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Differential expression of retinoic acid receptor beta (RARβ) and the AP-1 transcription factor in normal, premalignant and malignant human laryngeal tissues

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Abstract

The anticancer effects of retinoids are mainly mediated by their nuclear receptors. Recent studies have demonstrated that retinoic acid receptor beta (RARβ) plays a pivotal role from the early stages of laryngeal carcinogenesis; however, the exact mechanism of this detrimental effect has not yet been elucidated. One of the best-documented actions of retinoid receptors is the transrepression of activator protein-1 (AP-1) transcription factor activity, although this complex interplay has not been clarified. The present report is the first systematic morphological evaluation of the cross-talk of RARβ and AP-1 transcription factor in a large series of human laryngeal tissues containing normal epithelium, premalignant lesions (hyperplasia and/or dysplasia) and squamous cell carcinoma. Immunohistochemical methodology was performed on formalin-fixed, paraffin-embedded sections by using a panel of monoclonal and polyclonal antibodies against RARβ and the AP-1 components c-Jun, p-c-Jun (phosphorylated, active c-Jun) and c-Fos proteins. Their expression was screened and compared in 154 patients with various laryngeal histological entities. Nuclear expression of RARβ, c-Jun, p-c-Jun and c-Fos was detected in 81 (89.2%), 48 (52.8%), 66 (72.6%) and 73 (80.3%), respectively, out of 91 specimens with normal-appearing laryngeal epithelium; in 86 (87.8%), 94 (95.9%), 94 (95.9%) and 94 (95.9%), respectively, out of 98 specimens with hyperplastic laryngeal epithelium; in 58 (56.8%), 92 (90.2%), 96 (94.1%) and 96 (94.1%), respectively, out of 102 specimens with dysplastic laryngeal epithelium; in 10 (22.3%), 41 (91.2%), 44 (97.8%) and 41 (91.2%), respectively, out of 45 specimens with well-differentiated squamous cell carcinoma; in 13 (30.3%), 37 (86%), 39 (90.7%) and 41 (95.3%), respectively, out of 43 specimens with moderately-differentiated squamous cell carcinoma; and in 8 (66.7%), 10 (83.3%), 12 (100%) and 12 (100%), respectively, out of 12 specimens with poorly-differentiated squamous cell laryngeal carcinoma. Statistical analysis and correlation of the intensity of nuclear immunostaining of the studied proteins among the various histological entities revealed statistically significant results. The progressive upregulation of the AP-1 transcription factor constituents and downregulation of the RARβ protein detected from the onset of laryngeal tumorigenesis suggests an important role for the immediate-early AP-1/RARβ on/off "switch" in the process of laryngeal carcinogenesis.

Keywords: Retinoic acid receptor beta; Activator protein-1; Cross-talk; Normal laryngeal tissue; Laryngeal lesion; Carcinogenesis

1. Introduction

Laryngeal epithelium permits the phenotypic study of the genetic and epigenetic events occurring during the multistep process of carcinogenesis [1]. The main principle of this approach is the multifocal development of premalignant and malignant lesions within the entire carcinogen-exposed laryngeal epithelium, according to the already described "field cancerization" concept [2]. Laryngeal cancer accounts for approximately 2% of all malignant tumours. Despite improved detection and local control with surgery and/or radiotherapy and substantially active new chemotherapeutic regimens, the survival rates have been only marginally influenced in recent decades [3]. This grim prognosis underlines the urgent need for the early identification of new

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approaches for the prevention and treatment of laryngeal carcinoma.

Retinoids, a group of structural and functional derivatives of vitamin A, are physiological regulators of a wide array of essential biological processes including embryonic development, vision, reproduction, differentiation, proliferation and apoptosis [4]. Pharmacologically, they have been shown to suppress carcinogenesis in a variety of tissue types, e.g. skin, lung and oral cavity in many animal models [5], while clinically they seem able to reverse premalignant lesions and inhibit the development of second primary tumours in the head and neck region [6–8]. These findings further confirmed that retinoids might be useful in the chemoprevention of head and neck carcinomas.

Extensive research effort is still ongoing to elucidate the molecular and cellular mechanism of retinoid action [9]. It is generally accepted that retinoids exert their pleiotropic effects through nuclear retinoid acid receptors, which are members of the steroid hormone receptor superfamily [10]. Two types of retinoid receptors have been identified, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). There are three RAR isotypes and three RXR isotypes (α, β and γ), encoded by distinct genes, and for each isotype, there are at least two isoforms, which differ in their N-terminal A regions and are generated by differential usage of promoters and/or alternative splicing [11]. Like other members of this family, the retinoid receptors act as ligand-activated, DNA-binding, transcription factors through binding as RAR/RXR heterodimers to *cis*-acting RA response elements present in cognate genes [12].

The expression of nuclear retinoid receptors in normal appearing head and neck tissue, premalignant and malignant tissues has been analysed by *in situ* hybridisation and it was concluded that a selective loss of *RARβ* mRNA occurs at early stages of carcinogenesis [13,14]. Similar expression patterns of retinoid receptors have also been detected in other types of cancer (e.g. oesophageal, prostatic, lung and cervical cancers) [5,15–17]. The mechanisms responsible for the decreased expression of *RARβ* are still not well documented, although many hypotheses have been proposed [4]. Regardless of the exact mechanism, it is also not clear why *RARβ* suppression leads to or enhances malignant transformation.

Upregulation of gene expression by retinoid receptors requires interaction with additional cofactors that provide a direct link to the core transcriptional machinery and modulate chromatin structure (e.g. CBP/p300) [18]. In addition to their positive regulation of gene transcription, retinoid receptors can also function as negative transcription factors. One of the known transrepressive effects of retinoid receptors is their downregulatory effect on activator protein-1 (AP-1) transcription factor activity [19]. AP-1 is composed of the proto-oncoproteins, c-Jun and c-Fos, and its activity is often associated with cell proliferation and tumour progression. AP-1 activity is regulated by growth factors, cytokines, oncogenes and tumour promoters [20]. Enhanced AP-1 activation is a result of c-Jun overexpression, but also in the case of c-Fos-induced transformation, it is likely that formation of AP-1 through heterodimerisation with c-Jun is responsible for its transforming properties [21]. The mechanism by which activated retinoid receptors repress AP-1 activity remains largely unknown, although a direct protein-protein interaction between retinoid receptors and AP-1 [22], an inhibitory effect of retinoid receptors on the mitogen-activated protein kinase (MAPK) cascade [23] and a competition for a common coactivator have been proposed [18].

It has been demonstrated that competition of ligand-bound retinoid receptors and AP-1 for the common transcriptional coactivator cyclic adenosine monophosphate (AMP)-response element binding (CREB)-binding protein (CBP) may contribute, in part, to the mutual inhibition of their transcriptional activity [18]. CBP is one of the most important transcriptional integrators involved in a plethora of cellular events, contributing to the cross-coupling of distinct gene expression patterns in response to various stimuli [24]. We have recently shown that CBP overexpression might represent an immediate-early event during the laryngeal carcinogenesis process [25].

