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Heme Oxygenase-1 Expression Predicts Cervical Lymph Node Metastasis of Tongue Squamous Cell Carcinomas.

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Abstract

Heme oxygenase-1 (HO-1) is known as a stress-inducible protein. The presesnt study is designed to investigate the relationship between HO-1 expression levels and clinical features of tongue cancer by using HO-1 responsiveness to stress as a clinical indicator. One hundred and twelve biopsy samples from tongue squamous cell carcinomas were analyzed semiquantitatively by immunohistochemistry. Correlations between the expression level of HO-1 and the clinical features of tumors were statistically analyzed. Fifty four cases with surgical confirmation of lymph node metastasis were examined the association between cervical lymph node metastasis (pN) and other clinical features, including the HO-1 expression level, using logistic regression. The low HO-1 expression group contained significantly more undifferentiated samples (P = 0.04) and pN positive cases (P = 0.01) by univariate analysis. The low HO-1 expression group (odds ratio = 8.49; 95% confidence interval = 1.64-44.09, P = 0.01) and an endophytic shape (odds ratio = 16.79; 95% confidence interval = 1.77-159.53, P = 0.01) were significantly associated with an increased risk of developing lymph node metastasis by multivariate analysis. Low HO-1 expression was associated with lymph node metastasis. The expression profile suggests HO-1 could be used clinically as a marker for tumors possessing the potential for lymph node metastasis. This method could prove useful as an adjuvant method to detect lymph node metastasis and

may help reduce the number of surgeries by indicating when surgery is unnecessary.

Keywords: Heme oxygenase-1; squamous cell carcinoma; tongue cancer; lymph node metastasis

1. Introduction

Heme oxygenase (HO) is originally identified as an enzyme that catalyzes the initial reaction in heme catabolism: the oxidative cleavage of the α-meso carbon bridge of b-type heme molecules to yield equimolar quantities of biliverdin IXa, carbon monoxide (CO), and iron. HO has long been known to undergo adaptive regulation in response to heme. The effect is due to consequence of increased synthesis of a 32kDa protein, termed HO-1[1]. There are two isoforms: HO-1 and HO-2. HO-2 is the major isoform that presents under physiological conditions, localized in microsomes, and the stress inducible isoform HO-1 is localized in mitochondria. HO-1 and HO-2 are product of distinct genes. Human HO-1 gene (HMOX1) is localized to chromosome 22q12, while HO-2 gene (HMOX2) is localized to chromosome 16p13.3. HO-1 is classified as heat-shock protein 32k (HSP32) [2]. HO-1 expression is very sensitive to stress, and is induced by many stimuli, including heme, heavy metal, heat shock, endotoxin, inflammatory cytokines, prostaglandins, and oxidative stress. HO-1 is thought to help maintain cellular homeostasis [1, 3]. We considered whether we could use this characteristic of HO-1 as an indicator of the amount of stress on a cancer.

There are many reports concerning HSPs, including HSP 27 and 70, and associations with clinical findings of squamous cell carcinomas have been shown [4-7]. In the head and neck region, HSPs have also been investigated as clinical indicators and associations with clinical features have been reported

[8-12]. Although there are many reports on HSPs in the head and neck region, few are on HO-1. In a previous study the potential of using HO-1 as a marker for clinico-pathological features was reported [13]. However, the samples consisted of a small number from various regions of the head and neck and did not include pathological confirmation of cervical lymph node metastasis. Here, we collected samples from a uniform region, the tongue, and investigated the relationship between HO-1 expression and clinico-pathological features of tongue squamous cell carcinoma. We also discuss the usefulness of HO-1 as a clinical indicator of lymph node metastasis.

