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p53–HSP70 Complexes in Oral Dysplasia and Cancer: Potential Prognostic Implications

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We have previously shown overexpression of p53 and 70 kDa heat shock protein (HSP70) in potentially malignant, as well as malignant, oral lesions in an Indian population, suggesting that alterations of p53 and HSP70 expression may occur in the early stages of oral tumorigenesis. Herein we report immunological evidence for the specific association between p53 and HSP70 in potentially malignant and malignant oral lesions. This association was indicated by coimmunoprecipitation of p53 and HSP72/73 proteins observed with either an anti-p53 monoclonal antibody or an anti-HSP72/73 antibody. Furthermore, reciprocal blotting analysis showed that HSP72/73 proteins did not share an epitope with p53, confirming that the coimmunoprecipitation of p53 and HSP72/73 is a physical association of the proteins in potentially malignant lesions (dysplasia) and oral squamous cell carcinomas (SCCs). p53–HSP70 complex formation was observed in 19/52 cases of oral SCCs and 10/53 cases of potentially malignant lesions (leucoplakia). Normal oral mucosa did not show the presence of p53–HSP70 complexes (0/20 cases). p53–HSP70 complex formation may be one of the mechanisms of stabilisation of p53 protein resulting in its increased levels in potentially malignant and malignant oral lesions and may be implicated in oral carcinogenesis.

Keywords: p53, HSP70, p53–HSP70 complex, oral cancer, dysplasia

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INTRODUCTION

Oral cancer in the Indian population is a unique model system for the study of tumorigenesis; the development of malignancy is a multistep process such that a well-defined potentially malignant stage termed dysplasia often precedes frank malignancy [1]. Habitual betel quid and tobacco chewers often develop malignant tumours at the site where the tobacco folded betel quid is kept for prolonged periods (5–20 years). The genetic and molecular alterations associated with the development of an oral potentially malignant state or its progression to frank malignancy can thus be studied. We have previously shown p53 protein overexpression, not only in betel- and tobacco-related human oral squamous cell carcinomas (SCCs) but also in oral dysplastic lesions (leucoplakia), suggesting that alterations of p53 expression occur in early stages of oral tumorigenesis [2]. Several other groups have also reported p53 alterations at or before the stage of severe oral and laryngeal dysplasia [3–9]. Among the human cancers, the maximum frequency of p53 mutations has been observed in oral mucosal squamous cells (81%) [10]. Discordant p53 mutations among non-neoplastic respiratory mucosa, primary cancers and second upper aerodigestive primary lesions suggest that p53 is an important early target for mutations occurring at multiple mucosal sites [7, 11]. Despite the clinical importance of p53 in oral SCCs it is still unclear how the biochemical function(s) of mutant p53 is linked to transforming activity in oral tumorigenesis. Studies investigating the molecular mechanisms underlying the biological activity of p53 indicate that the p53 protein forms complexes with various cellular proteins and viral oncoproteins [12].

To understand the role of p53 in oral tumorigenesis we made an attempt to identify the proteins with which it interacts during the different stages of tumour progression, i.e. normal, potentially malignant and malignant cells. Unlike wild type p53, some mutant p53 proteins have the ability to bind HSC70 and to transactivate a number of promoters [13–17]. Transcriptional activation by mutated p53 genes has been shown to be closely correlated with transforming and HSC70 binding activity in oral SCC cell lines [17]. Our earlier results showed overexpression of p53 protein in oral dysplasias and SCCs [2] and differential expression of HSP70 during oral tumorigenesis [18]. These studies suggest that alterations in the expression of p53 and HSP70 occur during the oncogenic process possibly at the stage of premalignancy. Hence, the aim of the present study was to determine: (i) if there is an association between p53 and HSP70 in oral potentially malignant and malignant lesions; and (ii) whether there is a

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correlation between clinical status of these patients and the presence of p53–HSP70 complexes during oral tumorigenesis.