In our present study, we employed tissue specimens from patients with histological variations of laryngeal epithelium, including normal-appearing epithelium, premalignant lesions (hyperplasia and/or dysplasia) and squamous cell carcinomas, and examined the expression of RARβ and AP-1 transcription factor complex participants during laryngeal carcinogenesis, by immunohistochemical methods. The main aim of this analysis was to assess morphologically the expression of RARβ and AP-1 proteins during laryngeal carcinogenesis and to correlate it with our previous data on the CBP protein.

2. Materials and methods

2.1. Patients and histopathological classifications

A total of 154 laryngeal tissue specimens were selected from the archives of the Pathology Department of the University Hospital of Patras. These specimens were obtained from patients who had been examined and had undergone diagnostic biopsy and/or laryngectomy for premalignant lesions and/or invasive carcinomas of the larynx during the last decade, in the Oto-Rhino-Laryngology Department of the University Hospital of Patras. Five out of the 154 patients were female (3.2%)

and 149 were male (96.8%). The mean age of the patients was 60.21 ± 12.19 years (range, 22–83 years). No radiation or other pre-operative therapy was mentioned in the clinical notes of the patients. All histological slides were reviewed to identify normal-appearing, hyperplastic, dysplastic and tumour areas, and to determine the grading of the tumour. Hyperplasia was defined as an increased number of normal-appearing epithelial cells with subsequent thickening of mucosal epithelium, while dysplasia was defined as a loss of normal orientation of one epithelial cell to the other, accompanied by alterations in cell size and shape and hyperchromasia in the staining characteristics [25]. Tumours displaying prickle cells, orderly stratification and heavy keratinisation with keratin pearls were classified as well differentiated. Tumours showing prickle cells and some stratification with few or no keratin pearls were classified as moderately differentiated. Tumours exhibiting nuclear pleomorphism and hyperchromasia as well as many and atypical mitoses were classified as poorly differentiated. The distribution of premalignant and malignant epithelial lesions in the larynx of the patients is summarised in Table 1.

Table 1
Distribution of premalignant and malignant lesions in the laryngeal epithelium of 154 patients

Histological appearance[a]	Number of patients (*n*)
N	2
H	5
D	6
CaWD	4
CaMD	6
CaPD	1
N + H	8
N + D	8
H + D	10
N + H + D	12
N + CaWD	4
N + CaMD	3
N + CaPD	4
H + CaWD	2
H + CaMD	1
H + CaPD	2
D + CaWD	3
D + CaMD	3
N + H + CaWD	5
N + H + CaMD	2
N + D + CaMD	7
H + D + CaWD	8
H + D + CaMD	11
N + H + D + CaWD	11
N + H + D + CaMD	23
N + H + D + CaPD	3
Total	154

[a] N: Normal-appearing epithelium; H: Hyperplastic epithelium; D: Dysplastic epithelium; CaWD: Well-differentiated carcinoma; CaMD: Moderately-differentiated carcinoma; CaPD: Poorly-differentiated carcinoma.

2.2. Antibodies

The anti-RARβ antibody (Clone 336; Lab Vision-NeoMarkers, Fremont, CA, USA) is a mouse monoclonal antibody raised against a peptide corresponding to amino acids 11-25 of the human RARβ protein. It was applied at an 1:25 dilution overnight at room temperature. The anti-c-Fos antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) is a rabbit polyclonal antibody raised against a peptide mapping at the N-terminus of human c-Fos p62. The above antibody was applied at an 1:140 dilution overnight at room temperature. The anti-c-Jun antibody (sc-1694; Santa Cruz Biotechnology) is a rabbit polyclonal antibody against an epitope corresponding to amino acids 1-79 of human c-Jun p39. This antibody was applied at an 1:100 dilution overnight at room temperature. The anti-p-c-Jun antibody (sc-822; Santa Cruz Biotechnology) is a mouse monoclonal antibody raised against an epitope corresponding to amino acids 56-69 of human c-Jun, which specifically reacts with c-Jun p39 phosphorylated on Ser-63 (non-cross-reactive with JunB or JunD phosphorylated on the analogous Ser residues or with c-Jun non-phosphorylated at Ser-63). It is known that the phosphorylations on Ser-63 and Ser-73 are both necessary for c-Jun activation in response to a variety of extracellular stimuli [26]. This antibody was also applied at an 1:100 dilution overnight at room temperature.

2.3. Immunohistochemistry

All tissue specimens were fixed in 10% (v/v) buffered formalin and embedded in paraffin. Serial 5-μm sections were obtained for staining with haematoxylin and eosin and for immunohistochemical study, which was performed using the avidin-biotin-streptavidin complex (ABC) technique (Biogenex kit; Biogenex Laboratories, San Ramon, CA, USA). For c-Jun, p-c-Jun and c-Fos, antigen retrieval was performed additionally following a microwave-based method as previously described in Ref. [27], while for RARβ, antigen retrieval consisted of trypsinisation (Trypsin type II-484435; Immunon, Pittsburg, PA, USA) along with microwave irradiation as previously described in Ref. [28], in order to optimise the positive signal. Negative controls were processed by substituting the primary antibody with non-immune mouse/rabbit serum. Nuclear immunostaining was evaluated using light microscopy. Cases displaying immunostaining in more than 5% of tumour cells were considered as positive. A semi-quantitative method based on a four-point scale was chosen for the assessment of the intensity of immunostaining: (−), negative; (+), weak positivity; (++), moderate positivity; (+++), strong positivity. Two independent researchers randomly evaluated thirty specimens for each antibody using this scale. Cohen's coefficient of agreement

was estimated $k = 0.89$-0.92 (anti-RARβ: $k = 0.89$, vark (variance) $= 0.15$, $P < 0.01$; anti-c-Jun: $k = 0.92$, vark $= 0.18$, $P < 0.01$; anti-p-c-Jun: $k = 0.87$, vark $= 0.15$, $P < 0.01$; anti-c-Fos: $k = 0.91$, vark $= 0.17$, $P < 0.01$) and was found to be statistically significant.

2.4. Statistical analyses

Statistical analysis and the correlation study were performed using the Chi-square test, Mann–Whitney U-test and Kruskal-Wallis ANOVA by ranks. Results were considered statistically significant only if a P value of < 0.05 was determined.

3. Results

3.1. Expression of RARβ

Normal-appearing epithelium was found in 91 tissue specimens (Table 2). In 10 out of 91 specimens, there was no nuclear immunostaining for RARβ, while 33 specimens showed strong positivity and 24 specimens displayed moderate positivity for RARβ (Fig. 1a). Weak positivity for RARβ was observed in 24 specimens. Hyperplastic epithelium was found in 98 tissue specimens. In 51 out of 98 specimens there was weak positivity, 29 specimens showed moderate positivity (Fig. 1c) and 6 specimens exhibited strong positivity for RARβ, while 12 specimens were negative. There was a statistically significant difference in the intensity of nuclear staining for RARβ in normal-appearing laryngeal epithelium compared with hyperplastic epithelium ($P < 0.0001$).