2.Materials and methods

2.1. Patients

Tumor specimens were obtained by surgical biopsies of the tongue carcinoma from 112 patients who consulted the Division of Head and Neck Surgery, Chiba Cancer Center Hospital, during 1975-1999. The median age of the patients was 60, and the range was 27-88. The male:female ratio was 69:36. The tumors were staged according to the International Union Against Cancer (UICC) scheme [14]. The composition of the cases is shown in Table 1. No treatment for the malignant tumor was performed prior to biopsy. Fifty-four cases of the 112 patients underwent neck dissection and pN

was determined. The macroscopic shape of the tumors was classified into 4 groups, superficial, exophytic, endophytic, and combination type, as described previously [15] (Table 1). The maximum diameter was measured clinically. Three patients received surgical excision alone, 54 surgical excision and radiotherapy, and the remaining 55 patients received radiation therapy alone. For 109 patients treated with radiotherapy, 20 received liniac treatment alone (8-76 Gy), 31 received interstitial radiation alone (28-70 Gy), 1 electron beam therapy alone (42 Gy), and 57 a combination of these radiation therapies (10-85 Gy + 48.6-83 Gy).

2.2. Methods

Immunostaining was performed using the horseradish peroxidase-labeled streptavidin and biotin technique as described previously [13]. The samples were fixed with 10% neutral formalin buffered fixed specimens were embedded in paraffin and cut 5 μ m. The polyclonal antibody (Affinity Bioreagents, Inc, Golden, USA) was diluted 1:50 v/v in PBS, and the slides were incubated in the antibody solution at room temperature for 1 hour. The slides were reacted with biotinylated goat anti- rabbit IgG antibody, followed by horseradish peroxidase-conjugated streptavidin, and visualized with 3,3'-diaminobenzidine. One pathologist who was not informed of the patient's clinical status examined the immunostained slides.

The HO-1 immunostaining was semiquantified by a visual grading system in which the intensity of staining was categorized as Grade 0, 1+, 2+, or 3+, similar to a previously reported system [13]. Grade 0 was defined as the total

absence of HO-1 I immunostaining (Fig. 1A); Grade 1+ as < 25% of the tumor staining positive, or only faint staining (Fig. 1B); Grade 2+ as 25-50% of the tumor staining positive (Fig. 1C); and Grade 3+ as > 50% of the tumor staining positive (Fig. 1D).

For univariate analysis we used Student's t-test, Fisher's exact probability test, and the chi-square test. For multivariate analysis, multiple logistic regression analysis was used. The analyses were performed using the statistical software package StatView 5.0 (SAS Institute Inc. Cary, NC, USA.).

3. Results

3.1. Correlation between HO-1 expression and clinico-pathological features

HO-1 immunostaining of the tumor was classified into Grade 0, 10.7% (12 of 112); Grade 1+, 42.9% (48 of 112); Grade 2+, 27.7% (31 of 112); and Grade 3+, 18.8% (21 of 112). To simplify the correlation of HO-1 expression with clinical features, these groups were reclassified into low- (Grade 0, Grade 1+) and high- (Grade 2+, Grade 3+) HO-1 -expression groups. In addition, the T-categories were divided into T1 + T2 and T3 + T4 groups, and the N and pN-categories for lymph node metastasis as negative (N0) and lymph-node positive (N1 + 2) groups. The clinical stages were in addtion grouped into stage I + II and stage III + IV. The differentiation categories were divided into moderately or poorly differentiated (G2 + G3) and well differentiated (G1).

Table 2 shows the correlation between the HO-1 expression level and the clinico-pathological features of these cases. Comparison of the expression level of HO-1 with clinico-pathological features showed that high-expression groups included significantly more differentiated tumor cases. There were significant differences in the expression level of HO-1 between the G2 + G3 and G1 groups (P = 0.04), and between the pN0 and pN1 + 2 groups (P = 0.01). No significant difference in HO-1 expression was observed with respect to other factors, such as age, sex, T category, N category, stage, macroscopic shape, and maximum diameter.

3.2. Correlation between pN and clinico-pathological features including HO-1 expression

We then focused on the pN result and evaluated whether the HO-1 expression level could be a useful predictor of lymph node metastasis. The relationship between pN (N0 or N1 + 2) and clinico-pathological features was investigated by univariate analysis (Table 3). To simplify the classification, we divided the macroscopic shape classification into two groups, the endophytic type and the other types. To exclude the influence of irradiation, the irradiation type was used as a variable, dividing the cases into the following two groups: irradiation of the neck (the cases involving liniac irradiation) and cases without neck irradiation (the other cases, involving the interstitial therapy, no irradiation, etc.). This analysis showed that endophytic type (P = 0.03) and low HO-1

expression level (P = 0.01) were significantly associated with positive lymph nodes.

