overall survival between cyclin D1 positive and negative tumours either at univariate or multivariate analysis, although the levels of cyclin D1 were significantly higher in invasive than in *in situ* laryngeal carcinomas [121]. Similar results were obtained by Vielba et al. [122] and El-Naggar et al., although the latter considered both oral and laryngeal SCCs together [123].

As far as concerns the simultaneous involvement of these genes, it has been reported that there is an inverse correlation between p27 and cyclin D1 expression, thus suggesting that the absence of p27 expression may be due to sequestration by cyclin D1 and that the balance of these two opposing regulators of the cell cycle may be a determinant factor in cell proliferation. It has also been reported that high p27 levels are frequently associated with high cyclin D1 levels in cancer cell lines and tumours, and that the administration of anti-sense cyclin D1 cDNA in cancer cells with the aim of reducing cyclin D1 expression also reduced p27 levels. These data suggest the existence of a feedback loop between cyclin D1 and p27, the purpose of which is to maintain a homeostatic balance between the positive and negative regulators of the G1-S transition in the cell cycle [124-126] or that an alteration in the ubiquitine-proteasome pathway may affect the levels of p27 and cyclin D1 as both are degraded by the same pathway [127 128].

In vitro and *in vivo* studies have demonstrated an association between p27 and cyclin D1 expression in various neoplasms [129-131], and an association between p27/cyclin D1 co-expression and prognosis has been observed in extra-hepatic bile duct carcinoma [132], papillary thyroid carcinoma [49], ovarian tumours [133] and LSCCs [134].

Personal contribution

We have investigated the prognostic importance of cyclin D1 in 149 patients [135] and p27 in 132 patients [136] who underwent surgical treatment for primary LSCC at the Otorhinolaryngology Clinic of the Milan School of Medicine. Furthermore, we have also evaluated cyclin D3 immunoreactivity in 223 formalin-fixed and paraffin-embedded LSCC specimens [137].

Cyclin D1

Cyclin D1 protein expression was assayed by means of the avidin-biotin peroxidase complex method, with only those cases expressing strong immunoreactivity, in more than 5% of cells, being considered positive. Cyclin D1 immunoreactivity was observed in 48 cases (32.2%), with an exclusively nuclear pattern of immunostaining. Cyclin D1 overexpression was significantly higher in T_3-T_4 than in T_1-T_2 tumours ($p = 0.016$), tumours in stage III-IV than in those in stage I-II ($p = 0.026$), and in cases with lymph node metastases than in those without ($p = 0.014$). No significant correlation was found between cyclin D1 and age, anatomical site or histological grade. Univariate analysis showed that shorter disease-free and overall survival were significantly associated with anatomical site ($p = 0.0242$ and $p = 0.0196$), tumour extension ($p = 0.0001$ and $p = 0.0008$), clinical stage ($p = 0.0005$ and $p = 0.0028$) and cyclin D1 overexpression ($p = 0.0005$ and $p = 0.0152$), but multivariate analysis showed that only tumour extension ($p = 0.0008$) and cyclin D1 overexpression ($p = 0.0238$) were statistically significant predictors of disease-free survival. Overall survival significantly correlated with the anatomical site ($p = 0.0340$), tumour extension ($p = 0.0063$) and cyclin D1 ($p = 0.0622$, slightly over the threshold of significance).

p27

In order to evaluate p27 protein expression, tumour sections were immunostained with the anti-p27 1B4 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) monoclonal antibody, with a cut-off value of 50% of cells. Exclusively nuclear p27 overexpression was found in 82 cases (62.1%). High p27 levels (> 50% of neoplastic cells) were significantly more frequent in T_1 to T_2 tumours ($p = 0.005$) and in those presenting clinical stages I-II ($p = 0.018$), and were also more frequent in glottic tumours ($p = 0.080$, slightly above the threshold of statistical signifi-

cance). Univariate analysis showed that shorter disease-free and overall survival significantly associated with anatomic site ($p = 0.0417$ and $p = 0.0422$), tumour extension ($p = 0.0001$ and $p = 0.0042$), clinical stage ($p = 0.0004$ and $p = 0.0072$), and low p27 expression ($p = 0.0015$ and $p = 0.0127$). At multivariate analysis, p27 was the only statistically significant independent predictor of disease-free and overall survival ($p = 0.0001$ and $p = 0.0009$).

