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IMMUNOHISTOCHEMICAL STUDY OF p53, EGF, EGF-RECEPTOR, v-erb B AND ras p21 IN SQUAMOUS CELL CARCINOMA OF HYPOPHARYNX

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INDEXING WORDS

hypopharyngeal cancer; squamous cell carcinoma; EGF; EGF-receptor; p53; v-erb B; ras p21; immunohistochemistry

SYNOPSIS

We have characterized 24 hypopharyngeal squamous cell carcinomas and 5 normal hypopharyngeal tissues by immunostaining with antibodies against epidermal growth factor (EGF), EGF-Receptor (EGFR), p53, v-erb B, and ras p21. The Avidin-Biotin Complex (ABC) technique was employed. Overexpression of p53 appeared in 17 of 24 cases of squamous cell carcinoma of the hypopharynx (normal mucosa: none, well differentiated: 60%, moderately differentiated: 71.4%, poorly differentiated: 71.4%). Some dysplastic mucosae surrounding cancer lesions showed overexpression of p53. EGF and EGFR tended to be expressed more strongly in carcinoma [EGF: 29.1% (well differentiated: 30%, moderately differentiated: 28.6%, poorly differentiated: 28.6%); EGFR: 50% (well differentiated: 60%, moderately differentiated: 42.9%, poorly differentiated: 42.9%)] than in normal mucosa (EGF: 0%, EGFR: 20%). The v-erb B stained positively in carcinoma [62.5% (well differentiated: 70%, moderately differentiated: 71.4%, poorly differentiated: 42.9%)] but negatively in normal mucosa.

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These data suggest that genetic mutations of p53 probably play an important role at an early stage of tumorigenesis, and that the networks of EGF, EGFR and v-erb B probably are involved in the development of hypopharyngeal squamous cell carcinoma.

INTRODUCTION

Squamous cell carcinoma is the most common malignant neoplasm of the hypopharynx and patients with hypopharyngeal carcinoma generally have poor prognosis. Although its precise pathogenesis is unclear, much interest has been focused on the molecular mechanisms of the development of squamous cell carcinoma. Recently mutation of suppressor- and oncogenes and growth factors in carcinogenesis has drawn considerable attention, since the alteration of these genes seems closely related to the degree of malignancy. We have investigated the relationship between histologic types and grades of dysplasias and their positivity through immunohistochemical staining of hypopharyngeal cancers and estimated the expression or overexpression of oncogenes, suppressor genes and some growth factors in hypopharyngeal mucosae, dysplasia, and carcinoma. In this study, hypopharyngeal cancers are histologically classified to well differentiated, moderately differentiated and poorly differentiated squamous cell carcinomas, and are immunohistochemically studied to clarify the role of EGF, EGFR, p53, v-erb B, and ras p21 in the transformation and progression of hypopharyngeal cancer.

MATERIALS AND METHODS

Materials

Twenty-nine cases comprising 5 pharyngitis of the hypopharynx, 10 well differentiated squamous cell carcinoma, 7 moderately differentiated squamous cell carcinoma and 7 poorly differentiated squamous cell carcinoma were examined in this study. Those tissues were routinely fixed with 10 % buffered formalin solution and embedded in paraffin blocks. Four-micron-thick sections cut from the blocks were stained by routine immunohistochemical procedures for detecting growth factors (EGF and EGFR) and suppressor and oncogene products (p53, v-erb B and ras p21). The Avidin-Biotin Complex (ABC) technique was employed.

Methods: Antibodies

In preliminary studies, both frozen and paraffin-embedded sections were immunohistochemically stained, with appropriate reagents and under stable conditions.