Dysplastic epithelium was found in 102 tissue specimens. Most of them showed weak (49 specimens) (Fig. 1d) or no positivity (44 specimens) for RARβ, while 8 specimens displayed moderate positivity and 1 specimen showed strong positivity for RARβ. There was a statistically significant difference in the intensity of nuclear staining for RARβ in normal-appearing epithelium ($P < 0.0001$) and hyperplastic epithelium ($P < 0.0001$) compared with dysplastic laryngeal epithelium.

Laryngeal carcinoma was present in 100 tissue specimens. Well-differentiated squamous cell carcinoma was found in 45 specimens. Ten out of 45 specimens showed weak positivity for RARβ (Fig. 1e), while the remainder (35 specimens) were immunonegative. Moderately-differentiated squamous cell carcinoma was present in 43 specimens. Among them, 13 specimens exhibited weak positivity and 30 specimens no positivity for RARβ (Fig. 1f). Poorly-differentiated squamous cell carcinoma was found in only 12 specimens. Seven of them displayed weak positivity and 4 specimens showed no positivity for RARβ, while the remainder (1 specimen) exhibited moderate positivity. It is worth noting that no specimen with well/moderately-differentiated squamous cell carcinoma showed moderate or strong immunopositivity for RARβ. Overall, there was a statistically significant difference in the intensity of nuclear staining for RARβ in normal-appearing laryngeal epithelium ($P < 0.0001$), hyperplastic epithelium ($P < 0.0001$) and dysplastic epithelium ($P < 0.05$) compared with squamous cell laryngeal carcinoma. Interestingly, there was also a statistically significant difference in the intensity of nuclear staining for RARβ in poorly-differentiated carcinomas compared with well- ($P < 0.05$) and moderately-differentiated carcinomas ($P < 0.05$).

Table 2
Expression of retinoic acid receptor beta (RARβ) protein

Histological entity[a]	− *n* (%)	+ *n* (%)	++ *n* (%)	+++ *n* (%)
Normal-appearing Epithelium (91 specimens)	10 (10.8)	24 (26.4)	24 (26.4)	33 (36.4)
Hyperplastic Epithelium (98 specimens)	12 (12.2%)	51 (52)	29 (29.6)	6 (6.1)
Dysplastic Epithelium (102 specimens)	44 (43.2)	49 (48)	8 (7.8)	1 (1)
Well-differentiated Carcinoma (45 specimens)	35 (77.7)	10 (22.3)	0	0
Moderately-differentiated Carcinoma (43 specimens)	30 (69.7)	13 (30.3)	0	0
Poorly-differentiated Carcinoma (12 specimens)	4 (33.3)	7 (58.3)	1 (8.4)	0

[a] (−): Negative; (+): Weak positivity; (++): Moderate positivity; (+++): Strong positivity.

3.2. Expression of AP-1

3.2.1. c-Jun

In 43 out of 91 specimens with normal-appearing epithelium there was no nuclear immunostaining for c-Jun (Table 3, Fig. 2a), while 46 specimens showed weak positivity and 2 specimens displayed moderate positivity for c-Jun. No specimen exhibited strong positivity for c-Jun. In 50 out of 98 specimens with hyperplastic epithelium, there was weak positivity, 33 specimens showed moderate positivity (Fig. 2b) and 11 specimens exhibited strong positivity for c-Jun, while 4 specimens were negative. There was a statistically significant difference in the intensity of nuclear staining for c-Jun in hyperplastic epithelium compared with normal-appearing laryngeal epithelium ($P < 0.0001$).

Most of the specimens with dysplastic epithelium showed weak (42 specimens) or moderate positivity (26

specimens) for c-Jun, while 24 specimens displayed strong positivity (Fig. 2c) and 10 specimens showed no positivity for c-Jun. There was a statistically significant difference in the intensity of nuclear staining for c-Jun in dysplastic laryngeal epithelium compared with normal-appearing epithelium ($P < 0.0001$).

Eighteen out of 45 specimens of well-differentiated squamous cell carcinoma showed strong positivity for c-Jun, 17 specimens weak positivity and 6 specimens moderate positivity, while the remainder (4 specimens) were immunonegative. Among 43 specimens of moderately-differentiated squamous cell carcinoma, 16 specimens exhibited strong positivity (Fig. 2e), 15 specimens moderate positivity and 6 specimens weak positivity for c-Jun, while 6 specimens had no positivity. Five of the 12 poorly-differentiated squamous cell carcinomas showed moderate positivity, 3 specimens displayed weak positivity and 2 specimens showed strong positivity for