MATERIALS AND METHODS

Clinicopathological characteristics of patients

52 untreated patients (39 males and 13 females, aged between 22 and 85 years), diagnosed as having squamous cell carcinomas of the oral cavity and with TNM stages (UICC 1988), T_1 to T_4, N_0 to N_3 and M_0 to M_1, histological grading, moderate and poorly differentiated, tobacco and/or betel consumers were investigated for the expression of p53, HSP70 and p53–HSP70 complexes. The various sites included buccal mucosa (17 cases), tongue (13 cases), mouth floor (11 cases), alveolus (7 cases) and lip (4 cases). The diagnosis was based on clinical examination and histopathological analysis of tissue specimens. 53 oral dysplasia (leucoplakia) patients (38 males and 15 females, aged between 20 and 65 years) showing mild (12), moderate (21) or severe (20) dysplastic changes were analysed. The sites included buccal mucosa (29 cases), tongue (10 cases), mouth floor (9 cases) and lip (5 cases). These potentially malignant oral tissue specimens were collected from different patients who did not have any frank malignancy. 30 normal subjects (21 males and 9 females, aged between 25 and 60 years) were included in this study. The sites analysed were buccal mucosa (23 cases), tongue (3 cases), mouth floor (2 cases) and lip (2 cases).

Tissue specimens

Surgical specimens from squamous cell carcinomas, potentially malignant lesions and normal tissues of the oral cavity were obtained from the Department of Surgery, All India Institute of Medical Sciences, India. Specimens were collected in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) supplemented with 10% fetal calf serum (FCS) for immunoprecipitation and immunoblotting assays. A piece of tissue was placed in formalin for histopathological examination. The clinical and pathological data were recorded as described previously [2].

Antibodies

Monoclonal antibodies PAb1801 and PAb240 (Oncogene Science, Uniondale, New York, U.S.A.) are mouse anti-p53 monoclonal antibodies, human specific IgG_1s. PAb1801 recognises a denaturation resistant epitope between amino acids 32 and 79 of p53 protein. PAb240 recognises only mutant p53 under non-denaturing conditions. HSP72/73 (Ab-1, Oncogene Science) is a mouse monoclonal antibody that reacts with HSP70 in mammalian cells (clone w 27).

Immunoprecipitation

Tissue specimens suspended in methionine-deficient DMEM supplemented with 10% FCS were minced and filtered through fine nylon mesh to obtain a single cell suspension. Single cell suspensions obtained from oral SCCs, oral dysplasias and normal oral tissues were incubated in methionine-deficient DMEM supplemented with 10% FCS and antibiotics (streptomycin 50 μg/ml and penicillin 50 IU/ml) for 2 h at 37°C. Cellular proteins were labelled with ^{35}[S] methionine for 4 h and processed as described previously [2]. Equivalent amounts of TCA-precipitated radiolabelled proteins (1×10^6 cpm) from each sample were immunoprecipitated with the anti-p53 monoclonal antibody PAb1801, the anti-HSP70 monoclonal antibody or non-immune mouse serum for 2 h at 4°C. For immunoprecipitation analysis using the mutant p53-specific monoclonal antibody PAb240, equivalent amounts of radiolabelled cellular proteins from normal, dysplastic and cancerous oral tissues were used under non-denaturing conditions. Thereafter, 20 μl 10% pansorbin suspension was added and the mixture was allowed to react for 1 h. Immune complexes were collected and washed three times with SNNTE [5% sucrose, 1% (w/v) Nonidet P-40, 0.5 M NaCl, 50 mM Tris (pH 7.4) and 5 mM EDTA] and once with RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 1% (w/v) sodium deoxycholate] at 4°C. The pellets were collected and resolved by one-dimensional and two-dimensional electrophoresis. For one-dimensional SDS–PAGE, immune complexes were suspended in SDS-lysis buffer [21] and resolved on 10% SDS–polyacrylamide gels as described previously [2]. Gels were dried and autoradiographed.

