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The predictive value of p53, MDM-2, cyclin D1 and Ki67 in the progression from low-grade dysplasia towards carcinoma of the larynx

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Abstract

To evaluate the predictive role of the oncogenes p53, MDM-2 and cyclin D1, and the proliferative marker Ki67, in the progression from low-grade dysplasia to carcinoma of the larynx. We studied immunohistochemically a series of 32 low-grade pre-neoplastic laryngeal lesions, 10 of which progressed to invasive carcinoma. Immunoreactivity in more than 10 per cent of the dysplastic cells was detected in five cases immunostained with anti-p53 (≈ 15 per cent), in two with anti-MDM-2 (≈ six per cent), and 11 with anti-Ki67 antibodies (≈ 34 per cent), whereas none of the cases showed cyclin D1 overexpression. No significant association was found between p53 and MDM-2 immunoreactivity and the evolution to carcinoma; on the contrary, Ki67 expression was detectable in all but one of the 10 cases developing an infiltrative tumour (90 per cent), and in two of the 22 cases that did not progress (≈ nine per cent) ($p = 0.01$). These findings indicate that immunohistochemical assessment of the proliferative index in bioptic samples of dysplastic laryngeal mucosa may be useful in selecting patients who should undergo a more specific follow-up evaluation.

Key words: Oncogenes; Tumour markers, biological; Immunohistochemistry; laryngeal neoplasms

Introduction

The model of laryngeal tumorigenesis is based on the sequence of dysplasia-carcinoma, as confirmed by the fact that dysplastic changes in the laryngeal mucosa often precede frank malignancy (Shin *et al.*, 1994). Unfortunately, the histological assessment of the degree of dysplasia does not seem to be a sufficient means of accurately predicting the evolution of laryngeal preneoplastic lesions towards infiltrative carcinoma (Crissman and Fu, 1986; Blackwell *et al.*, 1995). This is particularly true in the case of low-grade lesions (mild and moderate dysplasia), given that only a small proportion of them progress towards malignancy (Blackwell *et al.*, 1995).

It has been suggested that the process of tumorigenesis in the larynx is based on the accumulation of various stages of genetic damage, which frequently affect the genes involved in cell cycle regulation (Bartkova *et al.*, 1995). The p53 and cyclin D1 genes play a central role in the G1 phase of the cell cycle: in response to genotoxic stress, p53 transcriptionally activates the p21 gene, which is able to inhibit the formation of cyclin-dependent kinases and thus block the cell cycle in G1 (Chen *et al.*, 1995). If the DNA damage is irreparable, p53 promotes programmed cell death by inducing an increase in Bax and a decrease in bcl-2 protein expression (Kernohan and Cox, 1996). These p53-regulated pathways are frequently inactivated by gene mutations and binding to viral or cellular proteins during the early stages of tumorigenesis (Vogelstein and Kinzler, 1992). In this context, there is evidence that the product of the human homologue of the murine double minute 2 gene (MDM-2), which is frequently amplified in human tumours, is capable of binding to p53 and inhibiting its ability to activate transcription by concealing its activation domain (Oliner *et al.*, 1993).

It has been suggested that the disruption of the pathways regulated by p53 and cyclin D1 may account for increased cell proliferation, which in turn may favour the occurrence of additional genetic lesions that have a potentially oncogenic effect (Quelle *et al.*, 1993). Proliferating cells express the non-histone nuclear Ki67 proteins of 345 and 395 kd (Gerdes *et al.*, 1992). Ki67 expression is lacking during the G0 phase of the cell cycle and it is

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therefore currently used for the immunohistochemical identification of the proliferative rate in human tumours (Brown and Gatter, 1990). There is evidence that tumours with a high proliferative index are more aggressive and have greater metastatic potential (Tunkegar *et al.*, 1991; Sarbia *et al.*, 1996). A number of studies have likewise indicated that Ki67 expression increases with the severity of dysplastic changes in pre-neoplastic lesions (Girod *et al.*, 1993).