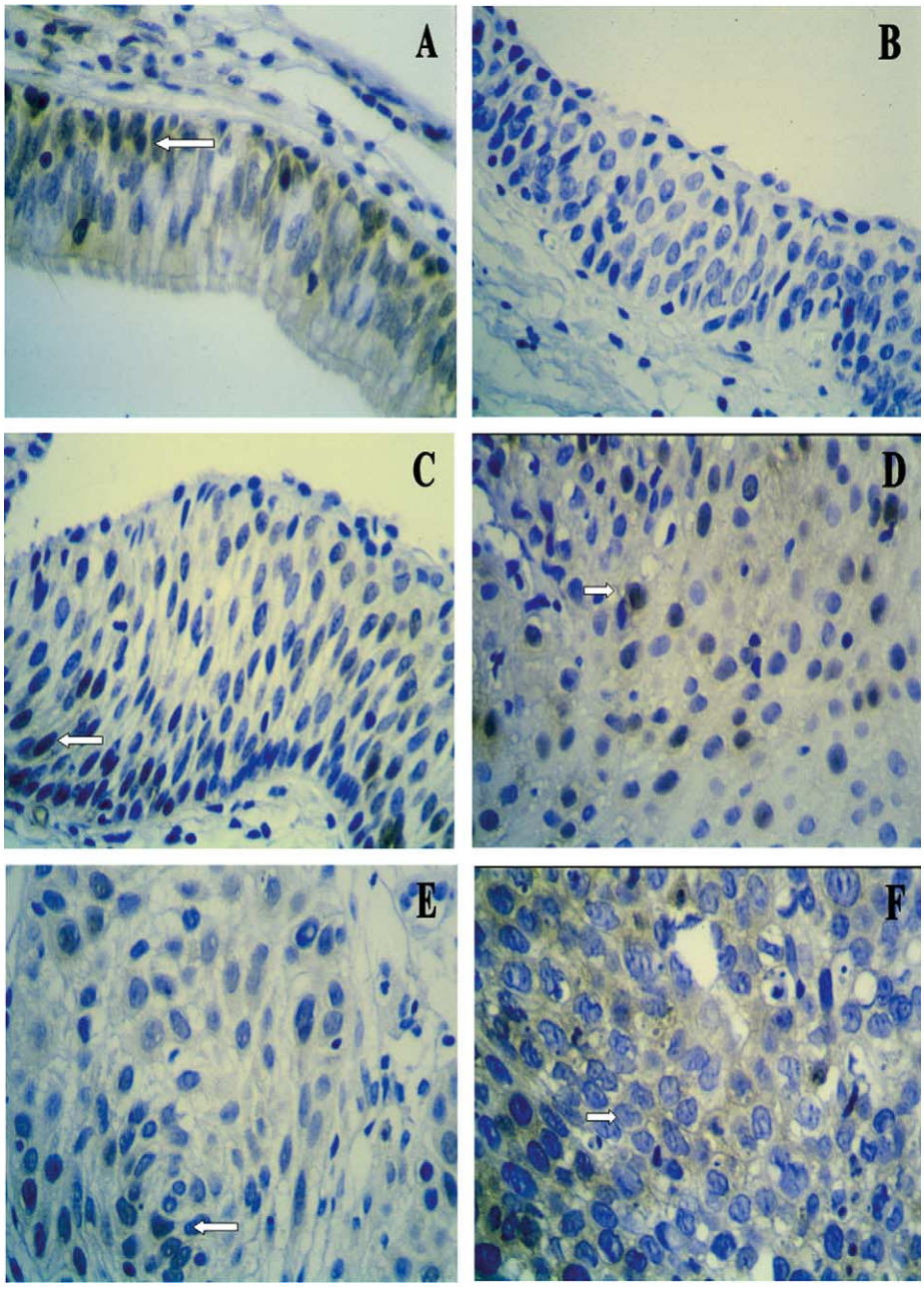

Fig. 1. Retinoic acid receptor beta (RARβ) expression in normal-appearing laryngeal epithelium, adjacent premalignant lesions and squamous cell carcinoma (diaminobenzidine (DAB), original magnification X40): (a) normal-appearing epithelium with moderate (++) nuclear positivity for RARβ; (b) negative control (i.e. no anti-RARβ antibody added) of the same tissue specimen; (c) hyperplastic laryngeal epithelium with moderate (++) nuclear immunostaining for RARβ; (d) dysplastic epithelium with weak (+) nuclear positivity for RARβ; (e) well-differentiated squamous cell laryngeal carcinoma with weak (+) nuclear positivity for RARβ; (f) moderately-differentiated squamous cell laryngeal carcinoma with negative (−) nuclear staining for RARβ. Arrows point to representative cells with nuclear detection of RARβ protein in each histological entity.

Table 3
Expression of c-Jun protein

Histological entity[a]	– *n* (%)	+ *n* (%)	++ *n* (%)	+++ *n* (%)
Normal-appearing Epithelium (91 specimens)	43 (47.2)	46 (50.7)	2 (2.1)	0
Hyperplastic Epithelium (98 specimens)	4 (4.1)	50 (51.1)	33 (33.6)	11 (11.2)
Dysplastic Epithelium (102 specimens)	10 (9.8)	42 (41.2)	26 (25.5)	24 (23.5)
Well-differentiated Carcinoma (45 specimens)	4 (8.8)	17 (37.8)	6 (13.4)	18 (40.0)
Moderately-differentiated Carcinoma (43 specimens)	6 (14)	6 (14)	15 (34.8)	16 (37.2)
Poorly-differentiated Carcinoma (12 specimens)	2 (16.7)	3 (25)	5 (41.7)	2 (16.7)

[a] (–): Negative; (+): Weak positivity; (++): Moderate positivity; (+++): Strong positivity.

Table 4
Expression of p-c-Jun (phosphorylated, active c-Jun) protein

Histological entity[a]	– *n* (%)	+ *n* (%)	++ *n* (%)	+++ *n* (%)
Normal-appearing Epithelium (91 specimens)	25 (27.4)	44 (48.4)	22 (24.2)	0
Hyperplastic Epithelium (98 specimens)	4 (4.1)	26 (26.5)	25 (25.5)	43 (43.9)
Dysplastic Epithelium (102 specimens)	6 (5.9)	24 (23.5)	25 (24.5)	47 (46.1)
Well-differentiated Carcinoma (45 specimens)	1 (2.2)	14 (31.2)	10 (22.2)	20 (44.4)
Moderately-differentiated Carcinoma (43 specimens)	4 (9.3)	7 (16.2)	8 (18.6)	24 (55.8)
Poorly-differentiated Carcinoma (12 specimens)	0	5 (41.7)	4 (33.3)	3 (25)

[a] (–): Negative; (+): Weak positivity; (++): Moderate positivity; (+++): Strong positivity.

c-Jun (Fig. 2f), while the remainder (2 specimens) were immunonegative. Overall, there was a statistically significant difference in the intensity of nuclear staining for c-Jun in squamous cell laryngeal carcinoma compared with normal-appearing laryngeal epithelium ($P<0.0001$) and hyperplastic epithelium ($P<0.05$), whereas there was no statistically significant difference in the intensity of nuclear staining for c-Jun among well-, moderately- and poorly-differentiated carcinomas ($P>0.05$).

3.2.2. p-c-Jun

In 25 out of 91 specimens with normal-appearing epithelium, there was no nuclear immunostaining for p-c-Jun, while 44 specimens showed weak positivity and 22 specimens moderate positivity for p-c-Jun (Table 4, Fig. 3a). No specimen exhibited strong positivity for p-c-Jun. In 43 out of 98 specimens with hyperplastic epithelium, there was strong positivity, 25 specimens showed moderate positivity (Fig. 3b) and 26 specimens displayed weak positivity for p-c-Jun, while 4 specimens were immunonegative. There was a statistically significant difference in the intensity of nuclear staining for p-c-Jun in hyperplastic epithelium compared with normal-appearing laryngeal epithelium ($P<0.0001$).

Most of the specimens with dysplastic epithelium exhibited strong (47 specimens) (Fig. 3c) or moderate positivity (25 specimens) for p-c-Jun, while 24 specimens showed weak positivity and 6 specimens no positivity for p-c-Jun. There was a statistically significant difference in the intensity of nuclear staining for p-c-Jun in dysplastic epithelium compared with normal-appearing laryngeal epithelium ($P<0.0001$).

Twenty out of 45 specimens of well-differentiated squamous cell carcinoma showed strong positivity for p-c-Jun (Fig. 3d), 14 specimens weak positivity and 10 specimens moderate positivity, while the remaining specimen was immunonegative. Among 43 moderately-differentiated squamous cell carcinoma specimens, 24 exhibited strong positivity, 8 specimens moderate positivity and 7 specimens weak positivity for p-c-Jun, while 4 specimens had no positivity. Five of the 12 poorly-differentiated squamous cell carcinoma specimens displayed weak positivity, 4 specimens showed moderate positivity and 3 specimens exhibited strong positivity for p-c-Jun (Fig. 3e), while no specimen was immunonegative. Overall, there was a statistically significant difference in the intensity of nuclear staining for p-c-Jun in squamous cell laryngeal carcinoma compared with normal-appearing laryngeal epithelium ($P<0.0001$), whereas there was no statistically significant difference in the intensity of nuclear staining for p-c-Jun among well-, moderately- and poorly-differentiated carcinomas ($P<0.05$).

