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ORIGINAL ARTICLE

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Coexpression of p53 and c-erbB-2 proteins is associated with histological type, tumour stage, and cell proliferation in malignant salivary gland tumours

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Abstract The development and progression of cancer are known to be regulated by various oncogenes and tumour suppressor genes. We analysed 63 primary malignant salivary gland tumours for the expression of p53 and c-erbB-2 proteins. Immunohistochemically, 7 of 63 tumours (11%) showed diffuse nuclear staining for p53 protein, and all 7 were also positive for c-erbB-2 protein. The overexpression of p53 protein correlated closely with the overexpression of c-erbB-2 protein ($P<0.001$). Overexpression of both p53 and c-erbB-2 proteins (coexpression) was found in tumours of certain histological types, such as adenocarcinoma, carcinoma in pleomorphic adenoma, and salivary duct carcinoma. Furthermore, it is noteworthy that coexpression was associated with high-grade carcinoma, advanced tumour stage, and a high Ki-67 labelling index (%) which is a marker of cell proliferation. In adenocarcinoma, we attempted to clarify the relationship between coexpression and histological grade. Coexpression was associated with histological grades showing high mitotic indices and necrotic areas, which reflected high cell-proliferative activity. These results suggest that the accumulation of genetic alterations, such as those involving p53 and c-erbB-2, plays an important part in the progression of malignant salivary gland tumours.

Key words Salivary gland · Tumour · c-erbB-2 · Ki-67 · Adenocarcinoma

Introduction

The development and progression of various malignant human neoplasms are known to be associated with multiple genetic changes. Recent studies have shown that activation of oncogenes such as *c-erbB-2* and mutation of the *p53* tumour suppressor gene are involved in the progression of malignant tumours. Mutation of the *p53* gene with resulting overexpression of the mutant *p53* protein has been found in several common human cancers, such as those arising from the lung [5], breast [3, 6], colon [28], oesophagus [21], and stomach [16]. Some studies have revealed that overexpression of p53 protein correlates with various histopathological variables suggesting a comparatively poor prognosis [4, 11]. On the basis of recent studies, it has also been asserted that immunohistochemical demonstration of the protein product encoded by *c-erbB-2* oncogene provides valuable prognostic information [24].

Salivary gland carcinoma (SGC) is a relatively rare neoplasm, and its molecular genetic alterations have not been well demonstrated. The present study was designed to elucidate the genetic alterations present in SGCs by focusing on the expression of p53 and c-erbB-2 proteins. Mutation of the *p53* gene is one of the most common genetic abnormalities in human malignancies, and amplification of the *c-erbB-2* gene has frequently been documented in human adenocarcinomas [15, 26]. With regard to SGCs, few previous studies have been reported, and in these DNA aneuploidy was closely related to *p53* immunoreactivity [13] and *c-erbB-2* immunostaining was associated with a comparatively poor prognosis [20]. However, there are no previous reports on the examination of coexpression of p53 and c-erbB-2 proteins in SGCs of various histological types.

SGC encompasses various histological groups. There are few reliable prognostic factors other than histological type, tumour size, anatomical extent of the disease, and lymph node status at the time of diagnosis. The diverse morphological features of SGC make it difficult to categorize and clarify their histology [9] and they remain a poorly categorized group of adenocarcinomas defined by the World Health Organization as "a carcinoma with glandular, ductal or secretory differentiation that does not fit into the other categories of carcinoma" [22]. It is suggested that this group of tumours has differing biological behaviour and different prognoses because it is morphologically heterogeneous. Definite criteria allow-

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ing prediction of biological behaviour would be of benefit for the treatment of patients. A few studies have been reported in which pathologists attempted to subclassify the adenocarcinomas, especially in terms of their behaviour and prognoses [25], but these morphological subclassifications have not been thoroughly established [9]. We have attempted to establish new criteria for adenocarcinomas of the salivary glands, which give information about their biological behaviour as well as their prognosis.

In this study, nuclear p53 accumulation and the expression of c-erbB-2 protein were investigated by means of an immunohistochemical technique. The results were evaluated for possible correlations with prognostic factors such as histological type, tumour stage (size and lymph node status), and Ki-67 labelling index (LI). Ki-67 has been shown to be a marker of cell proliferation, and a close correlation has been demonstrated between the frequency of Ki-67 positive cells and clinical outcome [8, 18]. We also attempted to clarify the association of expression of p53 and c-erbB-2 proteins with prognostic factors, the prognosis of individual patients, and histological details of the adenocarcinoma.

