Laryngeal squamous cell carcinoma (LSCC) is the most common type of head and neck squamous cell carcinoma (HNSCC). LSCC accounts for 1–2% of all malignancies diagnosed worldwide (Vokes et al, 1993; Licitra et al, 2003; Mao et al, 2004). Notwithstanding primary prevention, screening, surgical treatment, and radiotherapy, the long-term survival rate of LSCC patients has remained substantially unchanged in the last two decades (Hoffman et al, 1998). Survival of the patients depends on the stage of the disease; therefore, early detection and timely therapy are essential (Vokes et al, 1993; Hoffman et al, 1998; Licitra et al, 2003; Mao et al, 2004).

Laryngeal squamous cell carcinoma usually develops in a multistep process: normal mucosa – dysplasia (laryngeal intraepithelial neoplasia, LIN) – LSCC in situ – invasive LSCC (Rosai et al, 1992; Tabor et al, 2002; Zuckerberg, 2002; Johnson, 2003). Dysplasia is characterised by increased cell growth, cellular atypia (nuclear and nucleolar abnormalities, altered nuclear/cytoplasmic ratio, and altered cytoplasmatic differentiation), and architectural alteration of the epithelium. Conventionally, the dysplastic changes are graded as mild (LIN I: dysplasia limited to the basal third of the epithelium, few mitoses), moderate (LIN II: dysplasia involving the lower two-thirds of the epithelium, marked nuclear changes, prominent nucleoli, mitoses in the parabasal, and intermediate layers), and severe (LIN III: dysplasia involving more than two-thirds of the epithelial thickness, nuclear pleomorphism and hyperchromasia, prominent nucleoli, cell crowding, and atypical mitoses). Often, severe dysplasia and in situ carcinoma are grouped in the same category (Rosai et al, 1992; Tabor et al, 2002; Zuckerberg, 2002; Johnson, 2003). Early forms of dysplasia may be reversible if the initial stimuli (like smoke and volatile irritating substances) are removed, while severe dysplasia, if left untreated, is regarded as a precancerous lesion (Rosai et al, 1992; Tabor et al, 2002; Zuckerberg, 2002; Johnson, 2003). For patients with mild or moderate dysplasia, the reported rate of progression to invasive cancer is up to 11.5 and 45%, respectively. In severe dysplasia, higher rates of progression are commonly reported (Rosai et al, 1992; Tabor et al, 2002; Zuckerberg, 2002; Johnson, 2003). The molecular events that induce the evolution of dysplasia to carcinoma are still unknown (Cowan et al, 1992; Zuckerberg, 2002; Perez-Ordonez et al, 2006).

Osteopontin (OPN), also known as SPP1 (secreted phosphoprotein 1), is a highly acidic calcium-binding glycosylated phosphoprotein (Weber, 2001; Rittling and Chambers, 2004; Rangaswami et al, 2006). OPN can function both as cell adhesion molecule and as cytokine. It binds to the cell surface receptors $\alpha_v\beta_3$-containing integrins and CD44v6 (Weber, 2001; Rittling and Chambers, 2004; Rangaswami et al, 2006), thereby supporting proliferation, chemotaxis, attachment, and migration of many cell types. CD44 is a cell surface glycoprotein that is involved in regulating cell–cell and cell–matrix interactions, migration, and tumour growth and progression (Ponta et al, 2003). CD44 is overexpressed in laryngeal dysplasia and correlation with clinical outcome

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expressed as a standard receptor (CD44s) and in multiple splice isoforms (CD44v), whose expression is altered during tumour growth and progression. Expression of the ‘v6’ variant exon of CD44 is necessary for OPN binding (Ponta et al, 2003). OPN is overexpressed in many human tumours, for example, colon, breast, liver, prostate, gastric, ovarian, lung, thyroid, and kidney carcinomas (Agrawal et al, 2002; Kang et al, 2003; Ye et al, 2003; Schorge et al, 2004; Donati et al, 2005; Guarino et al, 2005; Matusan et al, 2006).

We recently reported that OPN and CD44v6 are overexpressed in full-blown LSCC (Celetti et al, 2005). Here, we have investigated the role of the OPN/CD44v6 axis in laryngeal dysplasia.