CYCLIN D3

Cyclin D3 protein expression was assayed on 3 µm thick sections penetrated with an antigen retrieval solution and then incubated with the anti-cyclin D3 monoclonal antibody DCS-22 (Novocastra, Newcastle upon Tyne, UK); only the cases showing ≥ 10% of immunoreactive neoplastic cells were considered positive.
A total of 88 (39.5%) out of the 223 tumours analysed were positive; of the remaining 135 cases, 70 (52%) showed cyclin D3 imunoreactivity in 1-9% of neoplastic cells and 65 (48%) were non-reactive.

Overall survival

Univariate analysis showed that an advanced clinical stage ($p < 0.0001$), low performance status ($p < 0.0001$), high tumour grade ($p = 0.001$), nodal metastases ($p = 0.0061$), a supraglottic site ($p = 0.0069$) and cyclin D3 immunoreactivity ($p = 0.0435$) were significantly associated with reduced overall survival; indeed, the 5-year overall survival probability for patients with tumours showing ≥ 10% and < 10% of immunoreactive neoplastic cells was, respectively, 0.61 and 0.70 (log rank $p = 0.0427$). At multivariate analysis, a low performance status ($p < 0.0001$), cyclin D3 immunoreactivity ($p = 0.0189$), an exophytic/ulcerating tumour type ($p = 0.0351$), and high tumour grade ($p = 0.0450$) were independent predictors of greater mortality.

Disease-free survival

Univariate analysis showed that an advanced clinical stage ($p < 0.0001$), low performance status ($p 0.0001$), high tumour grade ($p = 0.001$), nodal metastases ($p = 0.0043$), a supraglottic site ($p = 0.0065$), and cyclin D3 immunoreactivity ($p = 0.0545$) at borderline level of statistical significance, were associated with reduced disease-free survival; indeed, the 5-year disease-free survival probability for patients with tumours showing ≥ 10% and < 10% of immunoreactive neoplastic cells, was respectively, 0.61 and 0.69 (log rank $p = 0.0535$). At multivariate analysis, a low performance status ($p < 0.0001$), cyclin D3 immunoreactivity ($p = 0.0217$), high tumour grade ($p = 0.0223$), and an exophytic/ulcerating tumour type ($p = 0.0336$) were independent predictors of reduced disease-free survival.

CO-EXPRESSION

As far as concerns cyclin D1/p27 co-expression, low p27 levels were found in 22 of the 45 cyclin D1-positive cases (cyclin $D1^+/p27^-$), and in 28 of the 87 cyclin D1-negative cases (cyclin $D1^-/p27^-$), an inverse correlation that was slightly above the level of statistical significance ($p = 0.092$); of the 82 remaining patients, 23 had a cyclin $D1^+/p27^+$ phenotype and 59 a cyclin $D1^-/p27^+$ phenotype. Three classes of prognostic clinical relevance for disease-free and overall survival were obtained from the interaction of cyclin D1 and p27 expression in a Cox model using the 95% confidence interval (95% CI) of the relative risk (RR): cyclin $D1^-/p27^+$ had the best prognosis, cyclin $D1^+/p27^+$, cyclin $D1^-/p27^-$ an intermediate prognosis, and cyclin $D1^+/p27^-$ the poorest prognosis ($p = 0.0001$ and $p = 0.0001$ for trend for disease-free survival; $p = 0.0015$ and $p = 0.0008$ for trend for overall survival); in particular, the RR of tumour recurrence was 5.94 in the cyclin $D1^+/p27^-$ patients and 2.74 in the cyclin $D1^+/p27^+$ or cyclin $D1^-/p27^-$ patients versus the cyclin $D1^-/p27^+$ patients. Finally, with regard to overall survival, the RR was 3.60 in the cyclin $D1^+/p27^-$ patients and 1.72 in the cyclin $D1^+/p27^+$ or cyclin $D1^-/p27^-$ patients versus the cyclin $D1^-/p27^+$ patients.
In relation to cyclin D1/cyclin D3 co-expression, the patients with cyclin $D1^+$/cyclin $D3^+$ tumours experienced the poorest overall survival, those with cyclin $D1^-$/cyclin $D3^+$ or cyclin $D1^+$/cyclin $D3^-$ tumours – an intermediate course, and those with cyclin $D1^-$/cyclin $D3^-$ tumours – the most favourable outcome (log rank $p = 0.0002$). In a multivariate model adjusted for sex and clinical stage, the patients with cyclin $D1^-$/cyclin $D3^+$ or cyclin $D1^+$/cyclin $D3^-$ tumours had a risk of death of 2.71, and the patients with cyclin $D1^+$/cyclin $D3^+$ tumours of 4.11, in comparison with those with cyclin $D1^-$/cyclin $D3^-$ tumours. Similar data were observed for disease-free survival.