The specific aims of this study were to determine whether combinations of genetic alterations affecting oncogenes and/or tumour suppressor genes provide meaningful information about the role of these genes in the development or progression of tumours and whether the protein products of these genes might serve as new markers predicting the biological aggressivity of SGCs.

Materials and methods

Salivary gland tumours

We reviewed 63 primary malignant salivary gland tumours obtained from the pathological files of Keio University Hospital. These specimens had been surgically resected during the period from 1980 through 1995. Sections stained with haematoxylin and eosin were used for histological assessment. All tumours were classified histologically according to the WHO criteria published in 1991 [22]. In addition, SGCs were subdivided into two clinical grades: high-grade carcinoma (adenocarcinoma, carcinoma in pleomorphic adenoma, salivary duct carcinoma) and low-grade carcinoma (basal cell adenocarcinoma, polymorphous low-grade adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, acinic cell carcinoma). This classification was based on the clinical survival rate or growth rate, according to criteria used in previous reports [9, 22, 27] including that of the study by Ishii et al. [13].

Immunohistochemistry

The primary antibodies and staining methods used in this study are listed in Table 1. Immunohistochemical staining was done on 5-μm-thick sections cut from formalin-fixed, paraffin-embedded tissues, using primary antibodies recognizing p53, c-erbB-2, and Ki-67. Two murine monoclonal antibodies against p53, which recognize both wild-type p53 and mutant type p53, reacted with different denaturation-resistant epitopes. After deparaffinization of the sections and rehydration in ethanol, the endogenous peroxidase activity was blocked with hydrogen peroxide (0.3%) in absolute methanol. Indirect or avidin-biotin-peroxidase complex (ABC) methods were used, and the antigen retrieval method employing microwave or trypsinization was used for some antibodies. Negative controls were obtained by omission of the primary antibody.

Assessment of staining reaction

For p53 expression, only tumour cells with distinct nuclear immunostaining for p53 were regarded as positive. Expression of p53 was categorized into three groups: no expression, no positive nuclei; low expression, focal individual positive nuclei (focal pattern, Fig. 1b) or positive nuclei scattered throughout the tumour (scattered pattern, Fig. 1c); high expression, diffuse positive nuclei (diffuse pattern, Fig. 1a).

In evaluation of the expression of c-erbB-2 protein, intense cell surface membrane staining in the majority of cells was interpreted as positive (Fig. 1d). Diffuse cytoplasmic staining was ignored, although its significance remains uncertain [15].

For evaluation of the percentage Ki-67 labelling index (LI%), nuclei showing an intense homogeneous brown colour or granular staining were recognized as positive. The labelling index was defined as the ratio of the number of Ki-67-positive cells to that of total cells counted (about 500 cells in each section) on photographs of randomly selected areas in each individual tumour.

Assessment of histological grade of adenocarcinoma

Haematoxylin-eosin-stained sections were examined to evaluate the histological grades of adenocarcinomas, including one case of salivary duct carcinoma. Histological grades were evaluated according to two factors: cell-proliferative activity (mitotic index, area of necrosis) and morphological atypia (nuclear atypia, differentiation representing structural atypia). The mitotic index was assessed by counting mitotic figures in ten randomly selected high-power fields (400× magnification; field area, 0.188 mm^2).

Statistical analysis

The statistical significance of individual findings and associations was analysed using the Mann-Whitney U-test, Fisher's exact probability test, or the Chi-square test. Probability values lower than 0.05 were considered to be significant.

Table 1 Primary antibodies and staining methods used in this study (*O-S* Oncogene Science, Cambridge, USA, *Dako* Dako A/S, Glostrup, Denmark, *Novocastra* Novocastra Laboratories, Newcastle, UK, *Immunotech* Immunotech S.A., France; *antigen retrieval* antigen retrieval by microwave heating, *indirect* indirect method, *ABC* avidin-biotin-peroxidase complex method)

Antibody	Clone	Clonality	Source	Method	Dilution
p53	DO-1	Mono.	O–S	Antigen retrieval and indirect	×100
	PAb1801	Mono.	O–S	Antigen retrieval and indirect	×20
c-erbB-2		Poly.	Dako	Antigen retrieval and indirect	×100
	CB11	Mono.	Novocastra	ABC	×40
Ki-67	MIB-1	Mono.	Immunotech	Antigen retrieval and indirect	×100