MATERIALS AND METHODS

Study population

Patients (82 cases: 77 men and 5 women) underwent surgery at the Otolaryngology Department of the University Federico II of Naples between January 1993 and December 2001. The patients’ age ranged from 23 to 83 years, with a mean of 62.46 years. Paraffin blocks were retrieved from the files of the Department of Biomorphological and Functional Sciences, Pathology Section, University Federico II of Naples. Each patient agreed to and signed a consent for the treatment of clinical data and tissues for diagnostic and research purposes, according to the guidelines of the Institutional Ethic Committee. For all the patients, clinicopathologic and follow-up data were recorded (Table 1). Before surgery, patients underwent otolaryngological, fiberoptic, and radiological evaluation. The mean follow-up time was 10.1 years (range: 8 – 13 years). Follow-up consisted in clinical and radiological examination. The pathologic analysis was performed in a blinded fashion to the clinical informations. The cytological evaluation was fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Sections (4-mm thick) were stained with haematoxylin – eosin for histological examination. The pathologic analysis was performed in a blinded fashion to the clinical informations. The cytological evaluation was performed according to standard criteria (Evans et al, 1986; Gale et al, 2000; Tabor et al, 2003).

Table 1 Clinicopathological features of studied laryngeal intraepithelial neoplasia (LIN) patients

| Characteristics       | Total (%) |
|-----------------------|-----------|
| No. of subjects       | 82 (100)  |
| Male                  | 77 (94)   |
| Female                | 5 (6)     |
| Disease site          |           |
| Glottis – hypoglottis | 43 (53)   |
| Supraglottis          | 39 (47)   |
| Degree of dysplasia   |           |
| Mild                  | 21 (47)   |
| Moderate              | 8 (19)    |
| Severe                | 53 (64)   |
| Relapse               |           |
| No                    | 35 (43)   |
| LIN                   | 10 (12)   |
| SCC                   | 37 (45)   |

SCC = squamous cell carcinoma.

Immunohistochemistry

Four-micromolar thick serial sections, mounted on poly-l-lysine-coated glass slides, were dewaxed, rehydrated through multiple graded ethanol solutions, treated with 3% hydrogen peroxide for 5 min to inactivate endogenous peroxidases, and washed in distilled water. After antigen retrieval (microwave oven 5 min × 3 times, in 1% citrate buffer), nonspecific binding was blocked by incubation (2 h at room temperature) with 1.5% blocking serum. Slides were first incubated with anti-OPN (final concentration: 5 µg ml⁻¹) (10A16; Assay Designs, Ann Arbor, MI, USA) or anti-CD44v6 (dilution of 1 : 100) (NCL-CD44v6, clone VFF-7; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) monoclonal antibodies and then with biotinylated anti-IgG and the premixed avidin – biotin complex (overnight at 4°C) (Vectastain ABC kits; Vector Laboratories, Burlingame, CA, USA). The immune reaction was revealed with 0.06 mmol l⁻¹ diaminobenzidine (DAB-DAKO, Carpinteria, CA, USA) and 2 mmol l⁻¹ hydrogen peroxide. Finally, slides were counterstained with haematoxylin and coverslipped with a synthetic mounting media. Control slides in the presence of preimmune serum were included for each staining as an additional negative control. Anti-OPN antibody was preincubated with a fivefold molar excess of OPN peptide to ascertain specificity of the reaction.

The results of the immunohistochemical staining were evaluated separately and in a blinded fashion by two pathologists. Five representative microscopic areas at ×400 magnification were randomly selected for examination. Expression of OPN was semiquantitatively assessed as percentage of positive cells with respect to the total number of epithelial cells. The samples were assigned to one of the four following categories: 0 (absence of positive cells); + (<10% of positive cells); ++ (10 – 50% of positive cells); and +++ (>50% of positive cells). Staining of CD44v6 was classified as 'lower' (lower third) (L), 'lower and middle' (up to two-thirds of the thickness of the epithelium) (M), and 'full thickness' (F).

Statistical analysis

The Pearson’s χ² test was used to assess the statistical significance of the frequency distribution of all categories of OPN or CD44v6 expression by degree of dysplasia or by relapse. Differences were significant with P-value < 0.05.

Nonparametric Spearman’s correlation coefficient method was used to assess the statistical significance of the correlation between OPN expression vs CD44v6 positivity. A test was run for all the patients’ cohort combined or grouped by degree of dysplasia or by type of relapse. Correlations were significant when P-value was < 0.05. Disease-free survival curves of the patients were calculated using the Kaplan–Meier method, and analysis was performed by the log-rank test. Differences were significant when P<0.05. In this analysis, a group of 31 patients has been censored for lack of data. Statistical analysis was performed using the JMP software program (version 5.1.1; SAS Institute Inc., Austin, TX, USA).

RESULTS

Immunohistochemical detection of OPN and CD44v6 in laryngeal dysplasia

Eighty-two laryngeal samples with different degree of dysplasia (Table 1) and the matched normal mucosa were tested for OPN expression by immunohistochemistry with an anti-OPN-specific monoclonal antibody. Representative stainings are shown in Figure 1, and the entire data set is reported in Table 2. OPN was virtually undetectable (<2.0% of the cells) in normal tissue (n = 10). Dysplastic areas showed different degrees of OPN positivity. In most (76%) of mild dysplasia cases, only few cells
were positive (Figure 1A), while 50% of moderate and severe dysplasia samples had intense (++) OPN staining (Figures 1E and G). Only 20% cases of mild dysplasia were highly positive for OPN (+++); interestingly, in these samples, OPN expression coexisted with a diffuse CD44v6 staining (see below) (Figures 1C and D).