It has been demonstrated that the immunohistochemical approach may be a reliable method for detecting alterations in the genes regulating the cell cycle in laryngeal tumours: p53, cyclin D1 and MDM-2 overexpression has been found not only in infiltrative tumours, but also in adjacent preneoplastic lesions and histologically normal mucosa, thus prompting the hypothesis that p53 and cyclin D1 alterations may be early events in the multistep tumorigenesis process of laryngeal cancer (Dolcetti *et al.*, 1992; Pruneri *et al.*, 1996). Overexpression of p53 has also been found to be associated with progression from dysplasia to invasive carcinoma (Sauter *et al.*, 1994; Polkowski *et al.*, 1995). These data suggest that immunohistochemical analysis may be more reliable than traditional histological evaluation in the prediction of progression from dysplastic lesions towards infiltrative carcinoma.

In order to evaluate the putative predictive value of p53, MDM-2, cyclin D1, and Ki67 in relation to the progression from low-grade dysplasia towards carcinoma of the larynx, we studied their expression in pre-neoplastic lesions, and compared the results obtained in the cases that progressed with those in the cases that did not.

Materials and methods

Pathological samples

Bioptic samples of low-grade dysplastic laryngeal mucosa of the glottic region taken from 32 patients (14 with mild and 18 with moderate dysplasia) between 1982 and 1991 were selected from the files of Clinica Otorinolaringoiatrica I of the University of Milan. The patients were followed up for at least five years by means of clinical evaluation and/or direct laryngoscopy (every three months during the first year after the histological diagnosis of dysplasia and subsequently every year). The patients with a previous head and neck cancer or who had received radiation therapy were excluded. Smoking and drinking habits were also evaluated. The dysplastic changes were histologically assessed according to Shanmugaratnam (1991) and Blackwell (1995).

Immunohistochemistry

All of the samples were routinely fixed in 10 per cent buffered formalin and embedded in paraffin wax. For the immunolocalization of the p53, cyclin D1, MDM-2 and Ki67 proteins, a standard avidin-biotin peroxidase method was used as previously described (Pruneri *et al.*, 1997). The sources, clones and working concentrations of the antibodies used

TABLE I

CLINICAL AND IMMUNOHISTOCHEMICAL FEATURES OF THE LARYNGEAL LOW-GRADE PRE-NEOPLASTIC LESIONS INCLUDED IN THE STUDY

Case number	Gender	Age	Risk factors	p53	Cyclin D1	mdm2	Ki67	Progression
1	M	67	Yes	–	–	–	+	Y
2	M	55	Yes	–	–	–	–	N
3	M	56	Yes	–	–	–	+	N
4	M	64	Yes	–	–	–	+	Y
5	M	54	Yes	+	–	–	–	N
6	M	53	Yes	–	–	–	–	N
7	M	58	Yes	–	–	–	+	Y
8	M	41	Yes	–	–	–	–	N
9	M	68	Yes	–	–	–	+	Y
10	M	71	Yes	+	–	+	–	N
11	M	49	Yes	–	–	–	–	N
12	M	43	Yes	–	–	–	–	N
13	M	78	Yes	–	–	–	–	N
14	M	80	Yes	–	–	–	+	Y
15	M	75	Yes	–	–	–	–	N
16	M	48	No	–	–	–	–	N
17	M	72	Yes	+	–	–	+	Y
18	M	51	Yes	–	–	+	–	N
19	M	59	Yes	–	–	–	+	Y
20	M	69	Yes	–	–	–	+	Y
21	M	55	Yes	–	–	–	+	N
22	M	55	Yes	–	–	–	–	N
23	M	22	Yes	–	–	–	–	N
24	M	62	Yes	–	–	–	–	N
25	M	54	Yes	+	–	–	–	N
26	M	79	Yes	–	–	–	–	N
27	M	49	No	–	–	–	–	N
28	M	59	No	–	–	–	–	N
29	F	38	Yes	–	–	–	–	Y
30	M	69	Yes	+	–	–	–	N
31	M	46	Yes	–	–	–	–	N
32	M	50	Yes	–	–	–	+	Y

–: unreactive or less than 10 per cent of immunoreactive dysplastic cells; +: more than 10 per cent of immunoreactive cells; N: not progressed; Y: progressed in laryngeal squamous cell carcinoma.

