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Analysis of c-*erb*B-2 Amplification in Salivary Gland Tumours by Differential Polymerase Chain Reaction

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DNA samples extracted from 22 normal salivary glands, 38 salivary pleomorphic adenomas and 20 other salivary gland neoplasms were screened for amplification of the c-*erb*B-2 oncogene by a differential polymerase chain reaction (PCR). The samples were PCR amplified with primers specific for the c-*erb*B-2 oncogene and for a reference gene (interferon-γ). A breast carcinoma cell line SKBR-3 known to contain c-*erb*B-2 amplification was used as positive control. Following gel electrophoresis, the intensity of the amplified DNA bands was determined by laser densitometry and the level of amplification of the c-*erb*B-2 oncogene was assessed from the intensity of the c-*erb*B-2 specific band relative to that of the interferon-γ band. Of all the tumours detected, only the two poorly differentiated adenocarcinomas, two of the pleomorphic adenomas and one of the Warthin's tumours showed gene amplification at levels comparable to the breast carcinoma cell line. None of the normal salivary gland tissues was found to have amplification. Within the group of pleomorphic adenomas the average level of amplification was not significantly different from that observed in the normal salivary gland, or in total genomic DNA from unrelated tissue ($P \leqslant 0.001$, determined by a general linear model of statistical analysis). These results indicate that amplification of the c-*erb*B-2 oncogene is infrequent in salivary neoplasia. Thus, gene amplification alone cannot account for the high prevalence of c-*erb*B-2 overexpression demonstrated previously in salivary gland tumours. When present, c-*erb*B-2 amplification may be associated with a more aggressive behaviour.
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INTRODUCTION

SALIVARY GLAND neoplasia comprises probably the widest spectrum of morphologically complex and clinically unpredictable tumours afflicting humans [1]. Although in many other types of human neoplasias associations between tumour formation and progression, and oncogene aberration have been demonstrated, very few studies to date have addressed this problem in salivary gland tumours. This could be accounted for by the fact that, taken individually, the most malignant salivary gland tumours are relatively rare, as compared with the major cancers of the Western world (e.g. lung, breast and squamous cell carcinoma of the head and neck). However, the wide spectrum of tumours of the salivary gland can be regarded as the result of the deregulation of differentiation of only a few cell types [1]. In light of this view it is important to study the oncogenic mechanisms of all salivary gland neoplasias not only individually but also as a group.

The proto-oncogene c-*erb*B-2 also designated *neu* and *HER*-2, was originally identified as activated by a single point mutation in carcinogen induced rat neuroblastomas [2, 3]. This gene encodes a glycoprotein of the tyrosine kinase family [4] with a 50% amino acid homology with the epidermal growth factor receptor [5, 6]. Amplification of this oncogene has been reported in up to 46% of human breast carcinomas, primarily those of ductal origin [7–12]. Slamon *et al.* [13] originally reported a positive correlation between c-*erb*B-2 gene expression and the number of positive lymph nodes in breast cancer. Since then, several reports have been published to support the suggestion that amplification of this oncogene may be a useful indicator of poor prognosis in mammary cancer [7–24].

The mammary and salivary gland share several histogenetic and morphological similarities. Secretory units and excretory ductal trees can be identified in both. The acinar units of each tissue are composed of both secretory cells and basally located myoepithelial cells. Based on the assumption that similar tissues are targeted by carcinogens at similar molecular levels, it would be conceivable that amplification of c-*erb*B-2 is of significance in the development of salivary gland neoplasia as well. However, relatively few reports have addressed the role of oncogenes in general and of c-*erb*B-2, in particular in salivary gland neoplasia [5, 25–29]. Among 15 pleomorphic adenomas studied Kahn *et al.* [27] found one tumour to show amplification whereas Semba *et al.* [5] noted a 30-fold amplification in a single sample of adenocarcinoma of the

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salivary gland. It was speculated that, when present in salivary gland tumours, c-*erb*B-2 amplification is associated with a more aggressive behaviour. The objective of the present study was to determine the frequency of c-*erb*B-3 amplification in a larger sample and variety of tumours of the salivary gland.