For two-dimensional gel electrophoresis, immune complexes were suspended in urea lysis buffer and subjected to isoelectric focussing followed by 10% SDS–PAGE [20]. Isoelectric focussing was carried out using a mixture of ampholytes in the pH range 3–10 and pH 5–8 in the ratio of 1:4.

Detection of coimmunoprecipitated proteins by reciprocal immunoblotting

Coimmunoprecipitation of p53 and HSP70 in samples from oral SCCs, potentially malignant lesions and normal oral tissues was performed by first reacting unlabelled total cellular protein extracts (700 μg) prepared as described previously [2] with either PAb1801, PAb240 or anti-HSP70 antibodies. Six immunoprecipitation reactions, two with each antibody, were performed on proteins from each tissue specimen as described above. Thereafter, the first mouse antibodies were immobilised on protein A–Sepharose. Supernatants were electrophoreased on 10% SDS–polyacrylamide gels and proteins were transferred to nitrocellulose membranes. The membranes were then treated with a blocking solution (5% non-fat milk in Tris buffered saline containing 0.1% Tween 20) overnight at 4°C. p53 immunoprecipitate blots were probed for 2 h at 37°C with the anti-HSP70 monoclonal antibody. HSP70 immunoprecipitate blots were probed with the anti-p53 monoclonal antibody. Membranes were washed three times with TBS/Tween and incubated with HRP-conjugated rabbit anti-mouse total immunoglobulins for 1 h at 37°C, washed and proteins were detected by the enhanced chemiluminescence method (ECL) (Amersham).

RESULTS

Coimmunoprecipitation of p53 and HSP70 proteins

Immunoprecipitation of p53 protein from oral SCCs and potentially malignant tissues using anti-p53 monoclonal antibodies, PAb1801 as well as PAb240, resulted in the coimmunoprecipitation of p53 and 70 kDa proteins. The results shown in Fig. 1 were obtained with PAb1801. To ascertain the identity of the 70 kDa protein ^{35}[S]methionine labelled cell extracts were subjected to immunoprecipitation

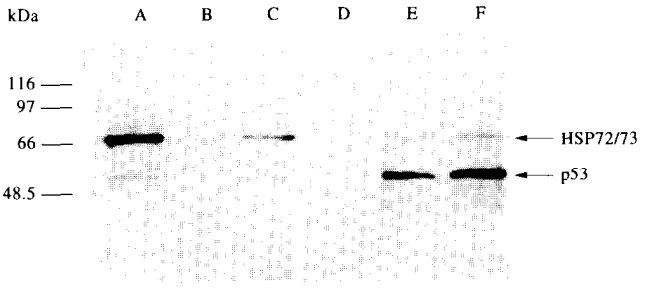

Fig. 1. Coimmunoprecipitation of p53 and HSP70. p53 and HSP70 immunoprecipitates were analysed on 10% SDS polyacrylamide gels. Lanes A, B and C represent HSP70 immunoprecipitates of oral squamous cell carcinomas, normal and potentially malignant oral tissues, respectively. Lanes D, E, and F represent p53 immunoprecipitates (using PAb1801) of normal, potentially malignant and malignant oral tissues, respectively.

with the anti-p53 monoclonal antibody PAb1801 or the anti-HSP70 monoclonal antibody (clone w 27). Immunoprecipitation of p53 with PAb1801 resulted in coimmunoprecipitation of a protein of a molecular weight of approximately 70 kDa in squamous cell carcinoma (Lane F) and oral dysplasia (Lane E). A similar observation was not found in normal oral tissues (Lane D). Anti-HSP70 monoclonal antibody precipitated, along with HSP70, a 53 kDa protein from oral SCCs (Lane A) and premalignant oral lesions (Lane C) but not from normal oral tissues (Lane B). Hence, coimmunoprecipitation of p53 and HSP70 proteins was observed in squamous cell carcinomas and oral dysplasias but not in normal oral tissues. Coimmunoprecipitation of p53 and HSP70 was also observed with PAb240 under non-denaturing conditions, indicating that mutant p53 was associated with HSP70. The complexes are resistant to a high salt concentration and the strong detergents used in the washing buffer, indicating that the p53–HSP70 complex is not an artefact of extraction. Coimmunoprecipitation analyses of p53–HSP70 complexes in normal, potentially malignant and malignant oral tissues are summarised in Table 1.