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are summarized in Table I. For antigen retrieval, the slides were treated with proteolytic digestion with 0.01 per cent Pronase solution (Dako S2013) (for cyclin D1), or with three (for p53 and Ki67) to six (for MDM-2) five-minute cycles of five minutes in a 780W microwave oven at 90°. The bioptic samples were considered positive if more than 10 per cent of the dysplastic cells proved to be immunoreactive to the antibodies used; the cases expressing only a few scattered immunoreactive cells were considered negative.

Statistical analysis

The statistical analysis was carried out by means of the Chi-square method with Yates' correction and/or Fisher's exact test (Armitage and Berry, 1991).

Results

The clinico-pathological features of the patients included in the study are summarized in Table I: all but one were male; and their mean age was 57.8 years (median 55.5). Moreover, all but three of them had risk factors. No significant association was found when comparing the clinical data of age ($\leq$50 years *vs* >50 years), gender and risk factors (smoking and drinking habit) with the progression towards malignancy.

Ten of the 32 patients developed an infiltrative squamous cell carcinoma within a mean period of 16.7 months from the first biopsy (range 2–41 months, median 11 months); the remaining 22 are still free of disease. The histological diagnosis of the first biopsy in the 10 patients who developed infiltrative carcinoma, was mild (four cases) and moderate dysplasia (six cases); of the 22 patients who did not progress, 10 were diagnosed as having mild, and 12, moderate, dysplasia ($p = 0.9$).

Five of the patients who developed infiltrative carcinoma underwent more than one laryngoscopy with biopsy (range 1–6, mean 1.4, median 1): evidence of high grade dysplasia was found in the subsequent biopsies of three of them (case numbers 17, 20 and 32). No difference in the immunohistochemical results was appreciable when the low-grade and high-grade pre-neoplastic lesions of the same patient were compared.

The immunohistochemical results are shown in Table I. Most of the analysed samples expressed only a few scattered cells that were immunoreactive to the antibodies used, but, nuclear immunoreactivity was detectable in more than 10 per cent of the dysplastic cells of five samples immunostained with anti-p53 (five out of 32, $\approx$ 15 per cent), two with anti-MDM-2 ($\approx$ six per cent) and 11 with anti-Ki67 antibodies (11/32, $\approx$ 34 per cent); furthermore, one of the two MDM-2 positive cases also showed p53 accumulation. Whatever the antibody used, the pattern of immunostaining was limited to the nucleus, with an intensity that ranged from weak to strong. None of the cases expressed cyclin D1 protein in more than 10 per cent of the dysplastic cells.

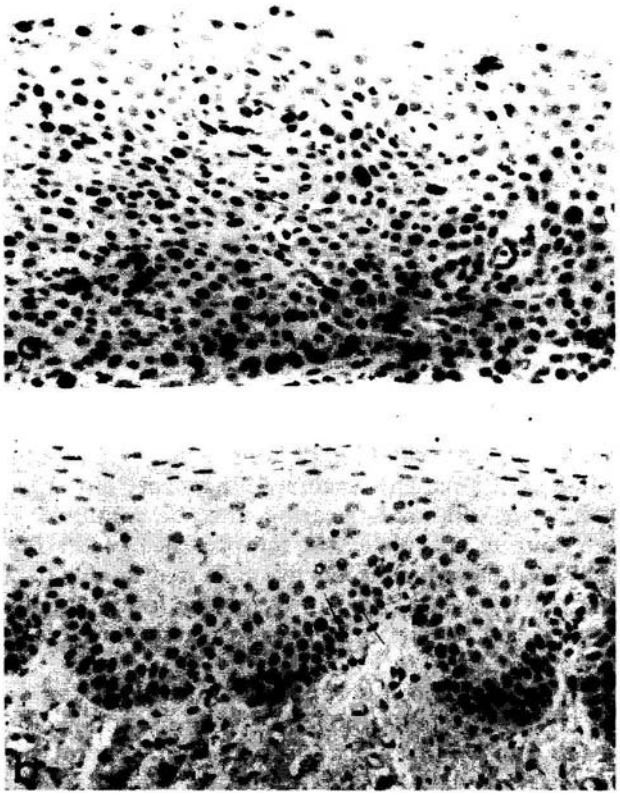

FIG. 1

An example of high (a) and low (b) Ki67 expression in samples of low-grade dysplasia (H & E; ×250).