Table 2 Clinicopathological features of salivary gland carcinomas (*maj* major salivary gland origin, *min* minor salivary gland origin, *LI* labelling index)

	No.	Sex, M:F	Mean age (years)	Site, maj:min	Ki-67 LI (%) (mean)	p53: positive		c-erbB-2: positive (%)
						High (%)	Low (%)	
Adenocarcinoma	11	8:3	54	8:3	48	4 (46)	0 (0)	6 (55)
Salivary duct carcinoma	1	1:0	71	1:0	68	1 (100)	0 (0)	1 (100)
Carcinoma in pleomorphic adenoma	3	2:1	66	3:0	64	2 (67)	0 (0)	2 (67)
Adenoid cystic carcinoma	21	11:10	48	11:10	14	0 (0)	2 (10)	0 (0)
Mucoepidermoid carcinoma	17	12:5	47	15:2	18	0 (0)	3 (18)	4 (24)
Acinic cell carcinoma	8	5:3	33	8:0	8	0 (0)	0 (0)	0 (0)
Basal cell adenocarcinoma	1	1:0	60	1:0	30	0 (0)	0 (0)	0 (0)
Polymorphous low-grade adenocarcinoma	1	1:0	33	0:1	13	0 (0)	0 (0)	0 (0)
Total	63					7 (11)	5 (8)	13 (21)

Results

Histological classification and clinicopathological features of SGCs

The histological classification and clinicopathological features of the SGCs examined are listed in Table 2.

The staining patterns obtained with DO1 and PAb1801 against p53 were similar in most tumours. In 7 of the 63 cases (11%) high expression of p53 protein was observed. These 7 were made up of 4 cases of adenocarcinoma, 1 of salivary duct carcinoma, and 2 of carcinoma in pleomorphic adenoma. There were some adenoid cystic carcinoma and mucoepidermoid carcinoma cases with low expression of p53. The scattered pattern of low expression was found in only 1 case of mucoepidermoid carcinoma. No detectable nuclear staining was found in unaffected salivary gland tissues surrounding the tumour.

The staining patterns with monoclonal and polyclonal antibodies against c-erbB-2 were similar, 13 cases (21%) being positive for c-erbB-2 protein. These 13 were 6 of adenocarcinoma, 1 of salivary duct carcinoma, 2 of carcinoma in pleomorphic adenoma, and 4 of mucoepidermoid carcinoma. No cell membrane immunoreactivity was observed in unaffected salivary gland tissues surrounding the tumour.

Histological types with mean Ki-67 LI (%) in excess of 50% were salivary duct carcinoma and carcinoma in pleomorphic adenoma.

Correlation between overexpressions of p53 and c-erbB-2 proteins

Table 3 shows that high expression of p53 protein correlates significantly with the expression of c-erbB-2 protein ($P<0.001$, Fisher's exact probability test). All 7 tumours that showed high p53 expression were also positive for c-erbB-2 proteins (coexpression). There were 50 tumours that were negative for both proteins.

Relationships of coexpression to histological type, clinical grade, tumour stage, cell proliferation, and prognosis