To ascertain the association of p53 and HSP70 proteins in oral squamous cell carcinomas and dysplasias, p53 immunoprecipitates; as well as HSP70 immunoprecipitates, from these lesions were analysed by two-dimensional gel electrophoresis. p53 immunoprecipitates showed the presence of a 70 kDa protein and HSP70 immunoprecipitates showed the presence of a 53 kDa protein in oral SCCs (Fig. 2a and b, respectively).

Table 1. *Analysis of p53-HSP70 complexes in normal, premalignant and malignant oral tissues*

	No. of cases	Positive	Negative
Oral SCCs			
p53	52	38	14
p53–HSP70	38	19	19
Premalignant lesions			
p53	53	30	23
p53–HSP70	30	10	20
Normal			
p53	30	3 (+/−)	27
p53–HSP70	20	—	20

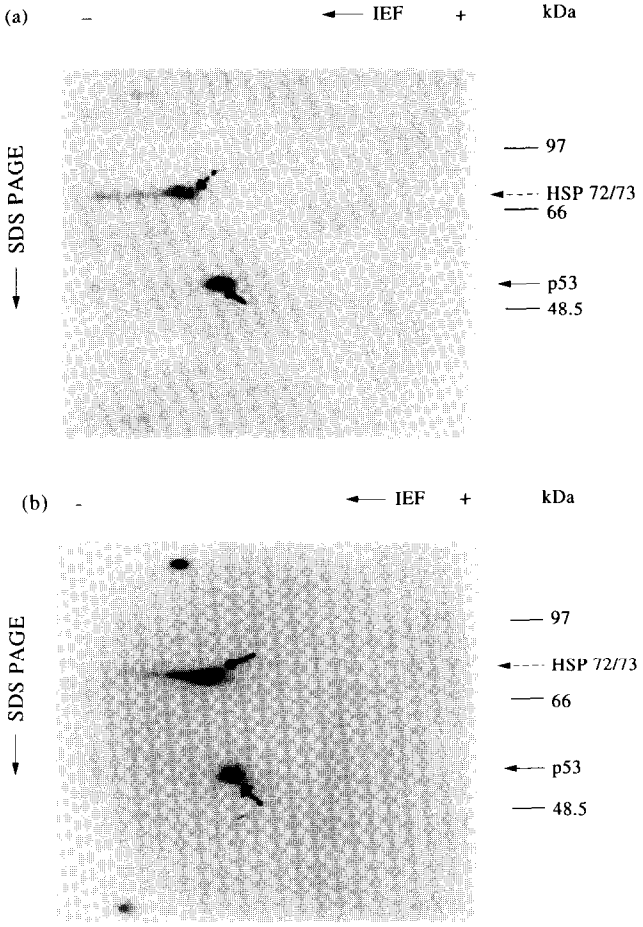

Fig. 2. Two-dimensional gel analysis of coimmunoprecipitates of p53 and HSP70 in oral squamous cell carcinomas. Immune complexes were analysed by isoelectric focussing (IEF) in the first dimension in the presence of 2% ampholytes (1.6% of pH 5.0–8.0, 0.4% pH 3.0–10.0) followed by 10% SDS–PAGE in the second dimension: (a) p53 immunoprecipitates (using PAb240) and (b) HSP70 immunoprecipitates.