3.2.3. c-Fos

In 18 out of 91 specimens with normal-appearing epithelium, there was no nuclear immunostaining for c-Fos, while 46 specimens showed weak positivity (Table 5, Fig. 4a), 24 specimens moderate positivity and 3 specimens strong positivity for c-Fos. In 46 out of 98 specimens with hyperplastic epithelium, there was strong positivity, 26 specimens showed moderate positivity (Fig. 4b) and 22 specimens displayed weak positivity for c-Fos, while 4 specimens were immunonegative.

There was a statistically significant difference in the intensity of nuclear staining for c-Fos in hyperplastic epithelium compared with normal-appearing laryngeal epithelium ($P < 0.0001$).

Most of the specimens with dysplastic epithelium showed strong (52 specimens) or moderate positivity (23 specimens) for c-Fos (Fig. 4d), while 21 specimens exhibited weak positivity and 6 specimens no positivity for c-Fos. There was a statistically significant difference in the intensity of nuclear staining for c-Fos in dysplastic laryngeal epithelium compared with normal-appearing epithelium ($P < 0.0001$).

Twenty five out of 45 specimens of well-differentiated squamous cell carcinoma showed strong positivity for c-Fos, 11 specimens moderate positivity and 5 specimens weak positivity (Fig. 4f), while the remainder

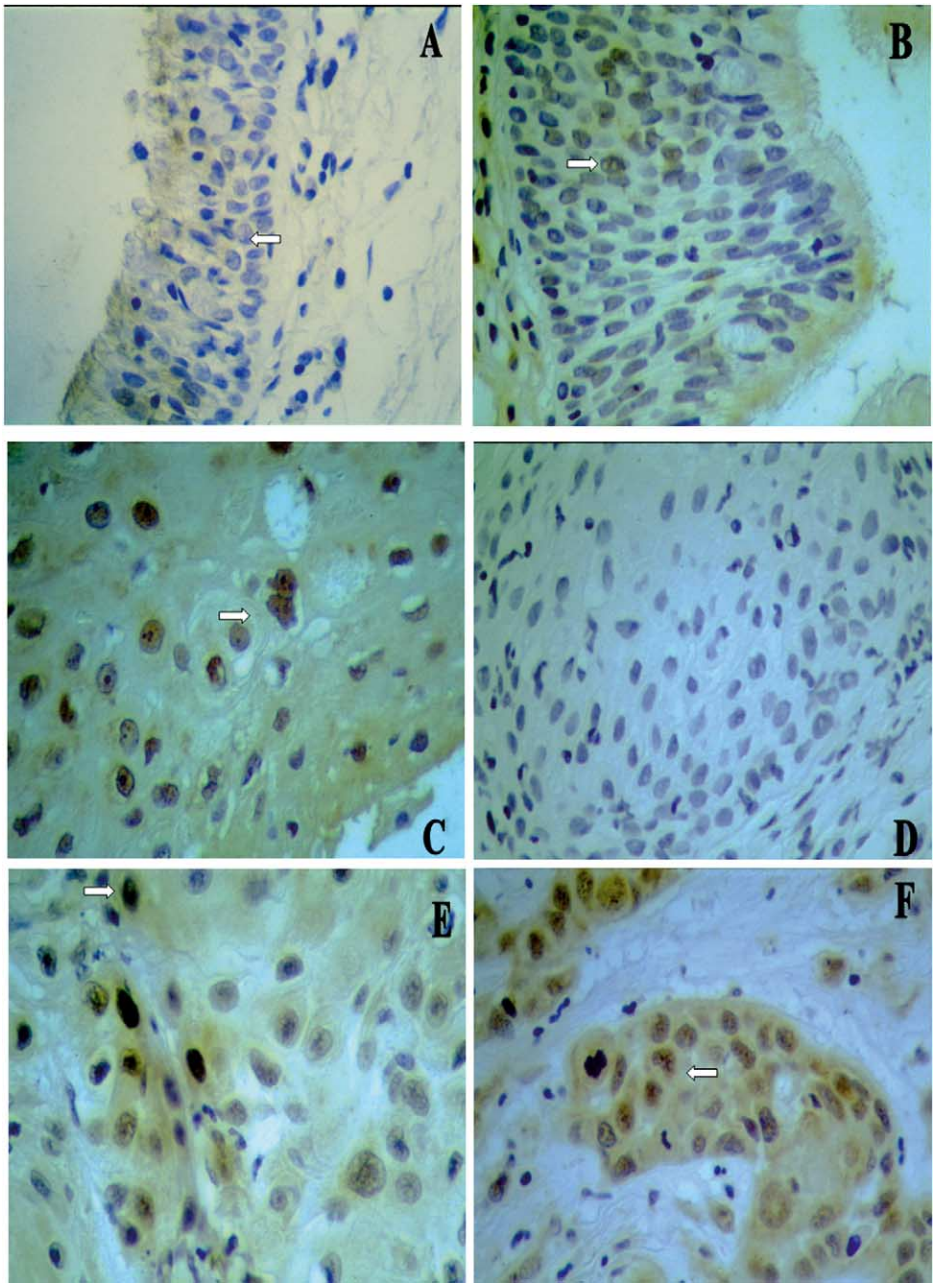

Fig. 2. c-Jun expression in normal-appearing laryngeal epithelium, adjacent premalignant lesions and squamous cell carcinoma (DAB, original magnification X40): (a) normal-appearing epithelium with negative (−) nuclear staining for c-Jun; (b) hyperplastic laryngeal epithelium with moderate (+ +) nuclear immunostaining for c-Jun; (c) dysplastic epithelium with strong (+ + +) nuclear positivity for c-Jun; (d) negative control (i.e. no anti-c-Jun antibody added) of the same tissue specimen; (e) moderately-differentiated squamous cell laryngeal carcinoma with strong (+ + +) nuclear positivity for c-Jun; (f) poorly-differentiated squamous cell laryngeal carcinoma with strong (+ + +) nuclear positivity for c-Jun. Arrows point to representative cells with nuclear detection of c-Jun protein in each histological entity.

(4 specimens) were immunonegative. Among the 43 specimens of moderately-differentiated squamous cell carcinoma, 25 specimens exhibited strong positivity, 8 specimens moderate positivity and 8 specimens weak positivity for c-Fos, while 2 specimens had no positivity. Five of the 12 poorly-differentiated squamous cell carcinoma specimens displayed strong positivity (Fig. 4e), 5 specimens moderate positivity and 2 specimens weak positivity for c-Fos, while no specimen was immunonegative. Overall, there was a statistically significant difference in the intensity of nuclear staining for c-Fos in squamous cell laryngeal carcinoma compared with normal-appearing laryngeal epithelium ($P < 0.0001$), whereas there was no statistically significant difference in the intensity of nuclear staining for c-Fos among well-, moderately- and poorly-differentiated carcinomas ($P > 0.05$).