Subsequently multivariate analysis was used. The predictor variables in 54 cases were used in a logistic regression model with negative or positive pN as the dependant variables. A logistic model for predicting pN was constructed using clinical variables, including age, sex, T or N category, stage, differentiation, with or without neck irradiation, maximum diameter, macroscopic shape, and HO-1 expression level. The adjusted odds ratios (OR) and 95% confidence intervals (CI) are shown in Table 4. The pathologically positive lymph nodes were significantly associated with an 8.49-fold increase in the low expression of HO-1 (OR = 8.49; 95% CI = 1.64-44.09, P = 0.01) and a 16.79-fold increase in the endophytic type (OR = 16.79; 95% CI = 1.77-159.53, P=0.01), but the other predictors were not significantly associated with pN.

4. Discussion

In the present study we found HO-1 expression in 112 cases of tongue SCC was associated with pN (P = 0.01) and differentiation (P = 0.04) by univariate analysis. We also found pN was significantly associated with HO-1 expression level (P = 0.01) and endophytic type (P = 0.01) by multivariate analysis. It has previously been found that the low-HO-1 expression group was correlated with N category and poor differentiation [13]. Here, the results of the differentiation are similar to those of the previous report. The correlation between pN and macroscopic

shape are also in agreement [15]. However, in the present study no correlation with the N category was found. This discrepancy may be due to a difference in the diagnostic methods, which have improved since 1970s, and the fact that the accuracy of determining the N category in the older cases was not as good as for recent cases. However, pN, which is a more accurate method for defining whether lymph node metastasis exists, correlated with HO-1 expression.

The reason the HO-1 expression level was associated with lymph node metastasis is still uncertain. However, following hypothesis could be suggested. One is the stress response of tumors against the immune recognition of the host. If a tumor suffers from stress caused by the human body, this finding, which suggests that metastasis is more difficult for the stressed cancer cell, is reasonable. HO-1 is a very sensitive stress-inducible protein that is up-regulated by very small stressors [1]. If the human immune system recognizes the tumor cell as a nonself antigen, tumor cells will be attacked by the defense system of the host, making metastasis to the peripheral regions difficult. To the contrary, however, grafts that had induced HO-1 expression survived rejection better than grafts that did not have elevated HO-1 expression [16, 17]. This may mean that cells that are rejected by the human body endure under stressful conditions by expressing stress proteins like HO-1, but that that there is no additional benefit to expressing stress-responsive gene products for cells that can easily escape from immune recognition.

Another possibility is genetic disturbance. The heme oxygenase gene is located in

22q12 of the human chromosome [18, 19]. There are interesting reports that indicate an association between allelic loss on chromosome 22 and clinico-pathological features in head and neck squamous cell carcinoma [20, 21]. If there is some genetic factor, like a tumor suppressor gene, that suppress carcinogenesis and invasiveness of the tumor near 22q, this could explain the coincidence between the loss of HO-1 expression and lymph node metastasis.

The control of cervical lymph node metastasis is a crucial factor for good prognosis in head and neck cancer treatment. In practice, computed tomography, magnetic resonance imaging, and ultrasonography are very useful for detecting lymph node metastases. Such methods are characterized by the morphological aspect for diagnosis and do not reflect the characteristics of the tumor biology. Furthermore, very small lymph nodes, i.e., less than 5 mm in diameter, cannot be identified using these imaging techniques [22]. In addition, although there are many indicators associated with the cell cycle, apoptosis, growth factors, cell adhesion, etc., that are correlated with prognosis or clinicopathological features [23, 24], the use of HO-1 expression is novel, and there are only a few reports of markers that indicate lymph node metastasis. If we can use this property of HO-1 expression to predict cervical lymph node metastasis clinically, it may prove helpful as an adjuvant method to detect lymph node metastasis and may help reduce the number of surgeries, like elective neck dissection, by indicating when such surgery is unnecessary.