Discussion

Traditional prognostic factors, such as the primary tumour site, tumour stage and histological grade, fail to predict the clinical outcome of individual patients with LSCCs. Consequently, new prognostic factors, such as the biological expression of cell-cycle regulators, have been investigated in an attempt to improve the accuracy of predicting tumour behaviour since malignant transformations may occur as a result of alterations in the genes, cyclins and cyclin-dependent kinase inhibitors that directly control the cell cycle and mitosis.
p27 is a potential prognostic factor since its down-regulation has been associated with poor prognosis in head and neck cancer. In keeping with reports in the

literature, we found that low p27 levels are significantly associated with the unfavourable clinicopathological parameters of tumour extension and an advanced clinical stage, as well as with tumour recurrence and reduced overall survival.

We found cyclin D1 over-expression in 32.2% of laryngeal carcinomas, which suggests a significant association with prognosis. It has been reported that cyclin D1 over-expression has an adverse effect on the clinical outcome of patients with various epithelial malignancies, and most of the studies investigating the clinical relevance of cyclin D1 aberrations in head and neck cancer have reported a significant association with reduced disease-free and overall survival. In agreement with others [138 139], we found a significant association between cyclin D1 over-expression and tumour recurrence, and a borderline statistical association with reduced overall survival.

As far as concerns cyclin D3, we provided the first evidence that cyclin D3 immunoreactivity is an independent predictor of poor disease-free and overall survival in LSCC patients. It has been reported that cyclin D3 immunoreactivity is an independent predictor of survival in patients with malignant melanoma [140] and non-Hodgkin's lymphoma [141], and that high levels of cyclin D3, measured by means of Western blotting, are significantly associated with a poor outcome in patients with breast cancer [142].

We also analysed, the clinical behaviour of patients classified on the basis of p27 and cyclin D1 expression, and found that those with low cyclin D1 and high p27 levels had the best disease-free and overall survival. These findings are further supported by the presence of a trend both in disease-free and overall survival, and lead to the speculation that multiple alterations in the genes involved in cell-cycle control are associated with more aggressive tumours.

We provide evidence that patients with tumours that are immunoreactive for one or both D cyclins are at an increasing risk of progression and death; in particular, the deregulation of both cyclin D1 and cyclin D3 is an even more powerful predictor of a dismal prognosis than node status and clinical stage.

In conclusion, our data suggest that the immunohistochemical evaluation of p27, cyclin D1 and cyclin D3 expression in LSCCs may be useful markers for selecting subgroups of patients with a poor prognosis who can be treated with more aggressive surgical approaches, adjuvant radiotherapy and chemotherapy, as well as for identifying those requiring a closer and more prolonged follow-up.

Various experimental studies and clinical trials have shown that the modulators of cyclin-dependent kinases enhance the radiosensitivity of tumoural cells [143], and may be able to arrest cells in the G1-G2 phases as a result of cyclin depletion and over-expression of cyclin-dependent kinase inhibitors [144].

It is now believed that altered and deregulated cyclin and cyclin-dependent kinase activity play a major role in the pathogenesis of head and neck SCC, and may, therefore, be suitable cell targets for pharmacological strategies and other therapeutic approaches. The observation that p27 gene transfer has a proliferation inhibiting effect on human head and neck SCC cell lines by adenoviral vector suggests the possibility of developing a new cancer gene therapy modality [145].

Moreover, flavoperidol (HMR 1275) and UCN-01 (7-hydroxy-staurosporine) have recently been identified as novel anti-neoplastic agents that exert their anti-tumoural activities by functionally subverting the pathways regulated by D-type cyclins and their cyclin-dependent kinase counterparts: flavoperidol inhibits most CDKS and has unique anticancer properties, and UCN-01 is known as a protein kinase C and CDK modulator, and has anti-proliferative and anti-tumour properties in many experimental tumour models. Interestingly, it has been shown that both compounds are effective in reducing tumour size in a mouse model of head and neck SCC [146-148], and that these effects are associated with a decrease in cyclin D3 and an increase in p27 levels. The fact that these provide appropriate surrogate markers of treatment efficacy *in vivo* make them suitable candidate for treating head and neck SCC patients. Accordingly, phase I trials of flavoperidol and UCN-01 combined with chemotherapy have already been designed in several tumour types, including head and neck SCC [149].

In brief, these preliminary results suggest that the administration of new molecular therapies may offer a further treatment approach for patients with refractory head and neck ACC, who could be recruited for clinical trials designed to monitor their efficacy.

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