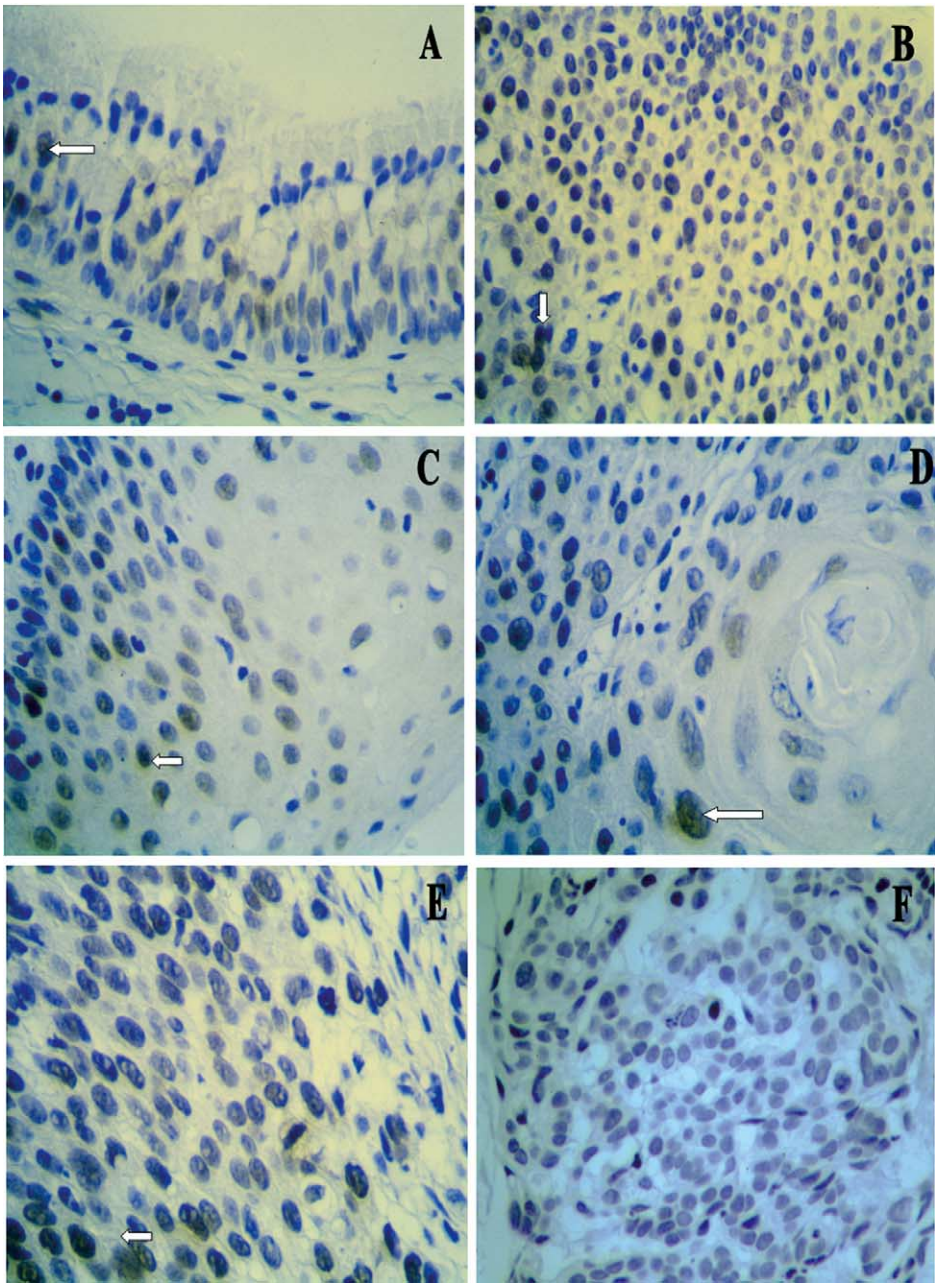

Fig. 3. p-c-Jun (phosphorylated, active c-Jun) expression in normal-appearing laryngeal epithelium, adjacent premalignant lesions and squamous cell carcinoma (DAB, original magnification X40): (a) normal-appearing epithelium with moderate (++) nuclear staining for p-c-Jun; (b) hyperplastic laryngeal epithelium with moderate (++) nuclear immunostaining for p-c-Jun; (c) dysplastic epithelium with strong (+++) nuclear positivity for p-c-Jun; (d) well-differentiated squamous cell laryngeal carcinoma with strong (+++) nuclear positivity for p-c-Jun; (e) poorly-differentiated squamous cell laryngeal carcinoma with strong (+++) nuclear positivity for p-c-Jun; (f) negative control (i.e. no anti-p-c-Jun antibody added) of the same tissue specimen. Arrows point to representative cells with nuclear detection of p-c-Jun protein in each histological entity.

Table 5
Expression of c-Fos protein

Histological entity[a]	– n (%)	+ n (%)	++ n (%)	+++ n (%)
Normal-appearing Epithelium (91 specimens)	18 (19.7)	46 (50.6)	24 (26.4)	3 (3.3)
Hyperplastic Epithelium (98 specimens)	4 (4.1)	22 (22.5)	26 (26.5)	46 (46.9)
Dysplastic Epithelium (102 specimens)	6 (5.9)	21 (20.6)	23 (22.5)	52 (51)
Well-differentiated Carcinoma (45 specimens)	4 (8.8)	5 (11.2)	11 (24.5)	25 (55.5)
Moderately-differentiated Carcinoma (43 specimens)	2 (4.7)	9 (18.6)	8 (18.6)	24 (58.1)
Poorly-differentiated Carcinoma (12 specimens)	0	2 (16.8)	5 (41.6)	5 (41.6)

[a] (–): Negative; (+): Weak positivity; (++): Moderate positivity; (+++): Strong positivity.

3.3. Correlation of AP-1 and RARβ expression in normal-appearing laryngeal epithelium

RARβ proved to have statistically more significant nuclear positivity compared with c-Fos ($P<0.0001$), p-c-Jun ($P<0.0001$) and c-Jun ($P<0.0001$) proteins in normal-appearing laryngeal epithelium. Among the AP-1 proteins, c-Fos and p-c-Jun exhibited statistically more intense nuclear positivity compared with c-Jun ($P<0.0001$ and $P<0.01$, respectively), while there was no statistically significant difference between them ($P>0.05$).

3.4. Correlation of AP-1 and RARβ expression in premalignant laryngeal lesions

3.4.1. Hyperplastic laryngeal lesions

c-Fos and p-c-Jun proteins displayed statistically more significant nuclear positivity compared with c-Jun ($P<0.0001$ and $P<0.001$, respectively) and RARβ ($P<0.0001$ and $P<0.0001$, respectively), while there was no statistically significant difference between them ($P>0.05$). No statistically significant difference was also detected between c-Jun and RARβ ($P>0.05$) in hyperplastic laryngeal epithelium.