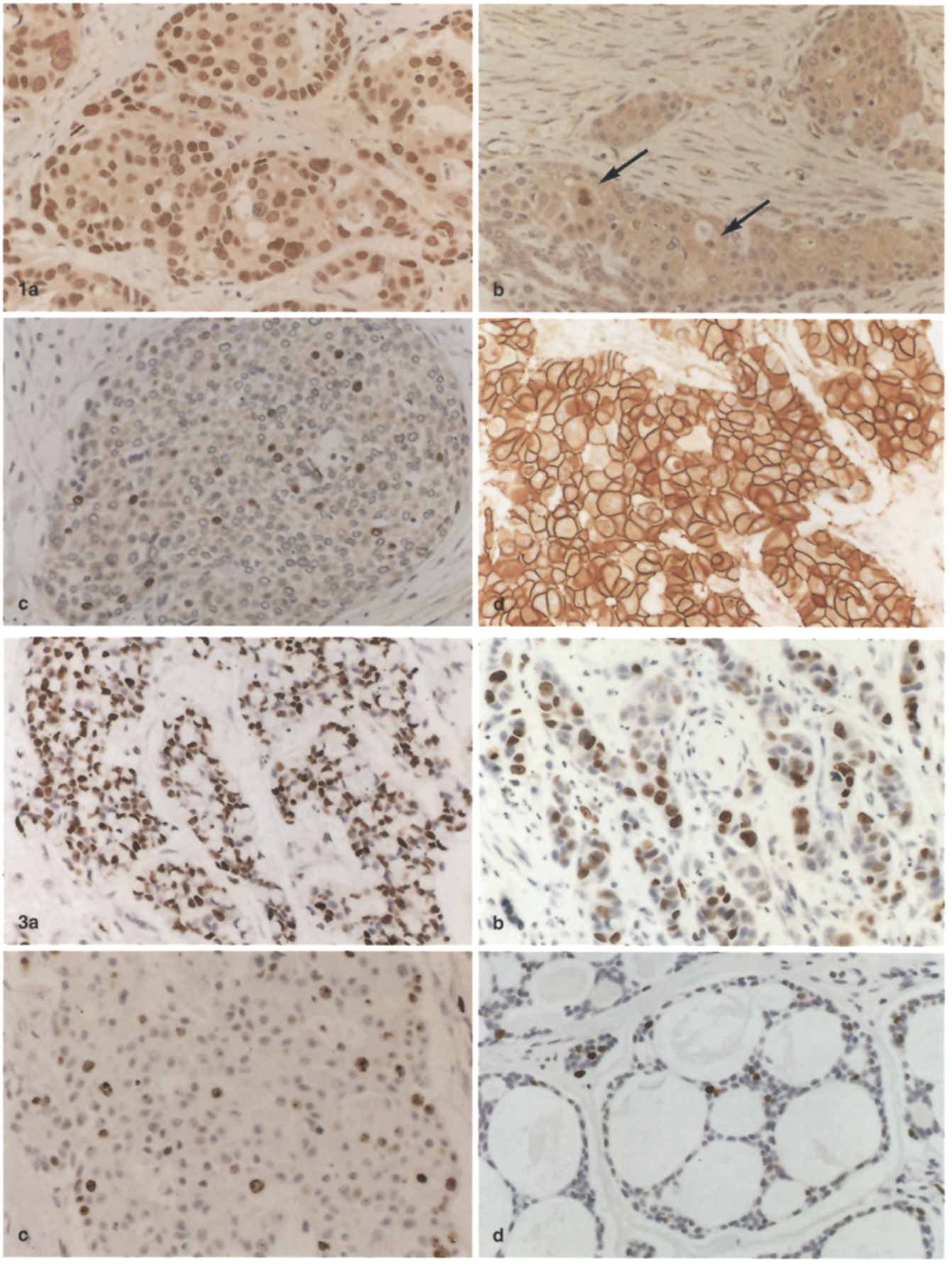
The relationships between coexpression and various prognostic factors are shown in Table 4 and 5. Coexpression was observed specifically in SGCs of certain histological types categorized as high-grade carcinoma: adenocarcinoma, salivary duct carcinoma, and carcinoma in pleomorphic adenoma (Table 4). Coexpression was significantly associated with high-grade carcinoma ($P<0.0001$), large tumour size ($P<0.001$), and positive lymph node metastasis ($P<0.001$) (Table 5). Although c-erbB-2 expression alone also correlated with large tumour size ($P<0.05$) and positive lymph node metastasis ($P<0.05$), coexpression was more closely associated with high-grade carcinoma than c-erbB-2 expression alone ($P<0.05$) (Table 5).

Correlations between Ki-67 LI (%) and the expressions of the two proteins are illustrated in Fig. 2. Coexpression showed a significantly stronger association with a high Ki-67 LI (%) than c-erbB-2 expression alone ($P<0.05$) or no expression of either protein (no expression) ($P<0.0001$; Fig. 3a–d).