MATERIALS AND METHODS

Tissues specimens and cell culture

Fresh normal and neoplastic salivary gland tissues were collected at the time of surgical excision. The normal (control) tissues were obtained from sialoadenectomy specimens adjacent to tumour tissue or areas of mild sialoadenitis with or without sialolithiasis. Quick frozen sections from the tissues were examined under the microscope to confirm the diagnoses and to help dissect portions of the specimens corresponding to cellular areas of the tumours or the salivary gland. Such portions of tissues were frozen rapidly in liquid nitrogen and stored in desiccated form at −70°C until DNA extraction.

The human breast carcinoma cell line SKBR-3 [30] was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The cells were cultured in Leibowitz's alpha-50 medium (Gibco/BRL, Burlington, Ontario, Canada) supplemented with fetal calf serum (15%; Gibco/BRL), according to the specifications of the suppliers of the cells.

DNA extraction and differential polymerase chain reaction (PCR)

DNA was extracted from the cultured cells and from the tissues by standard procedures [31]. Human placental DNA for controls was obtained from Oncor (Gaithersburg, Maryland, U.S.A.).

The differential PCR method of Frye *et al.* [30] was employed with the *HER-2/neu* Onco-Lyzer® kit and oligomer amplification primers (Amplimer set for Gene Amp® PCR) supplied by Clontech (Palo Alto, California, U.S.A.). The oligonucleotide primers corresponded with the first 20 sense and last 20 antisense bases of sequence regions 2122–2219 of the c-*erb*B-2 gene and 4582–4731 of the interferon-γ gene (as cited in Ref. 30). The PCR mixture contained the target DNA sample (1.25 μg/ml), 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 0.20 mmol/l of each dNTP, 0.01% porcine skin gelatin (Sigma, St. Louis, Missouri, U.S.A.), 0.5 μmol/l of each amplimer and 2.5 U of *Taq* polymerase in 50 μl total volume with a 50 μl mineral oil overlay. The automated thermal cycling device was from Cetus/Perkin-Elmer. Before preparing the PCR mixture, the DNA samples were heated at 94°C for 5 min to inactivate any contaminating proteases. The PCR cycles included 1 min at each temperature (94, 55, and 72°C). After the PCR, 10 μl of each sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide. The UV-illuminated gels were photographed using Polaroid 665 film. Densitometry of the PCR amplified DNA bands was performed on the negative, with the aid of an LKB-Ultroscan XL laser densitometer.

RESULTS

Tissues from 72 patients (22 normal salivary glands, 42 benign and 8 malignant salivary gland tumours; see Table 1) were available for analysis by differential PCR. The level of gene amplification in each sample was assessed by the intensity of the c-*erb*B-2 specific (target gene) band relative to the interferon-γ (reference gene) band, i.e. the c-*erb*B-2/interferon-γ ratio. Each sample was analysed at least twice. Duplicate and triplicate analyses yielded similar ratios (less than 0.05 difference between ratios obtained for the same sample). Examples of PCR amplified DNA bands and corresponding c-*erb*B-2/interferon-γ ratios obtained from normal salivary gland, from control genomic DNA from placenta, from the control SKBR-3 carcinoma cell line and from salivary gland tumour samples are illustrated in Fig. 1.

The PCR amplification ratios obtained in the negative control (genomic DNA from placenta) were found to be less than 1.1, whereas in the positive control cell line they ranged from 1.5 to 2.9. Because this cell line is known to carry c-*erb*B-2 gene amplification consistently [30], a PCR ratio of more than 1.5 in our samples was interpreted as indicating amplification of the c-*erb*B-2 oncogene.

Of all the tumours analysed, only the two poorly differentiated adenocarcinomas, two of the pleomorphic adenomas and one Warthin's tumour showed c-*erb*B-2 amplification. In a representative set of analyses, the PCR amplification ratios in

Table 1. *The differential PCR amplification of the c-*erb*B-2 oncogene in normal salivary gland and in salivary gland*

Diagnosis	No. of samples	Mean c-*erb*B-2/IFN-γ ratio
Normal salivary gland	22	0.8±0.1
Pleomorphic adenoma	38	0.9±0.1
Acinic cell carcinoma	4	2.4±0.8
Mucoepidermoid tumour	2	0.9±0.0
Warthin's tumour	4	0.8±0.5
Adenocarcinoma (poorly differentiated)	2	2.0±0.3
SKBR-3	5	2.4±0.8
Placenta	8	0.7±0.3

DNA was extracted from the tissues and amplified by differential PCR with primers specific for the c-*erb*B-2 oncogene and the interferon-γ gene as described in Materials and Methods. Aliquots of the samples were electrophoresed on agarose gels. The gels were stained by ethidium bromide. Densitometry of the photographic negatives of UV-induced fluorescence was performed using an LKB-Ultroscan XL laser densitometer. The data points represent the ratio between the densitometric intensity of the c-*erb*B-2 DNA band and the interferon-γ DNA band for each sample.