Acknowledgements

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Figure legend

Fig. 1. Representative photomicrographs of immunohistochemical staining with HO-1 antibody according to the visual grading system. Grade 0 (a total absence of HO-1 I immunostaining) (A), Grade 1+ (< 25% of tumor staining positive) (B), Grade 2+ (25-50%) (C), Grade 3+ (> 50%) (D). (original magnification, x100)

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TABLE 1

Characteristics for Oral Squamous Cell Carcinoma Case Patients

Clinical features		
Median age		60
Range		27-88
Sex ratio f/m		36 /76
Macroscopic shape		
Exophytic		20
Combination		26
Endophytic		45
Superficial		21
Maximum diamater of tumor		
Mean±S.D.		3.19±1.47
Radiation		
Liniac		20
Interstitial therapy		31
Electron therapy		1
No irradiation		3
Liniac+other radiation therapies		57
Surgery		
Without surgery		55
Excision with neck dissection		54
Excision alone		3

TNM classification		n
T category	T1	32
	T2	47
	T3	26
	T4	7
N category	N0	78
	N1	14
	N2a	3
	N2b	12
	N2c	5
M category	M0	111
	M1	1
Stage	Stage I	33
	Stage II	33
	Stage III	22
	Stage IV	24
pN	N0	19
	N1	11
	N2a	3
	N2b	17
	N2c	4

TABLE 2

Correlation with HO-1 Expression and Clinicopathological Features

	High Expression	Low Expression	P value	
Age				
>60	28	31	0.82	c
60>=	24	29		
Sex				
Female	13	23	0.13	c
Male	39	37		
T category				
T1+2	41	38	0.07	c
T3+4	11	22		
N category				
N0	39	38	0.18	c
N1+2	13	22		
Stage				
I+II	34	32	0.20	c
III+IV	18	28		
Differentiation				
Moderately or poorly	15	29	0.04	c
Well	37	31		
Macroscopic shape				
Exophytic	8	12	0.68	c
Combination	14	12		
Endophytic	19	26		
Superficial	11	10		
Maximum diamater of tumor (n)	52	60		
Mean±S.D.	3.09±1.44	3.28±1.50	0.75	t
pN				
pN0	13	6	0.01	c
pN1+2	11	24		

c: chi square test
F: Fisher's exact test
t: Student's t-test

TABLE 3

Correlation with pN and Clinicopathological Features including HO-1 expression

	pN negative	pN positive	P value	
Age				
>60	9	18	0.78	c
60>=	10	17		
Sex				
Fmale	5	10	0.86	c
Male	14	25		
T category				
T1+2	13	23	0.84	c
T3+4	6	12		
N category				
N0	12	17	0.30	c
N1+2	7	18		
Stage				
I+II	10	15	0.49	c
III+IV	9	20		
Differentiation				
G2+3	8	22	0.14	c
G1	11	13		
Macroscopic shape				
Endophytic	4	18	0.03	F
Other	15	17		
Maximum diamater of tumor	19	35		
	3.17±1.30	3.28±1.67	0.24	t
HO-1 expression				
High	13	11	0.01	c
Low	6	24		
Radiation				
With neck irradiation	14	27	0.78	c
Without neck irradiation	5	8		

c: chi square test
F: Fisher's exact test
t: Student's t-test

TABLE 4

Odds ratios (OR) and 95% Confidence Intervals (CI) for pN-positive cases associated with clinicopathological features including HO-1 expression

	Adjusted OR	95% CI	P value
Age (60≦:60＞)	1.34	0.24-7.37	0.74
Sex (female : male)	0.53	0.09-3.36	0.50
T category (T1+2: T3+4)	0.28	0.02-4.62	0.37
N category (negative : positive)	3.61	0.20-64.17	0.38
Stage (I+II : III+IV)	0.28	0.02-17.77	0.80
Differentiation (G2+3 : G1)	0.61	0.13-2.86	0.53
Radiation (with : without neck irradiation)	0.74	0.11-4.89	0.75
Macroscopic shape (the others : endophytic type)	16.79	1.77-159.53	0.01
Maximum diamater of tumor	0.86	0.35-2.12	0.74
HO-1 expression (high : low)	8.49	1.64-44.09	0.01