Of the 63 cases studied, 32 were followed up either for more than 5 years or until death. These 32 cases were made up of 4 with coexpression (3 adenocarcinomas, 1 carcinoma in pleomorphic adenoma), 3 with c-erbB-2 expression alone (2 adenocarcinomas, 1 mucoepider-

Table 3 Associations between p53 and c-erbB-2 protein expressions

		No. of tumours c-erbB-2		Total
		(−)	(+)	
No. of tumours p 53	(−)	50	6	56
	(+)	0	7	7
	Total	50	13	63


1a
b
c
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3a
b
c
d

Table 4 Relationship of coexpression to histological type in salivary gland carcinoma

Histological type	No. of tumours (%)		
	p53:High (+) c-erbB-2(+)	p53 (−) c-erbB-2(+)	p53 (−) c-erbB-2(−)
Adenocarcinoma	4 (57)	2 (33)	5 (10)
Salivary duct carcinoma	1 (14)	0 (0)	0 (0)
Carcinoma in pleomorphic adenoma	2 (29)	0 (0)	1 (2)
Adenoid cystic carcinoma	0 (0)	0 (0)	21 (42)
Mucoepidermoid carcinoma	0 (0)	4 (67)	13 (26)
Acinic cell carcinoma	0 (0)	0 (0)	8 (16)
Basal cell adenocarcinoma	0 (0)	0 (0)	1 (2)
Polymorphous low-grade adenocarcinoma	0 (0)	0 (0)	1 (2)
Total	7 (100)	6 (100)	50 (100)

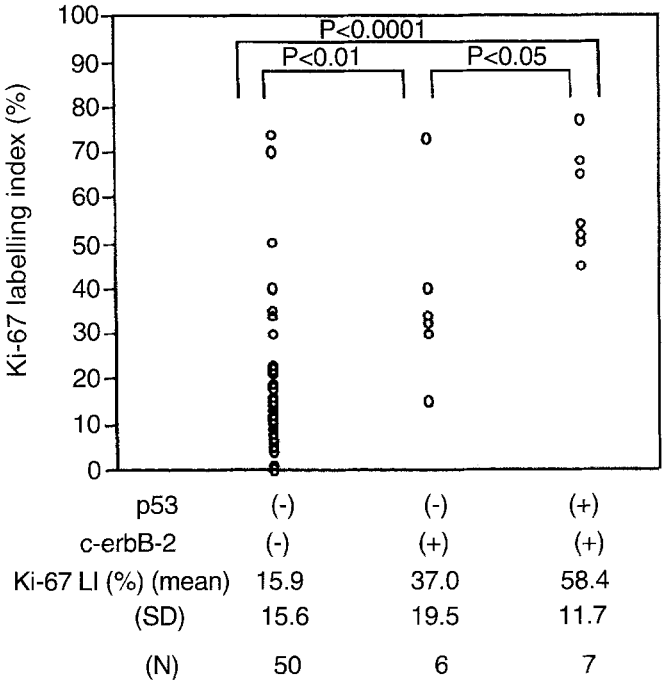

Fig. 1a–c p53 immunostaining (DO-1). **a** High expression, **b** low expression, focal pattern, **c** low expression, scattered pattern. **d** c-erbB-2 immunostaining. (**a** Adenocarcinoma, **b–d** mucoepidermoid carcinoma). ×80

Fig. 2 Coexpression associated with Ki-67 labelling index (%). Mann-Whitney U-test. Coexpression is associated with a higher Ki-67 LI (%) than c-erbB-2 expression alone or no expression

Fig. 3a–d Ki-67 immunostaining (MIB-1). **a** Case with coexpression (carcinoma in pleomorphic adenoma), **b** case with coexpression (adenocarcinoma), **c** case with c-erbB-2 expression alone (mucoepidermoid carcinoma), **d** case with no expression (adenoid cystic carcinoma). Tumours in the cases with coexpression contain a large number of Ki-67-positive cells compared with those in cases with c-erbB-2 expression alone or no expression. ×80

moid carcinoma), and 25 with no expression (4 adenocarcinomas, 1 carcinoma in pleomorphic adenoma, 1 basal cell adenocarcinoma, 10 adenoid cystic carcinomas, 7 mucoepidermoid carcinomas, and 2 acinic cell carcinomas). Mortality rates within 5 years after the primary surgery were 4 out of 4 cases (100%) with coexpression, 2 out of 3 (67%) with c-erbB-2 expression alone, and 14 out of 25 (56%) with no expression.