3.4.2. Dysplastic laryngeal lesions

c-Fos and p-c-Jun proteins proved to have statistically more profound nuclear positivity compared with c-Jun ($P<0.001$ and $P<0.001$, respectively) and RARβ ($P<0.0001$ and $P<0.0001$, respectively) in dysplastic laryngeal epithelium, while there was no statistically significant difference between them ($P>0.05$). c-Jun protein also exhibited statistically more intense nuclear positivity than RARβ ($P<0.0001$).

3.5. Correlation of AP-1 and RARβ expression in squamous cell laryngeal carcinomas

c-Fos protein proved to have statistically more significant nuclear positivity compared with c-Jun ($P<0.01$) and RARβ ($P<0.0001$) in squamous cell laryngeal carcinomas, while p-c-Jun protein showed statistically more intense nuclear positivity compared with RARβ ($P<0.0001$), but not with c-Jun ($P>0.05$). No statistically significant difference between c-Fos and p-c-Jun was observed. Finally, c-Jun protein displayed statistically more significant nuclear positivity than RARβ ($P<0.0001$) in laryngeal carcinomas.

4. Discussion

The present study represents the first systematic morphological investigation of the cross-talk of RARβ and AP-1 transcription factor proteins during early laryngeal carcinogenesis in a large series of human laryngeal tissues comprising normal-appearing epithelium, premalignant lesions (hyperplasia and/or dysplasia) and squamous cell carcinoma. The current findings expand our previous observations and provide additional insight into the biological role of this complex protein interplay during laryngeal carcinogenesis.

In our analysis, a gradually reduced expression of RARβ protein was demonstrated for the first time by immunohistochemistry in various laryngeal histological entities, while an opposite, increasing expression of c-Jun, p-c-Jun and c-Fos proteins was detected, especially in premalignant laryngeal lesions, implying that this pattern of protein overexpression might reflect the detrimental effect of RARβ/AP-1 cross-talk during early laryngeal carcinogenesis.

Clinicopathological investigations on laryngeal carcinogenesis are scarce due to the low incidence of this type of malignancy compared with other carcinomas, e.g. breast and lung carcinomas. However, the laryngeal epithelium provides a unique model for studying the multistep process of carcinogenesis as there can be coexistence of normal-appearing epithelium, premalignant lesions and invasive squamous cell carcinoma in laryngectomy specimens and/or laryngeal biopsies. Moreover, in spite of recent treatment advances, survival rates of patients with laryngeal carcinoma remain quite poor [3]. Therefore, new approaches, such as chemoprevention, need to be developed. During recent years, many clinical and experimental studies have established the retinoids as potent regulators of epithelial cell growth with reported benefit in the treatment of

premalignant lesions and prevention of second primary tumours of the head and neck region [9].

Many factors can contribute to tumorigenesis, including inherited and acquired genetic changes, chromosomal rearrangements, epigenetic phenomena and chemical carcinogenesis. Retinoids interfere with these events at several levels, with their main known actions being induction of differentiation and/or apoptosis of tumour cells and inhibition of tumour proliferation [4]. These effects are mediated by retinoid nuclear receptors. It has been established that RAR/RXR heterodimers are the functional units that transduce the retinoid signal. These heterodimers activate transcription by binding to RA response elements located in the promoter region of retinoid-dependent target genes [29]. However, the mechanisms through which retinoids suppress carcinogenesis are complex and poorly elucidated. Up to now, the use of retinoids in clinical trials has been

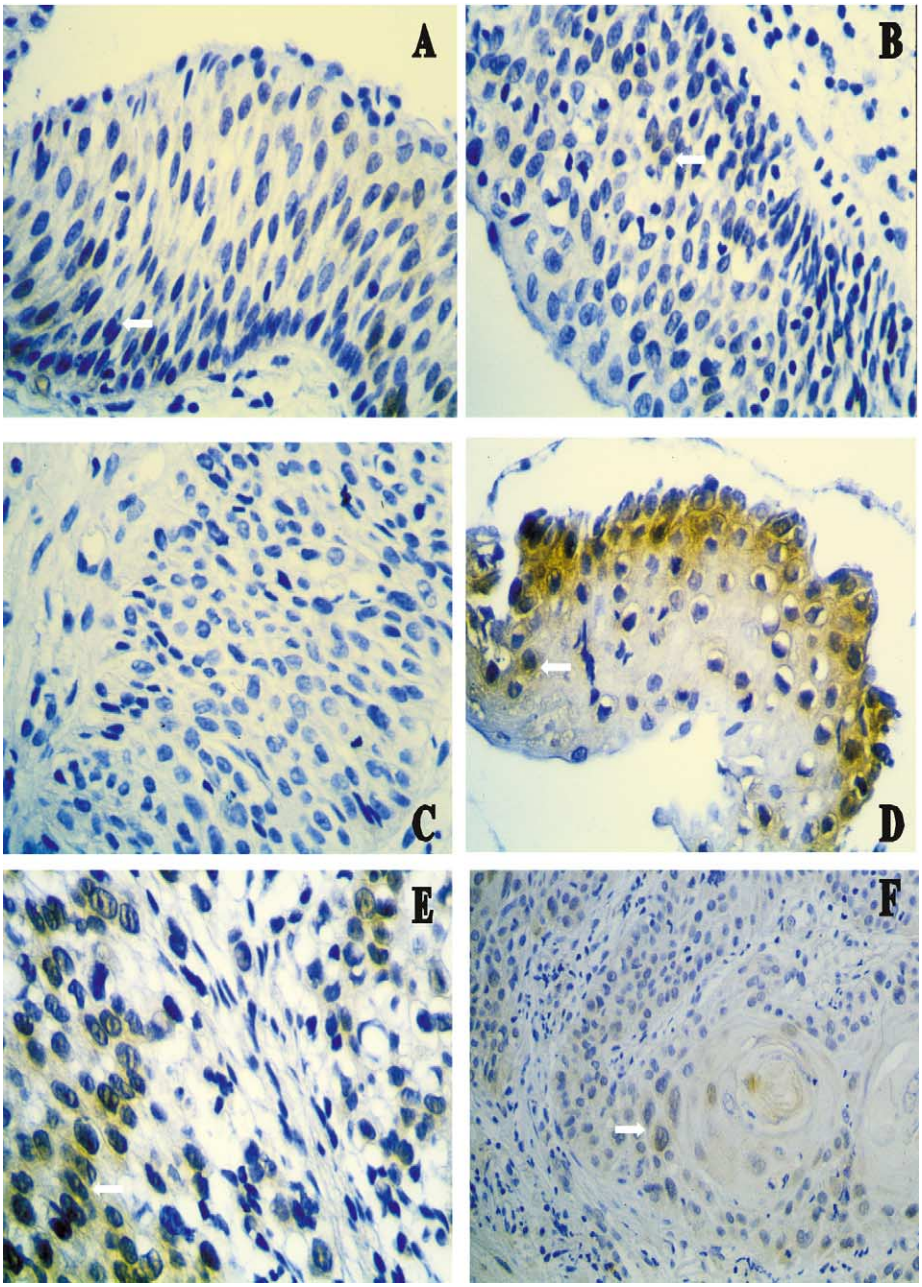

Fig. 4. c-Fos expression in normal-appearing laryngeal epithelium, adjacent premalignant lesions and squamous cell carcinoma (DAB, original magnification ×40): (a) normal-appearing epithelium with weak (+) nuclear staining for c-Fos; (b) hyperplastic laryngeal epithelium with moderate (++) nuclear immunostaining for c-Fos; (c) negative control (i.e. no anti-c-Fos antibody added) of the same tissue specimen; (d) dysplastic epithelium with moderate (++) nuclear positivity for c-Fos; (e) poorly-differentiated squamous cell laryngeal carcinoma with strong (+++) nuclear positivity for c-Fos; (f) well-differentiated squamous cell laryngeal carcinoma with weak (+) nuclear positivity for c-Fos. Arrows point to representative cells with nuclear detection of c-Fos protein in each histological entity.