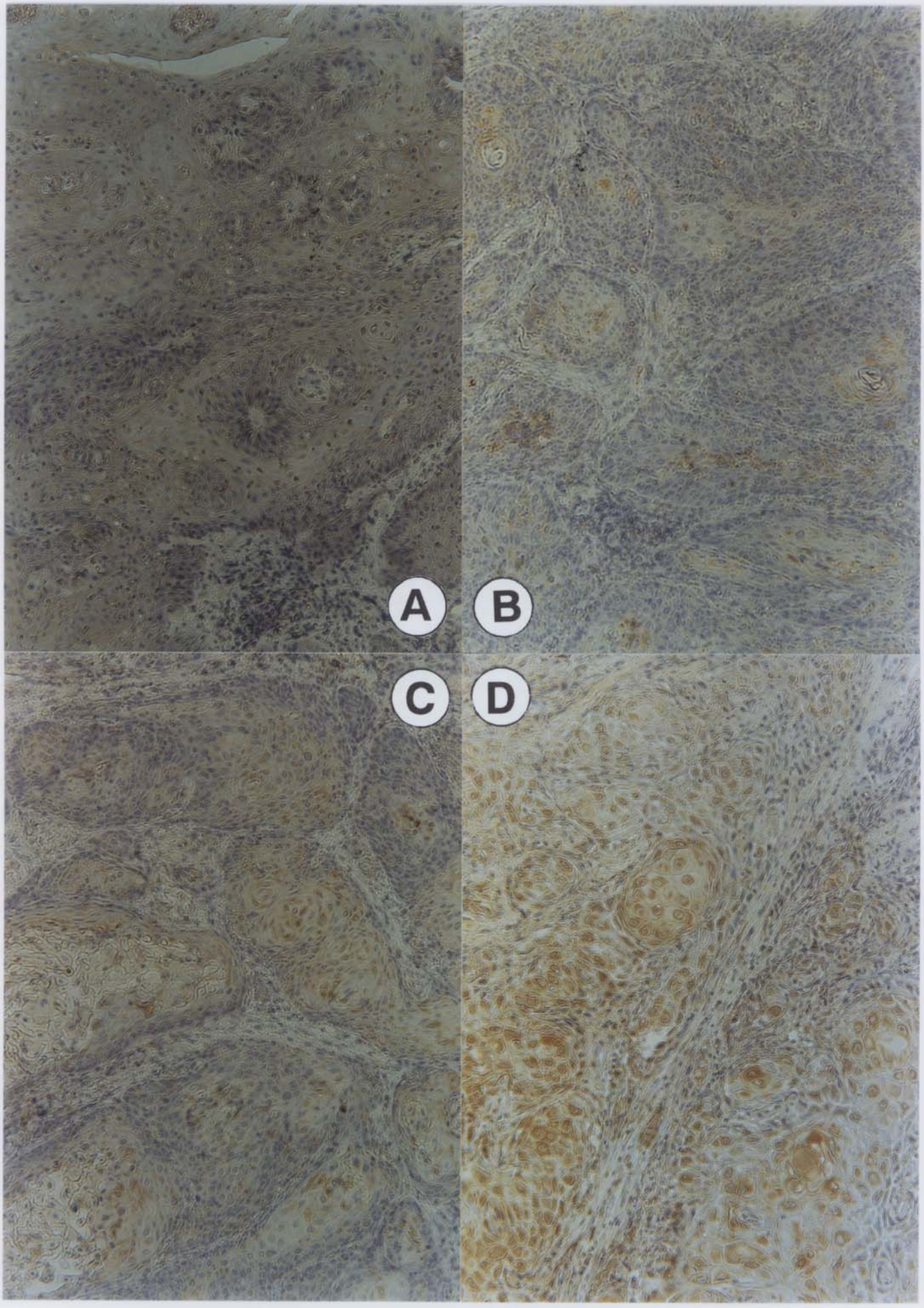

Figure 1