Detection of p53–HSP70 complexes by reciprocal immunoblotting

Coimmunoprecipitation of p53 and HSP70 proteins can be due to either shared epitopes or the physical association of the proteins. Reciprocal immunoblot results showed that the anti-HSP70 monoclonal antibody and PAb1801 did not cross react with p53 and HSP72/73 protein, respectively. The anti-HSP70 monoclonal antibody coimmunoprecipitated a 53 kDa protein that was recognised by PAb1801 in oral dysplasias and oral SCCs (Fig. 3, lanes A and B, respectively) on nitrocellulose membranes; there was no revelation of any detectable HSP72/73 proteins on these membranes. The p53 protein band was not detected in normal tissue (Fig. 3, lane C). Reciprocally, PAb1801 coimmunoprecipitated a 70 kDa protein in potentially malignant oral lesions and oral SCCs which was identified as HSP70 when the nitrocellulose membrane was probed with the anti-HSP70 monoclonal antibody (Fig. 4, lanes B and C, respectively). Although p53 was present in the extract and on the blot it was not detectable when the anti-HSP70 monoclonal antibody was used as a Western blot probe. Normal oral tissue did not show the HSP70 band (Fig. 4, lane A). These results confirm that p53 and HSP72/73 proteins contain distinct epitopes recognised

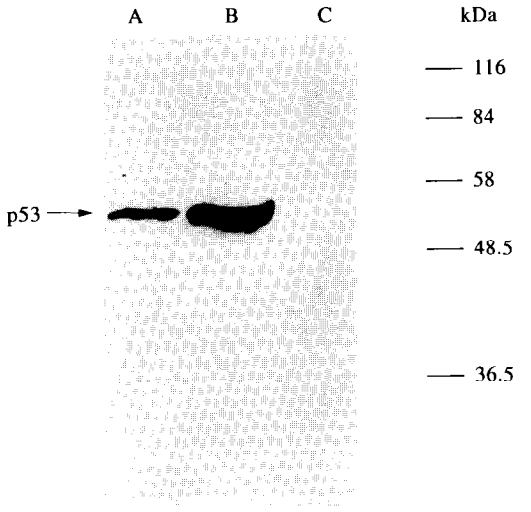

Fig. 3. Detection of p53 protein by reciprocal immunoblotting. Equal proteins (700 μg) from tissue specimens were incubated with the anti-HSP70 monoclonal antibody. Immune complexes were resolved on 10% SDS–PAGE, transferred to a nitrocellulose membrane, probed with PAb1801 and detected using the ECL method. Lane A, potentially malignant lesions; B, oral squamous cell carcinoma; C, normal oral tissue.

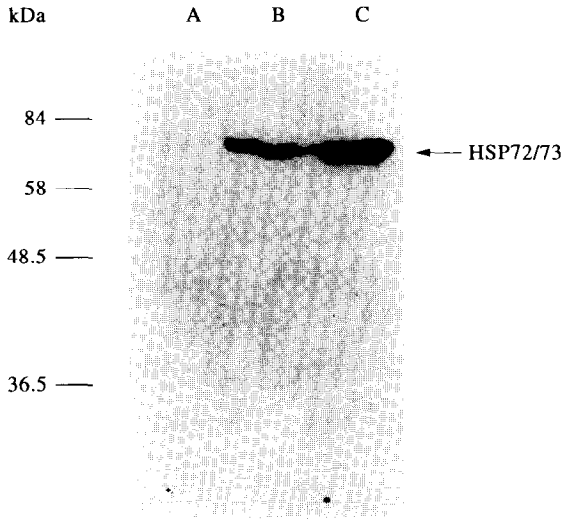

Fig. 4. Reciprocal immunoblotting for detection of HSP70 in p53 immunoprecipitates. Equal amounts of protein (700 μg) from tissue specimens were incubated with the anti-p53 monoclonal antibody PAb1801. Immune complexes were resolved on 10% SDS–PAGE and transferred to a nitrocellulose membrane and probed with the anti-HSP70 monoclonal antibody and detected using the ECL method. Lane A, normal oral tissue; B, potentially malignant lesion; C, oral squamous cell carcinoma.

by anti-p53 and anti-HSP70 monoclonal antibodies. Therefore, the coimmunoprecipitation data shown here suggests a physical association between p53 and HSP72/73 in oral squamous cell carcinomas and in potentially malignant oral lesions but not in normal oral tissues.