The antibodies used were anti-EGF, anti-EGFR, anti-p53, anti-v-erb B and anti-ras p21. The anti-EGF monoclonal antibody (MAb) was obtained from the ascites fluid of Balb/c mice injected with a hybridoma formed by the fusion of mouse myeloma cell (p3UI) with spleen cells from a Balb/c mouse immunized with synthesized hEGF (Wakunaga, Japan, dilution 1:100).[38] The anti-EGF receptor MAb (referred to as No. 528) was raised against the EGF receptor on A431 cells derived from an epidermoid carcinoma of the vulva (Oncogene Science, New York, dilution 1:20).[22,23] The anti-p53 MAb (referred to as BP53-12) was the product of mouse hybridomas cloned from a polyethylene glycol-induced fusion of splenocytes; it reacts with wild and mutant forms of p53 products (Japan Tanner Corp., Japan, dilution 1:10). The anti-retroviral-erb B (v-erb B) oncogene product polyclonal antibody (PAb) was raised against a bacterially expressed erb B DNA restriction fragment (BamHI/BamHI); the antiserum immunoprecipitates the erb B protein from AEV-transformed chicken fibroblasts recognizes the EGF Receptor protein (Medac Molecular Biology, dilution 1:400).[15] The NCC-RAS-001, anti-ras oncogene product p21 antibody, kindly supplied by Dr. S. Hirohashi (National Cancer Center Research Institute, Japan), was raised against recombinant p21 containing valine substituted for glycine at position 12[19]; it reacts with both normal p21 and point-mutated p21 (dilutions 1:500 and 1:1000).[21,15]

Methods: Immunohistochemistry

The sections were deparaffinized with xylene and routinely dehydrated through a series of graded alcohols. Prior to EGF, EGFR and ras p21 staining, histologic sections were digested with a 0.05 % trypsin solution for 10 minutes at 37 °C, treated with a methanol solution containing 3 % H_2O_2 for 5 minutes to eliminate endogenous peroxidase, washed in Tris buffer for 5 minutes, incubated for 5 minutes at room temperature in normal goat serum, washed in Tris buffer for 5 minutes, and incubated in primary antibodies at room temperature overnight for p53 and EGFR, and for 30 minutes for EGF, v-erb B and ras p21. After two times washes with Tris buffer each for five minutes, the sections were incubated in biotinylated anti-mouse immunoglobulins at room temperature: EGFR and p53 for 60 minutes, and EGF and ras p21 for 30 minutes; the v-erb B sections were incubated in biotinylated anti-rabbit immunoglobulin; all the sections were

washed two times in Tris buffer for 5 minutes each. Reactions with the avidin-biotin peroxidase complex (DAKO Laboratories) were performed at room temperature: EGFR and p53 for 60 minutes and EGF, v-erb B and ras p21 for 30minutes; the sections were then rinsed with Tris buffer for 15 minutes. After color development with 0.03% diaminobenzidine (including 0.01% H_2O_2), the sections were counterstained with Mayer's hematoxylin or methyl green for 30 seconds. When more than one quarter of the neoplastic cells were stained, results were interpreted as positive. As negative control, the primary antibody was replaced by phosphate-buffered saline (PBS).

Histological slides were viewed by light microscopy, and staining intensity was graded on a scale of - to ++.

RESULTS

Twenty male and four female cases ranged between ages of 41 to 83 years were choosen for this study. Histo- and clinico-pathologic data of the twenty-four squamous cell carcinoma and five cases of pharyngitis are listed in Table I. Twenty four squamous cell carcinomas are classified into three histologic subtypes; well differentiated type: 10 cases, moderaterly differentiated type: 7 cases, and poorly differentiated type : 7 cases. The positive rates for p53, EGF, EGFR, v-erb B, and ras p21 of the twenty-nine cases are shown in Table II. EGF, v-erbB and ras p21 stained diffusely in the cytoplasms (Fig. 1,3,4), p53 products stained in the nuclei, and EGFR stained on their cell membranes (Fig. 2) of squamous cell carcinomas. The p53 product stained positively in 16 cases of squamous cell carcinoma (66.6%), but stained neither in pharyngitis nor in normal mucosae of carcinoma cases.

EGFR showed positive on cell membranes of twelve carcinomas (50%) and stained faintly on the membranes of the basal layer of normal mucosa in only one of the five cases of pharyngitis (20%). Although this difference was not significant, EGFR showed positive in more cases of well differentiated than in those of moderately- or poorly- differentiated carcinoma. In the cytoplasm of the 24 cases of squamous cell carcinoma, ras p21, EGF and v-erb B showed positive in 8 (33.3%), 7 (29.2%), and 15 (62.5%) cases respectively. EGF, ras p21 and v-erbB showed at a relatively higher frequency in well- and moderately-differentiated than in poorly differentiated carcinoma; this differences between tumor differentiation and the positive rate was, however, not significant. EGFR and p53 were

Table I. Clinical and histologic features of the hypophryngeal tumors.