Table 5 Relationships[a] of coexpression to clinical grade and tumour stage in salivary gland carcinoma

	No. of tumours		
	p53:High(+) c-erbB-2(+)	p53 (−) c-erbB-2(+)	p53 (−) c-erbB-2(−)
	+++		
Clinical grade	+	NS	
Total	7	6	50
High-grade carcinoma	7	2	6
Low-grade carcinoma	0	4	44
Tumour stage	**		
Tumour size	NS	*	
Total	7	6	50
>6 cm	3	2	2
>4 to 6 cm	2	0	3
>2 to 4 cm	2	4	19
≦2 cm	0	0	26
	++		
Lymph node (pathologic)	NS	+	
Total	5	4	24
Metastasis (+)	5	3	3
(−)	0	1	21

[a] Fisher's exact probability test: +++ $P<0.0001$, ++ $P<0.001$, + $P<0.05$; Chi-square test: ** $P<0.001$, * $P<0.05$; *NS* not significant

Relationships of coexpression with histological grade, Ki-67 LI (%), tumour stage, and prognosis in the cases of adenocarcinoma

Table 6 shows the relationships of coexpression to histological grade and Ki-67 LI (%) in adenocarcinoma cases, including 1 of salivary duct carcinoma. Coexpression tended to be seen in tumours with high mitotic indices,

Table 6 Relationships of coexpression to histological grade and Ki-67 LI (%) in adenocarcinoma

	No. of tumours		
	p53:High(+) c-erbB-2(+)	p53(−) c-erbB-2(+)	p53(−) c-erbB-2(−)
Histological grade			
Mitotic index (MI)			
10 <MI	3	1	
5<MI≦ 10	1	1	2
MI≦ 5	1		3
Total	5	2	5
Necrosis			
(+)	4		1
(−)	1	2	4
Total	5	2	5
Differentiation			
Poorly	5	1	2
Moderately		1	2
Well			1
Total	5	2	5
Nuclear atypia			
Severe	5	1	3
Mild		1	2
Total	5	2	5
Ki-67 LI (%)			
>50	5	1	1
>30 to 50		1	
≦30			4
Total	5	2	5

necrotic areas, and high Ki-67 LI (%). In addition, coexpression tended to be more closely related than no expression to advanced tumour stage (large tumour size and positive lymph node metastasis).

Of the 12 cases of adenocarcinoma studied in terms of clinical outcome, 2 were not followed up, and 1 case was followed up for only 19 months. The remaining 9 cases were followed up for a minimum of 65 months. Evaluation of the prognosis was based on the death rate during the 5 years after surgery. Death supervened within 5 years after surgery in 3 out of 3 (100%) with coexpression, 1 out of 2 (50%) with c-erbB-2 expression alone, and 3 out of 4 cases (75%) with no expression.

Figure 4 illustrates a case diagnosed as adenocarcinoma; this tumour contained of distinct two histological patterns, a cribriform type of adenoid cystic carcinoma and a poorly differentiated adenocarcinoma with a solid pattern showing central necrosis resembling that seen in ductal carcinoma of the breast (Fig. 4a). The latter region showed high expression of p53 (Fig. 4c) and c-erbB-2 proteins (Fig. 4e), although the former region was negative for both proteins. In addition, the latter region contained a higher number of Ki-67-positive cells than the former (Fig. 4d). A transitional region was recognized between the two portions of the tumour (Fig. 4b).

Discussion

The development and progression of cancer are regulated by the expression of various oncogenes and tumour suppressor genes. Among prognostic factors determining the course of SGCs, genetic alterations such as those affecting *p53* and *c-erbB-2* have been the focus of considerable interest in recent years, although neither has been studied as extensively as those of other carcinomas.

The Human *p53* gene is inactivated in several malignant tumours. Inactivation is due to mutations generally localized in a highly conserved region (exons 5–9). These mutations result in the formation of a p53 protein with a longer half-life, which is immunohistochemically detectable [12]. Recent studies have demonstrated a variety of p53 staining patterns and shown that strong staining in the majority of cells is frequently associated with a *p53* gene mutation [2, 17]. The role and regulation of p53 expression may vary among malignant neoplasms. Though previous studies have shown significant associations between high levels of p53 protein expression and advanced disease stage and metastatic tumour spread, p53 overexpression has also been shown to be associated with the transition from dysplastic lesions to carcinoma [12]. In colon carcinogenesis, *p53* gene alterations seem to play an important part in malignant conversion from adenoma to adenocarcinoma [10]. Furthermore, several reports have suggested that p53 expression is associated with high cell-proliferative activity in tumours arising from the liver, breast, and urinary bladder [19]. In a study of SGCs, Ishii et al. [13] demonstrated that p53 accumulation was demonstrated immunohistochemically in high-grade carcinomas, all of which showed aneuploidy in a nuclear DNA histogram pattern. Their results suggest that an abnormality of p53 is associated with cell proliferative activity. Until recently only a few studies have indicated that a p53 mutation may be involved in the malignant transformation of pleomorphic adenoma [1, 7].