The samples were also analysed for the expression of CD44v6, the receptor that is involved in OPN binding (Table 2). Only basal cells (L category) were CD44v6 positive in 76% of mild dysplasia samples (Figure 1B). Instead, in moderate dysplasia, CD44v6 positivity was found in the basal two-thirds (M category) (Figure 1F) or even full thickness (F category) (Figure 1H). The association between OPN and CD44v6 immunoreactivity resulted significant when analysed by the Spearman’s rank correlation test (Table 3A). The frequency distribution of OPN positivity or CD44v6 immunoreactivity by degree of dysplasia resulted highly significant at Pearson’s \( \chi^2 \) test (Table 4A).

Foci of squamous metaplasia of laryngeal cylindric-cell-lined areas were almost constantly present in our samples. Metaplastic areas (\( n = 20 \)) were almost constantly negative for OPN and CD44v6 staining (Figures 2A and B). Only in few (5%) cases, we observed an intense (+++) OPN staining paralleled by full-thickness CD44v6 positivity in the squamous metaplastic cells (Figures 2C and D). Although the ultimate statistical relevance of this finding is still to be verified on larger series of cases, it is interesting to note that these OPN- and CD44v6-positive metaplasia areas were found in patients with a history of development of SCC at the follow-up.

OPN and CD44v6 expression levels in laryngeal dysplasia negatively correlate with disease-free survival

The disease-free survival rate in patients affected by laryngeal dysplasia negatively correlated with intense OPN staining and full-thickness CD44v6 staining. As shown by the Kaplan–Meier

### Table 2

| LIN | OPN positivity | CD44v6 positivity |
|-----|----------------|-------------------|
| Mild (21) | 16/21 (+) | 16/21 (L) |
| Moderate (8) | 3/8 (+) | 3/8 (L) |
| Severe (53) | 16/53 (+) | 16/53 (L) |

OPN = osteopontin. *Osteopontin and CD44v6 expression were assessed by immunohistochemistry and scored respectively as follows: + = < 10% positive cells; ++ = 10–50% positive cells; and +++ = 50–100% positive cells; L = lower; M = lower and middle; and F = full-thickness involvement of the epithelial layers.
survival curves reported in Figure 3A, the 8-years disease-free survival was 94 and 91% for OPN (+-) and OPN (+ + -) positive cases, respectively, and 33% for OPN (+ + +) positive cases (two-sided log-rank test, P<0.0001; Figure 4A). Relative to CD44v6 expression, the probability of recurrence was 94 and 75% for cases that showed basal (L), or basal and middle (M) staining, respectively, and 38% for patient that had full-thickness (F) positivity (two-sided log-rank test, P = 0.007; Figure 4B).

At the Pearson’s test, the frequency distribution of OPN and CD44v6 expression levels were significantly correlated with relapse (Table 4B).

Moreover, the correlation between OPN and CD44v6 expression in patients with absence of relapse, with recurrence of dysplasia, or with progression to LSCC resulted very significant at the Spearman’s rank correlation test (Table 3B). Finally, the contingency analysis showed that the frequency distribution of OPN by CD44v6 were highly significant in combined or grouped types of relapse (Table 4C).

DISCUSSION

An in-depth understanding of the factors involved in the initial steps of LSCC development will facilitate the prevention and diagnosis of this condition. Currently, histological grading and the

Table 3A Correlation of osteopontin and CD44v6 expression in all laryngeal intraepithelial neoplasia (LIN) patients combined or grouped by degree of dysplasia

| LIN           | rs  | P  |
|---------------|-----|----|
| Combined (82) | 0.8231  | <0.0001 |
| Mild (21)     | 0.9941  | <0.0001 |
| Moderate (8)  | 0.2622  | =0.5304  |
| Severe (53)   | 0.7957  | <0.0001 |

Note: Correlation between osteopontin and CD44v6 expression in LIN patients analysed by Spearman’s rank correlation test. Correlation coefficient (rs) and Ps are shown (Ps <0.05 was considered significant).

Table 3B Correlation of osteopontin and CD44v6 expression in all laryngeal intraepithelial neoplasia (LIN) patients combined or grouped by relapse

| Relapse       | rs  | P  |
|---------------|-----|----|
| Combined (82) | 0.8231  | <0.0001 |
| No (35)       | 0.8133  | <0.0001 |
| LIN (10)      | 0.5976  | =0.0734  |
| SCC (37)      | -0.7892 | <0.0001 |

Note: Absence of relapse (no), recurrence of dysplasia (LIN), progression to carcinoma (SCC). Correlation between osteopontin and CD44v6 expression in LIN patients analysed by Spearman’s rank correlation test. Correlation coefficient (rs) and Ps are shown (Ps <0.05 was considered significant).