limited and quite disappointing, because of their profound side-effects and the development of retinoid resistance [30]. Nevertheless, there are many ongoing efforts for the development of more potent, selective and safe synthetic retinoids [31,32].

Accumulating evidence indicates that altered expression of RARβ protein is involved in the pathogenesis of a diverse range of solid tumours [5,33], albeit lost expression of RARα or RARγ has also been reported [34]. Loss of RARβ expression has been associated with human tumour progression and is frequently found in carcinomas of the head and neck, lung, oesophagus, breast, pancreas and prostate [15,16]. This loss is also found in premalignant lesions of the head and neck, lung and mammary gland [15,17,35], suggesting that such a deregulation could represent an initiative mechanism through which tumour cells escape from normal cellular homeostasis. Our study confirmed these observations since RARβ expression was found reduced in premalignant and malignant human laryngeal lesions, while there was RARβ overexpression in normal-appearing laryngeal epithelium with similar percentages obtained using an *in situ* hybridisation technique [13,14].

Although loss of RARβ expression appears to be a common event during laryngeal carcinogenesis, the causative mechanism is largely unknown. Loss of heterozygosity at chromosomal region 3p21-3p24, that contains the *RARβ* gene, has been detected in lung, but not in laryngeal carcinomas [36]. Inactivation of nuclear retinoid receptor coactivators or activation of their corepressors may also account for altered expression of RARβ [37]. Loss of RARβ expression in premalignant oral lesions has been correlated with a low cellular level of retinoids, as RARβ expression is dependent on cellular levels of retinoids [38]. Finally, aberrant methylation of the *RARβ* gene promoter has been recently documented in lung carcinomas [39]. Overall, these observations imply that multiple mechanisms may be engaged in the loss of *RARβ* gene expression.

Induction of the *RARβ* promoter by retinoids and/or expression of RARβ seem to correlate with the growth-inhibitory effect of retinoids in solid tumours. Indeed, several retinoid-unresponsive tumour cell lines can be made retinoid-responsive by introducing exogenous recombinant RARβ [40]. The exact mechanism that is regulated by the RARβ protein is unknown, but it is challenging to speculate that it might be related to two functions that seem to distinguish RARβ from the other retinoid receptors. First, RARβ interacts only inefficiently with corepressors so that RARβ/RXR heterodimers are more responsive to retinoids than RARα/RXR heterodimers [41]. Second, RARβ is able to transrepress the AP-1 transcription factor reversing established tumour promotion, because anchorage-independent growth of transformed cells is efficiently inhibited [19]. Although the precise mechanism of the anti-AP-1 activity of retinoids remains elusive, the importance of this cross-talk for growth control is increasingly recognised and considered as a phylogenetically highly conserved function [23].

AP-1 plays a pivotal role in carcinogenesis. The AP-1 complex is a homo/heterodimer of Jun- and Fos-related proteins, which binds to a promoter element termed the TPA-responsive element (TRE). The activity of this transcription factor complex is modulated by various stimuli including growth factors, cytokines and tumour promoters [42]. Changes in the expression levels of AP-1 family members, post-translational modifications (i.e. phosphorylation and oxidation/reduction) and dimer composition alter both DNA-binding affinity and transactivation potential [43]. Overexpression of AP-1 has been implicated in the pathogenesis and in the malignant progression of several human tumours [26], but there has been no report of its expression pattern in laryngeal premalignant and malignant lesions. One of the main findings of our study was the observation that c-Fos overexpression parallels p-c-Jun (phosphorylated, active c-Jun) upregulation during early laryngeal carcinogenesis. With regard to the statistically more significant expression of the p-c-Jun protein compared with total c-Jun in normal-appearing epithelium, premalignant and malignant laryngeal lesions, we propose it is most likely due to the different nature (monoclonal versus polyclonal) of the two antibodies used.

The transrepression of AP-1 by ligand-bound retinoid receptors has been demostrated previously, both *in vitro* and *in vivo*. It has also been shown that downregulation of AP-1 by retinoids correlates with the inhibition of tumour promoter-induced transformation and tumour development [9]. The mechanism of AP-1 transrepression is presently unknown. No binding of ligand-bound retinoid receptors to the TRE sequence has been observed, hence excluding competition for DNA binding as a potential mechanism [19]. Several other mechanisms have been proposed including binding of the ligand-bound retinoid receptor to a partner of the AP-1 dimer, which would thus interfere with dimerisation, a prerequisite for AP-1-mediated transcriptional control [44]. Interference with the Jun N-terminal kinase (JNK) signalling pathway represents another possible mechanism [23]. Our data seem to argue for the JNK-inhibition scenario of RARβ/AP-1 cross-talk, since nuclear overexpression of the activated (phosphorylated) c-Jun species is in accordance with c-Fos upregulation, implying that formation and activation—via c-Jun phosphorylation—of AP-1 dimers remain unaffected through all stages of laryngeal carcinogenesis. However, a recent report suggested that transcriptional activation of AP-1-regulated target genes through the MAPK cascade is an equally significant event with recruitment of cofactors [45]. Competition for the limited amounts of the coactivator CBP/p300, which is

necessary for the transcriptional activity of both nuclear retinoid receptors and AP-1, may contribute, in part, to the mutual retinoid and AP-1 inhibition of transcriptional activity [18]. In this vein, we have recently shown that CBP overexpression represents an immediate-early event during laryngeal carcinogenesis [25].

Taking all these findings together, an indirect rational conclusion is that, in the early stages of laryngeal carcinogenesis, genetic instability of carcinogen-exposed laryngeal epithelium permits the epigenetic gradual downregulation of RARβ, which combined with overexpression of CBP and AP-1 proteins favours AP-1 upregulation, thus triggering tumour progression and inhibiting differentiation and/or apoptosis of transformed cells. Immunohistochemical evaluation of biopsy specimens from premalignant laryngeal lesions is a simple and quite reliable procedure to identify patients with this expression profile. In addition, based on the ''field-cancerization'' concept of the upper aerodigestive tract, these results might be of great importance for establishing new prognostic and predictive intermediate biomarkers in future laryngeal and lung chemoprevention clinical trials.

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