The *c-erbB-2* oncogene encodes a membrane receptor with homology to the epidermal growth factor receptor (EGFR). Although most studies have analysed either gene amplification or protein expression, a close correlation between the results of the two detection methods has been reported [29]. Amplification of the *c-erbB-2* oncogene and/or overexpression of its products have been recognized as being associated with a poor prognosis in ovarian, endometrial, and both node-positive and node-negative breast carcinomas [20]. In SGCs, earlier studies of c-erbB-2 expression are few and results have been contradictory. Press et al. [20] and Sugano et al. [26] demonstrated that overexpression of c-erbB-2 protein was associated with a poor prognosis. Another study, however, suggested that this oncoprotein was infrequently expressed [15, 23] and found no clear evidence that it reflected the biological behaviour of the tumour [15].

The present study revealed that high p53 protein expression is present in certain histological types categorized as high-grade carcinomas. All cases with high p53

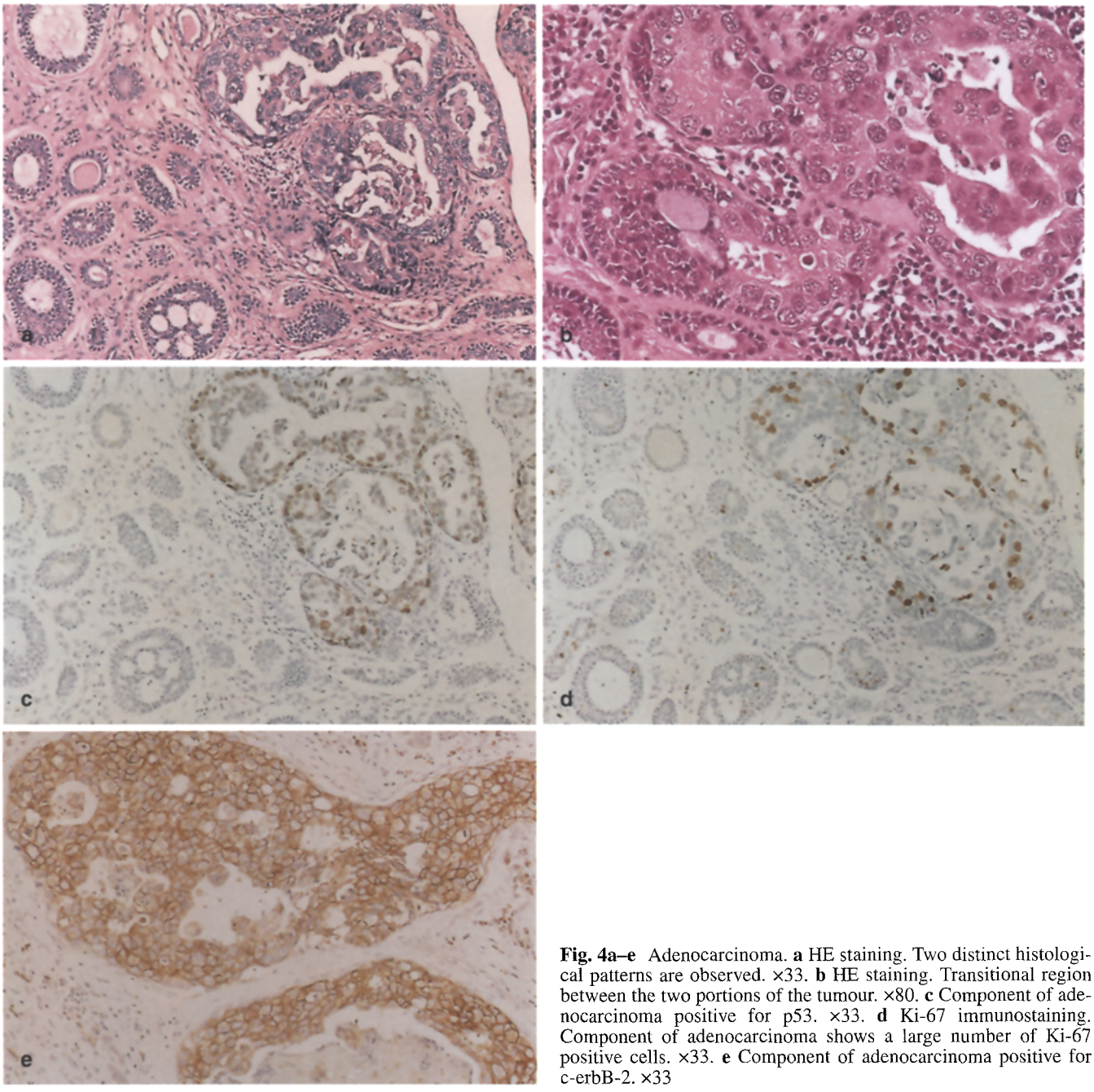

Fig. 4a–e Adenocarcinoma. **a** HE staining. Two distinct histological patterns are observed. ×33. **b** HE staining. Transitional region between the two portions of the tumour. ×80. **c** Component of adenocarcinoma positive for p53. ×33. **d** Ki-67 immunostaining. Component of adenocarcinoma shows a large number of Ki-67 positive cells. ×33. **e** Component of adenocarcinoma positive for c-erbB-2. ×33

protein expression were also positive for c-erbB-2 protein. The results of this study reveal that p53 protein is significantly associated with c-erbB-2 positivity ($P<0.001$). Some earlier studies have reported that expression of p53 and c-erbB-2 proteins occurs independently during the development of breast tumours [11]. In contrast, other reports have demonstrated an unfavourable prognosis for breast carcinomas coexpressing the two proteins [14] or a close correlation between expressions of the two oncogene products [29]. However, no previous studies have demonstrated the close relationship between these two proteins in SGCs.

This study showed that c-erbB-2 expression alone was significantly more closely associated with advanced tumour stage and high Ki-67 LI (%) than was expression of neither protein (no expression). Coexpression was, however, significantly more closely related to high-grade carcinoma and high Ki-67 LI (%) than c-erbB-2 expression alone. The fact that coexpression was found in high-grade carcinomas and advanced tumour stages showing high cell-proliferative activity suggests that coexpression plays an important part in the late stage of tumour progression. However, it should be emphasized that these two oncogene and tumour suppressor gene proteins were