Case	Age	Sex	Histo- and clinico-pathologic Diagnosis	p53	ras p21	v-erb B	EGF	EGFR
1	63	male	chronic pharyngitis	−	−	−	−	−
2	74	male		−	−	−	−	−
3	66	male		−	−	−	−	−
4	49	male		−	−	−	−	−
5	72	male		−	−	−	−	±
6	41	male	well differentiated s.c.c.	++	−	+	−	+
7	70	male		++	±	++	−	+
8	74	male		+	−	+	−	±
9	54	male		+	−	−	−	+
10	59	female		±	−	−	−	−
11	58	male		±	−	−	−	−
12	67	male		±	±	++	±	++
13	73	male		−	−	±	−	±
14	55	male		−	+	−	+	−
15	62	male		−	+	+	+	−
16	57	female	moderately differentiated s.c.c.	++	−	+	−	+
17	77	male		++	±	++	±	±
18	62	female		+	+	+	+	++
19	57	female		±	±	−	−	−
20	56	male		±	−	±	−	−
21	49	male		−	−	−	−	−
22	65	male		−	−	±	−	−
23	51	male	poorly differentiated s.c.c.	++	−	−	+	±
24	39	male		++	−	+	−	−
25	63	male		++	−	−	−	−
26	51	male		±	+	±	+	±
27	72	male		±	−	−	−	−
28	83	male		−	−	+	−	±
29	65	male		−	−	−	−	−

s.c.c.: squamous cell carcinoma

Table II. Histological features and the positive rate for specific stains.

	p53	ras p21	v-erb-B	EGF	EGFR
Non-carcinoma	**0/5**	**0/5**	**0/5**	**0/5**	**1/5**
Carcinoma	**17/24**	**8/24**	**15/24**	**7/24**	**12/24**
Well differentiated	7/10	4/10	7/10	3/10	6/10
Moderately differentiated	5/7	3/7	5/7	2/7	3/7
Poorly differentiated	5/7	1/7	3/7	2/7	3/7

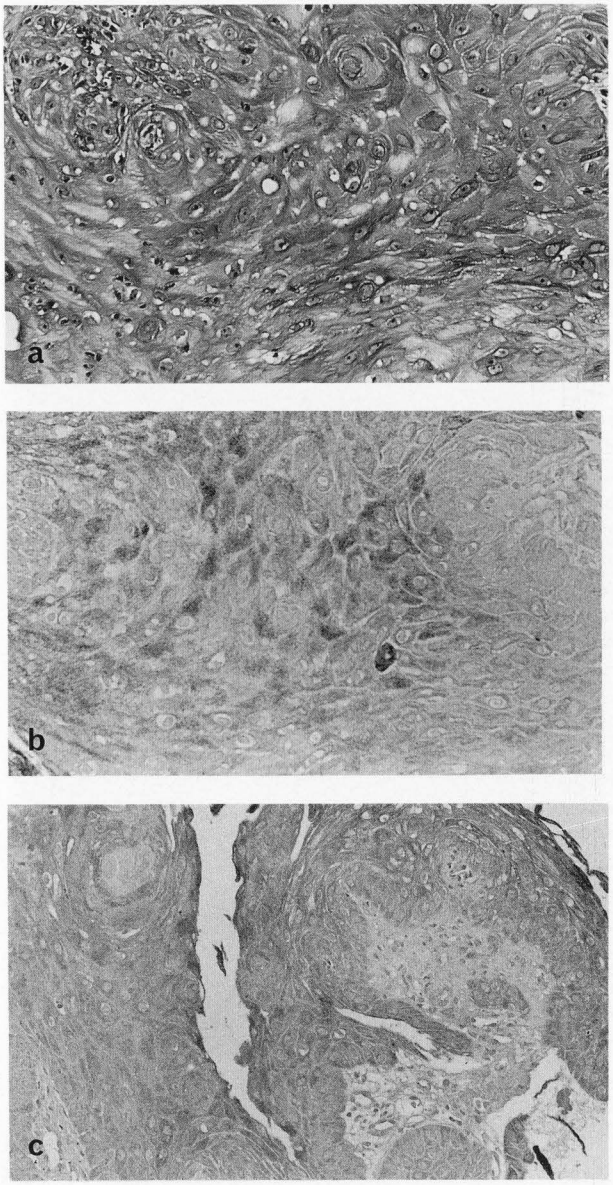

Fig. 1. Expressions of EGF and ras in paraffin sections of squamous cell carcinoma, Case No 14. The positive staining was diffuse in the cytoplasms by ras and EGF stains. (**a**: Hematoxylin-Eosin stain, magnification x 300; **b**: ras stain, magnification x 300; **c**: EGF stain, magnification x 150)