Ki67 expression was detectable in all but one of the 10 patients who developed an infiltrative tumour (nine out of 10, 90 per cent), and in two of the 22 who did not progress ($\approx$ nine per cent). This finding was statistically significant when tested using the Chi-square method with Yates' correction ($p = 0.01$). The immunoreactivity of p53 in more than 10 per cent of the dysplastic cells was found in one of the cases which progressed towards infiltrative carcinoma (10 per cent) and in three cases which did not ($\approx$ 13 per cent). Finally, none of the MDM-2 positive cases progressed towards carcinoma.

Discussion

It has been suggested that the traditional morphological evaluation of the laryngeal pre-neoplastic lesions may not be sufficient to predict their evolution to infiltrative carcinoma (Blackwell *et al.*, 1995). An important component of the multistep tumorigenesis process may be the accumulation of genetic lesions involving genes related to the cell cycle such as p53, cyclin D1, MDM-2. On the basis of these considerations, we investigated which of these molecular alterations might be clinically useful to evaluate the progression of laryngeal low-grade dysplasia.

In the present series, five cases were positive for p53 ($\approx$ 15 per cent), two for MDM-2 ($\approx$ six per cent), and 11 for Ki67 antibodies ($\approx$ 34 per cent), whereas none of the cases showed cyclin D1 overexpression.

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Our data indicate that p53 overexpression is not useful for predicting the progression from dysplasia to infiltrative carcinoma: p53 expression was present in 10 per cent of the patients who progressed to carcinoma and ≈ 13 per cent of those who did not. This finding is partly in contrast with those of others (Sauter *et al.*, 1994; Uhlman *et al.*, 1996) who have found that p53 overexpression may identify cases prone to neoplastic progression. This discrepancy may be reasonably ascribed to the fact that we established a threshold for the evaluation of the immunohistochemical results. Commercially available antibodies recognize both mutated and wild-type forms of the p53 protein, and it is known that the latter increase in response to genotoxic stresses, such as tobacco smoking (Ahomadegbe *et al.*, 1995). Most of the patients included in the present study were smokers and, bearing this in mind, it is tempting to speculate that the presence of the few scattered p53 immunoreactive cells may simply be a paraphysiological event in dysplastic lesions of the larynx, rather than a hallmark of neoplastic transformation.

It has been recently reported that overexpression of MDM-2 protein, which is capable of binding and inactivating p53, frequently occurs in hyperplastic and pre-neoplastic lesions of the oral mucosa (Girod *et al.*, 1993). We have also recently found that MDM-2 overexpression is significantly associated with p53 protein accumulation in dysplastic lesions adjacent to infiltrative squamous cell carcinomas of the larynx (Pruneri *et al.*, 1997). In the present series, one of the two cases expressing MDM-2 protein in more than 10 per cent of the neoplastic cells also showed p53 accumulation. Given our small number of MDM-2 positive cases, we believe that further studies of larger series are needed to establish the putative predictive role of p53 and MDM-2 coexpression in the progression of dysplastic lesions to invasive carcinoma.

Our results indicate that Ki67 expression in low-grade dysplastic lesions of the larynx is significantly associated with neoplastic progression: a high proliferative index was detectable in all but one of the 10 patients who developed an infiltrative tumour, and in only two of the 22 who did not ($p = 0.01$). It has been suggested recently that the immunohistochemical evaluation of Ki67 expression may help in classifying dysplastic lesions of the cervical mucosa (Bulten *et al.*, 1996), and there is also evidence that Ki67 levels increase with the severity of dysplastic changes in laryngeal, oesophageal and oral epithelium (Girod *et al.*, 1993; Polkowski *et al.*, 1995; Zidar *et al.*, 1996). Nevertheless, this is the first report in which the role of Ki67 expression in predicting the progression from laryngeal preneoplastic lesions towards carcinoma has been investigated. Although obtained in a relatively small number of cases, our data indicate that the immunohistochemical assessment of the proliferative index in the bioptic samples of dysplastic laryngeal mucosa may be a useful means of identifying subgroups of patients who should undergo further follow-up evaluation.

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