not invariably associated with progression, as a number of cases with advanced-stage tumours and high Ki-67 LI (%) showed no expression. In other words, it is possible that unknown mechanisms other than these two gene alterations are related to progression. Furthermore, the relationship between coexpression and prognosis with SGCs was investigated. Both the prognostic study in all available cases of SGCs and that in the cases of adenocarcinoma only showed that coexpression tended to correlate with a poorer prognosis than did no expression, although statistical analysis was not available owing to the small number of cases. The accumulation of cases with strict follow-up is awaited.

There remains the problem of adenocarcinomas belonging to the unsubclassified group, although these consist of tumours with extremely diverse morphological and clinical features. According to Spiro et al. [25], the biological behaviour of an adenocarcinoma is influenced by its clinical stage, the site of involvement, and its histological grade. Histological grades, in terms of mitotic index and the presence of necrosis, reflect cell proliferative activity as well as the Ki-67 LI (%). The present results indicate that coexpression is more closely associated with high cell-proliferative activity than is no expression. Among the series of SGCs in this study, there was a rare and interesting case of adenocarcinoma with two distinct histological patterns. This tumour contained a transitional area between these two histological types, which suggests tumour progression. These two regions showed different staining for p53, c-erbB-2 proteins, and Ki-67, suggesting that coexpression was associated with transformation from adenoid cystic carcinoma to poorly differentiated adenocarcinoma, from low- to high-grade carcinoma, or low to high cell-proliferative activity. The findings in this case indicated that coexpression was involved in the late stage of multistage carcinogenesis (multistage carcinogenesis, although multistage carcinogenesis had not previously been demonstrated in the SGCs.

The present study is the first to demonstrate a close correlation between p53 protein overexpression and c-erbB-2 protein overexpression in the SGCs. Furthermore, coexpression was shown to be associated with certain histological types, advanced tumour stage, and high cell-proliferative activity. In adenocarcinoma, coexpression was associated with histological grades showing high mitotic indices and necrotic areas reflecting high cell-proliferative activity. These results suggest that accumulation of oncogene and tumour suppressor gene alterations plays an important part in SGC tumour progression.

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