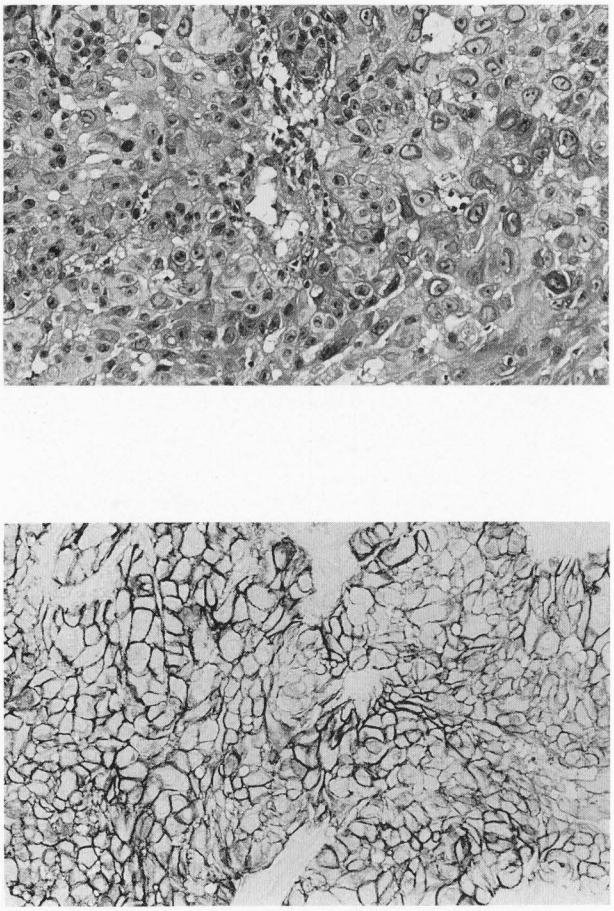

Fig. 2. Expressions of EGFR in a paraffin section of well differentiated squamous cell carcinoma, Case No 12. Cancer cell stained positively for EGFR. The positive staining was seen on cell membrane by EGFR. (above: Hematoxylin-Eosin stain, magnification x 300; below: EGFR stain, magnification x 300)

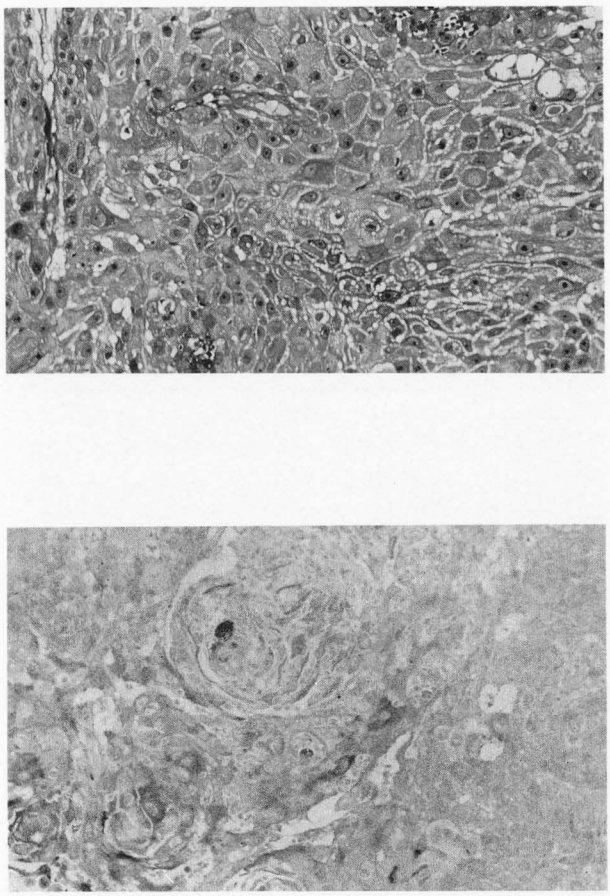

Fig. 3. Expressions of v-erb B in a paraffin section of well differentiated squamous cell carcinoma, Case No 12. Cancer cell stained positive for v-erb-B. The positive staining was diffuse in the cytoplasm for v-erb B (above: Hematoxylin-Eosin stain, magnification x 300; below: v-erb B stain, magnification x 300)