Table 4 Pearson’s test

| A. Contingency analysis of osteopontin (OPN) and CD44v6 positivity by degree of dysplasia | | |
|-----------------------------------------------|-----|----|
| OPN                                           | 13.425  | 0.0094 |
| CD44v6                                        | 16.198  | 0.0028 |

| B. Contingency analysis of OPN and CD44v6 positivity by relapse (no, LIN, SCC) | | |
|-----------------------------------------------|-----|----|
| OPN                                           | 21.780  | 0.0002 |
| CD44v6                                        | 9.567  | 0.0484 |

| C. Contingency analysis of OPN positivity by CD44v6 expression in all laryngeal intraepithelial neoplasia combined or grouped by relapse | | |
|-----------------------------------------------|-----|----|
| Combined (82)                                | 73.026  | <0.0001 |
| No (35)                                      | 32.694  | <0.0001 |
| LIN (10)                                     | 10.000  | 0.0067 |
| SCC (37)                                     | 34.857  | <0.0001 |

LIN = laryngeal intraepithelial neoplasia; SCC = squamous cell carcinoma. Note: Absence of relapse (no), recurrence of dysplasia (LIN), and progression to carcinoma (SCC).
Osteopontin is able to engage several cell surface receptors, including integrins and CD44 variants. In particular, OPN binds CD44 proteins that contain v6-encoded sequences, and OPN/CD44v6 binding has been implicated in carcinogenesis (Ponta et al., 2003). Here, we show that OPN expression levels were paralleled by intense expression of CD44v6; at contingency analysis, the frequency distribution of OPN expression by CD44v6 positivity resulted highly significant at Pearson’s test; moreover, the association between OPN and CD44v6 immunoreactivity was highly significant at the Spearman’s correlation coefficient test, suggesting that CD44v6 is at least one of the functional OPN receptors in laryngeal dysplasia.

To investigate whether the OPN/CD44v6 overexpression was causally related with dysplasia, cytological changes induced by OPN stimulation of primary human keratinocytes, obstructed by CD44 blockade, have been observed (Celetti A et al, in preparation). Addressing CD44 as a functional receptor for OPN would be important to explore the molecular mechanism underlying dysplastic changes induced by the OPN/CD44 axis. It is known that CD44 triggering stimulates diverse signalling pathways, including activation of ERK (Bourguignon et al., 2005), RAC (Teramoto et al., 2005), and RHO (Bourguignon et al., 2003), as well as secretion of soluble factors, like cytokines and metalloprotei-nases (Zhang et al., 2002; Bourguignon et al., 2003; Murphy et al., 2005). These pathways are potentially involved in dysplastic changes induced by OPN/CD44v6.

A model for the initiation and progression of colorectal cancer has become a paradigm for other human solid tumours (Fearon and Vogelstein, 1990). Like colorectal cancer, HNSCC is thought to
progress through a series of well-defined clinical and histopathological stages. While not all of the specific mutations required for progression have been delineated, a working molecular model has been proposed (Silverman, 2003). The loss of chromosomal regions 3p and 9p21 are among the first identified genetic changes (Mao et al, 1996; Sanz-Ortega et al, 2003). In particular, loss-of-heterozygosity (LOH) at 9p21 in conjunction with promoter hypermethylation results is the inactivation of the CDKN2A gene, coding for the cyclin-dependent kinase inhibitor 2A (p16INK4A). This alteration occurs prior to the development of histologic atypia and is associated with the transition from normal to hyperplastic/metaplastic mucosa (Papadimitrakopoulos et al, 2001; Sanz-Ortega et al, 2003). Subsequent LOH at 17p with mutation of the TP53 tumour suppressor gene is associated with progression to dysplasia (Boyle et al, 1993). The overexpression of the EGF receptor is also an early event in carcinogenesis (Rubin Grandis et al, 1998). Amplification and overexpression of the CCND1 gene, encoding cyclin D1 is a common late event in HNSCC formation (Michalides et al, 1995; Izzo et al, 1998; Chatrath et al, 2006). Our findings suggest that the upregulation of the OPN/CD44v6 axis is an additional early event during the progression of laryngeal dysplasia. Thus, early immunocytochemical detection of OPN and CD44v6 can be exploited to set a screening test for laryngeal dysplasia. Moreover, perturbation of OPN/CD44v6 signalling may represent a promising novel strategy to prevent progression of laryngeal preneoplastic lesions.

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