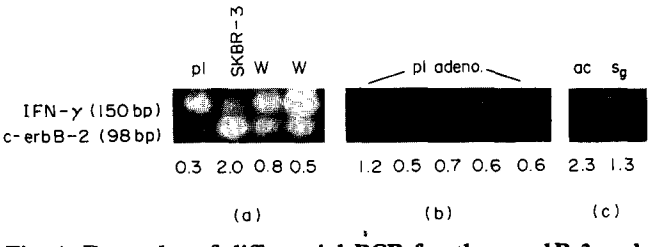

Fig. 1. Examples of differential PCP for the c-*erb*B-2 and IFN-γ (interferon-γ) genes from various tumour samples (pleomorphic adenoma, pl.adeno; Warthin's tumour, W; adenocarcinoma, ac.), SKBR-3 breast carcinoma cells, normal salivary gland tissue (sg.), and genomic DNA from placenta (pl.). The numbers below the bands represent the corresponding c-*erb*B-2/IFN-γ ratios.

these tumours were as follows: 1.7 and 2.2 in the adenocarcinomas, 1.7 and 1.6 in the pleomorphic adenomas and 1.9 in the Warthin's tumour. None of the normal salivary gland tissues were found to have amplification of this oncogene. The average PCR amplification ratios calculated for each sample group, in a representative set of analyses, are presented in Table 1. Within the group of pleomorphic adenomas, the average level of amplification was not significantly different from that found in the normal salivary gland or in genomic DNA from unrelated tissue ($P \leqslant 0.001$, determined by a general linear model of statistical analysis).

DISCUSSION

We have employed the simple and rapid method of differential PCR, as described originally by Frye *et al.* [30], to detect c-*erb*B-2 gene amplification in salivary gland neoplasia. This method is semiquantitative and the actual c-*erb*B-2/interferon-γ ratio may be underestimated due to possible overlaps, on the electrophoresis gel, between the reference gene band and *erb* specific single-strand byproducts of the PCR [30]. However, the differential PCR is sensitive enough to detect gene amplification at a sensitivity level as high as 2-fold [30] and allows for the estimation of the relative degree of amplification. Thus, our data indicate that some salivary gland tumours carry amplification of the c-*erb*B-2 oncogene. Because the target/reference gene ratio in these tumours was found to be of the same magnitude or lower than in the SKBR-3 cell line known to carry 2–8-fold amplifications of c-*erb*B-2 [30, 32], we estimate that in the salivary gland tumours the level of amplification of this oncogene lies below 8-fold. The most malignant salivary gland tumour type examined, i.e. the poorly differentiated adenocarcinoma showed the highest level of amplification. Interestingly, in a previous study, Samba *et al.* [5] examined c-*erb*B-2 in a single salivary adenocarcinoma and found it to be amplified, albeit at a much higher level (30-fold). One might speculate that a higher level of amplification of this oncogene in salivary gland tumours is indicative of malignancy or a more aggressive behaviour. However, in our study the oncogene was found to be amplified in some benign tumours as well. Further extensive, clinically prospective studies are needed if one is to establish firm correlations between tumour behaviour and oncogene amplification in salivary gland neoplasia.

We have shown previously that while the c-*fos* oncogene is underexpressed [28], c-*erb*B-2 is consistently overexpressed in a variety of salivary gland neoplasias [33]. Because gene overexpression is often but not always attributable to gene amplification, it was of particular interest to determine whether c-*erb*B-2 overexpression in salivary gland neoplasia is the result of gene amplification. Our data indicate that c-*erb*B-2 amplification is rare in salivary gland tumours. If so, gene amplification alone cannot acount for the ubiquitous overexpression of this oncogene in salivary gland neoplasia. Therefore, it is reasonable to conclude that other molecular events are responsible for c-*erb*B-2 overexpression. The nature of such molecular events remains to be investigated. Studies on the nature of c-*erb*B-2 oncogene aberrations in salivary gland tumours are ultimately relevant in selecting the appropriate target for new treatment strategies based on oncogenes [34–36].

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