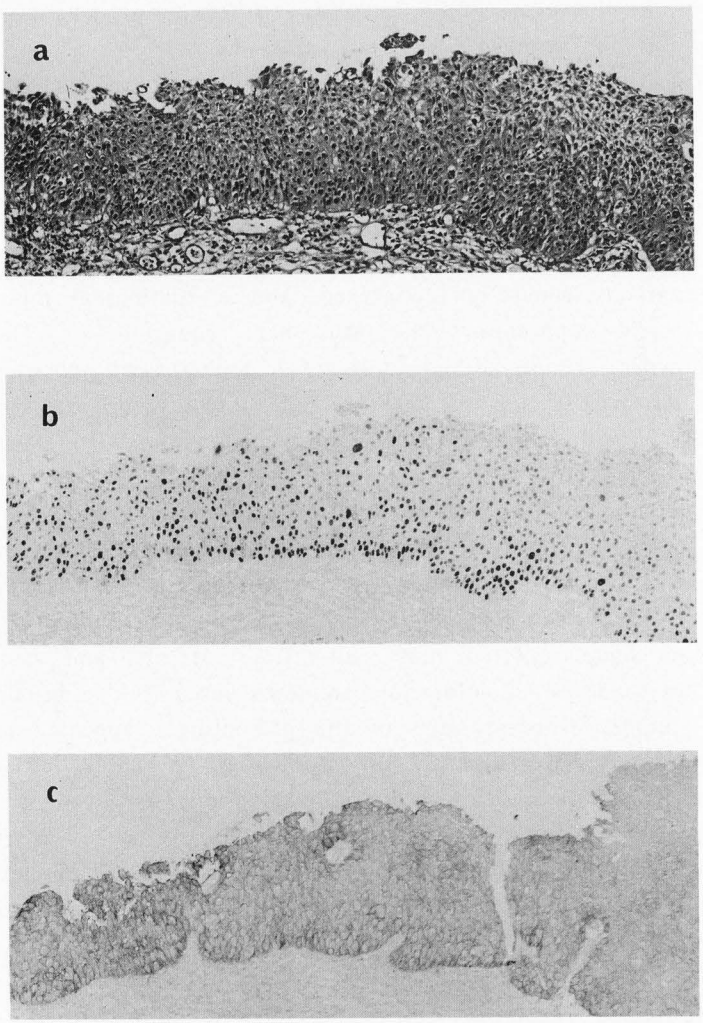

Fig. 4. Expressions of p53 product and EGFR in a paraffin section of the dysplastic epitherium in Case No 7. Dysplastic cells were strongly positive for p53, and EGFR. (**a**: Hematoxylin-Eosin stain, magnification x 75; **b**: p53 stain, magnification x 75; **c**: EGFR stain, magnification x 75)

overexpressed in some dysplastic mucosae surrounding cancer lesions (Fig.4); in contrast, ras p21, EGF, v-erb B were negative in dysplastic lesions. In this case, EGFR and p53 stained strongly positive in cancer cells, but did not stained in non-cancerous and non-dysplastic lesions; they did not always manifest the same staining pattern in the serial section study.

DISCUSSION

In the oncogenesis of various organs, the concept of multi-step carcinogenesis is generally recognized, and chronological mutagenesis of oncogenes and suppressor genes has been reported.[7] There are few reports, however, about carcinogenesis of hypopharyngeal mucosae. We estimated the expression or overexpression of oncogenes, suppressor genes and some growth factors in hypopharyngeal mucosae, dysplasia, and carcinoma.

Overexpression of p53 protein, well known for its expression of the mutated, long-life form in most cases, has been found in primary lung carcinoma,[16] breast carcinoma,[2,3,6] basal cell carcinoma of the human skin,[33] esophageal carcinoma,[17,31] and gastric carcinoma.[17] Some investigators have reported that alterations of p53 and ras genes are found in up to 50 % of colorectal carcinoma cases.[7] In our and others' previous studies, overexpression of the p53 protein appeared at advanced stages of tumorigenesis in the colon and liver[28] and at initial stages of tumorigenesis in thymomas.[14] In this study, the dysplastic epithelium of the hypopharynx showed overexpression of p53, which may be suggestive of mutation of p53 appearing at an early stage of carcinogenesis.