To determine if the p53–HSP70 complex formation in potentially malignant oral lesions is implicated in carcinogenesis, i.e. progression of preneoplastic lesions to malignancy, we conducted regular follow-up studies of these patients. Of the 53 oral dysplasia (leucoplakia) cases examined, 30 cases showed overexpression of p53 protein. Among these cases, p53–HSP70 complexes were detected in 10 patients. Five of these 10 potentially malignant lesions progressed to malignancy within a period of less than 2 years. In contrast, 20 oral dysplasia (leucoplakia) cases which did not show the presence of p53–HSP70 complexes were also followed up and only one of these cases progressed to malignancy during this period. This follow-up study is still being continued. The prognostic significance of p53–HSP70 complexes in oral cancer patients was determined. 38 of the 52 oral SCC cases examined showed detectable levels of p53 protein. The p53 positive cases were analysed for p53–HSP70 complexes. 19 of the 38 cases showed the presence of p53–HSP70 complexes. Follow-up studies of these cases revealed that 14 of the 19 cases showed poor prognosis with respect to histological grading, recurrence (within 8 months to 2 years) and/or metastasis.

DISCUSSION

We report here immunological evidence for the specific association between p53 and HSP72/73 heat shock proteins in oral squamous cell carcinomas, as well as potentially malignant oral lesions. This is the first demonstration of p53–HSP70 complex formation, not only in oral tumours but also in potentially malignant lesions, suggesting that association of mutant p53 with HSP70 is an early event in oral tumorigenesis. Similar association of p53 with HSP70 has been observed in breast [19] and ovarian carcinomas, as well as in osteosarcoma [22] and lung carcinoma cell lines [23]. In breast cancer patients a close correlation has been observed between the presence of circulating antibodies and bad prognosis. Antibodies to p53 in sera have been detected only in those breast cancer patients in which p53–HSP70 complex formation was observed. This in turn implies that the presence of the p53–HSP70 complex may be correlated with the prognosis. It has also been suggested that HSPs may be implicated in the antigenic presentation of p53 and immune survelliance [19].

The most intriguing feature of our study is the detection of p53–HSP70 complexes not only in oral SCCs but also in potentially malignant oral lesions. A good correlation exists between high levels of p53, HSP70 and the presence of p53–HSP70 complexes in oral dysplastic and cancerous lesions. Similar co-ordinate regulation of p53 and HSP70 suggests that these proteins could be involved in similar processes related to control of cell growth and/or division. Coimmunoprecipitation of p53 and HSP70 proteins by the mutant specific p53 monoclonal antibody PAb240 suggests that structural alterations of p53 may mediate the association of HSP70 and mutant p53, which is a functionally normal response of HSP70 to an altered tumour suppressor gene product. Alternatively, the association of p53 with HSP70 in potentially malignant, as well as malignant oral lesions, suggests that p53–HSP70 complex formation may be an important event in the multistep process of the development of oral tumours.

In oral cancer patients, the mutant p53–HSP70 complexes formed may elicit an effective immune response to the antigen, mutant p53, and may account for the presence of circulating antibodies against p53 in the serum of these patients. In view of the potential prognostic significance of the detection of

p53–HSP70 complexes in human oral SCCs and in potentially malignant lesions, our laboratory is currently involved in the analysis of circulating antibodies against p53 in these patients. Additional studies which aim to show more clearly the relationship between detection of p53 and HSP70 proteins, circulating p53 antibodies, p53 gene mutations and clinical course in patients with oral dysplasia (leucoplakia) and oral SCCs are currently underway.

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