On the other hand, the expression of EGF, EGFR and erb B can be associated with a worse prognosis in some tumors, e.g. gastric carcinoma,[37] breast carcinoma[35] and squamous cell carcinoma of the oral mucosa,[8] squamous cell carcinoma of the maxillary sinus,[25] esophageal squamous cell carcinoma,[27,30] sebaceous and sweat gland carcinoma of the skin,[13] and primary laryngeal carcinoma.[32] EGF, a polypeptide (molecular weight, 6,045) consisting of 53 amino acids,[10] is distributed in the submandibular gland, parotid gland, and duodenal Brunner's gland, stomach, pancreas, and bone marrow,[20] and it promotes growth and differention, not only in epithelial, but also in mesenchymal cells.[12] EGFR is encoded by a proto-oncogene erb B which produces the erb B gene of a similar, but not identical, structure.[15,35] In this study,

EGF and EGFR tended to express more strongly in cases of hypopharyngeal carcinoma (EGF: 29.1%; EGFR: 50%) than in those of non-carcinoma (EGF: 0%; EGFR: 20%). These results suggest that the networks of EGF and EGFR probably play an important role in the progression of squamous cell carcinoma. V-erb B, which relates to EGFR but represents a truncated EGFR whose extracellular part is lost, can accelerate cell cycles without ligands, e.g. EGF and TGF a; it stained positively in hypopharyngeal squamous cell carcinoma (62.5%), but was negative in all of the non-cancerous lesions. EGFR and EGF are not directly related to carcinogenesis, but it can be hypothesized that cancer cells develop with autocrine or paracrine systems in the presence of EGF, EGFR and v-erb B. Some authors have reported that EGFR expresses more intensely in poorly differentiated carcinoma than in the well differentiated type,[9] although other reported that overexpression of EGFR was obsereved immunohistopathologically in well differentiated as well as poorly differentiated squamous cell carcinoma of the head and neck.[18] In this study, the relationship between the positive rate of EGFR and the histologic types of hypopharyngeal squamous cell carcinoma was not significant. Taken together, further studies are required to elucidate the causal relationship between EGFR positivity and histologic types of hypopharyngeal squamous carcinoma. EGFR has been reported positive in cholesteatoma,[5] which is hyperkeratotic non-neoplastic epithelium, and higher immnoreactivity of EGF in the epidermis was demonstrated in active than in inactive cholesteatoma.[11] Our study has demonstrated that EGFR showed positive in some basal cells of the normal hypopharyngeal mucosa. Thus, whenever EGFR is used as a carcinoma marker, its grade of staining should be estimated carefully.

Ras p21 showed positive at early stages of tumorigenesis in the colon and have some relationship with poor prognosis in thymomas.[14,26] Oku et al.[29] showed high positivity of ras p21 in oral squamous cell carcinoma with poor prognosis. In our 24 cases of hypopharyngeal squamous cell carcinoma, ras p21 showed positive in 8 cases and negative in all of non-cancerous lesions, but there is no difference between staining pattern and tumor differentiation. Dysplastic mucosae showed the overexpression of p53 and EGFR; ras p21, EGF, and v-erbB were both negative in all of the dysplastic lesions but positive only in cases of cancer of the hypopharynx. In contrast, EGF and EGFR were sometimes positive in non-cancerous lesions.[4,24]

A difficult problem sometimes encountered in deciding ways of treating dysplastic mucosae of the hypopharynx. Our results suggest that combined immunohistochemical stainings with antibodies against tumor suppresser and oncogenes may be new helpful markers to predict the biologic behaviour of dysplastic mucosae in the hypopharyngeal region. Further, our data suggest that p53-, ras p21-, EGF-, and/or v-erb B-positive dysplastic mucosa may have a biological nature similar to that of carcinoma.

The present study strongly indicates that the mutation of p53 probably plays an important role at the early stages of tumorigenesis, and that the networks of EGF, EGFR and v-erb B may be important in the progression and/or proliferation of squamous cell carcinoma. Our staining result presents new prognostic factors for histologic diagnoses of hypopharyngeal neoplasms or dysplasia.

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