Circulating p53 Antibodies as Early Markers of Oral Cancer: Correlation with p53 Alterations[1]

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ABSTRACT

p53 aberrations are early events in the pathogenesis of betel- and tobacco-related oral malignancies. Accumulation of p53 protein in oral lesions may elicit a humoral immune response against p53 protein in these patients. p53 antibodies (Abs) were analyzed in 183 sera obtained from patients with premalignant or malignant oral lesions and normal individuals by enzyme-linked immunoassay using recombinant p53 protein as antigen. These results were correlated with accumulation of p53 protein in patients' matched oral tissue specimens. Circulating p53 Abs were observed in 24 of 70 (34%) cancer patients and 15 of 50 (30%) patients with premalignant oral lesions. p53 Abs showed a significant association with increase in tumor size and dedifferentiation of tumors, factors indicative of poor prognosis. Expression of p53 protein was analyzed in 43 matched oral lesions (18 premalignant and 25 malignant cases). All the p53-seropositive patients (7 leukoplakia and 11 squamous cell carcinoma) showed elevated levels of p53 protein in matched oral lesions. However, the total number of patients seropositive for p53 Abs was lesser than that of patients exhibiting p53 protein accumulation in oral lesions. Four of the 63 normal healthy individuals who were heavy consumers of tobacco (smoking/chewing) and betel were found to be positive for p53 Abs. Detection of circulating p53 Abs in patients with premalignant oral lesions suggests that humoral immune response against p53 protein is an early event in oral oncogenesis and may be a surrogate marker for both p53 alteration and preclinical cancer.

INTRODUCTION

Management of oral cancer is one of the greatest challenges in medical oncology because of its rising incidence worldwide (1). In India, where the habit of chewing tobacco and betel nut is frequent, ~40% of the malignant oral lesions culminate in mouth and oropharynx (2, 3). Early premalignant oral lesions such as leukoplakia are clinically distinct in the Indian population, and 5–10% progress toward frank malignancy (4). Therefore, in this high-risk population, identification of a biological marker is of utmost importance, to complement clinicopathological findings for a more accurate prediction of individual patients' prognoses and to help clinicians in planning more effective therapeutic strategies.

Alterations in the tumor suppressor gene *p53* have been reported in 53–93% of HNSCCs[3] (5–7). We and others have shown that p53 protein is overexpressed not only in primary and recurrent oral SCCs but also in premalignant lesions (8). Abnormal accumulation of the mutant p53 protein in tumor cell nuclei has been significantly associated with the presence of p53 Abs in patients with a diverse range of cancers (9–16). In HNSCCs, significant association has been observed between p53 Abs and poor clinical outcome, *i.e.*, increased risk of relapse and death (17). However, the stage at which p53 Abs appear in the serum during the pathogenesis of the disease is not yet known, as most of the studies on p53 Abs have been carried out on malignant lesions.

We have recently shown that serum p53 Abs are present in a limited number of primary and recurrent oral SCCs by immunoblotting. To elucidate the mechanisms by which a humoral immune response against p53 protein is elicited, the presence of serum p53 Abs was correlated with the levels of p53, HSP70, and p53-HSP70 complexes in matched oral lesions (18). Here, our objective was to ascertain the stage of appearance of p53 Abs during the pathogenesis of oral cancer and determine its correlation, if any, with protein accumulation in the tobacco-abused oral cancer patients in context of the Indian population. We also determined the potential clinical significance of p53 Abs as early serological markers for identifying patients with premalignant lesions who are at a high risk of transition to malignancy or oral cancer patients with poor prognosis.

MATERIALS AND METHODS

Serum samples were obtained from patients with histopathologically confirmed oral SCCs from Institute Rotary Cancer Hospital, All India Institute of Medical Sciences (New Delhi, India). A total of 183 sera were analyzed. Seventy sera were obtained from patients with oral SCCs. The mean age of

Received 12/10/97; revised 6/10/98; accepted 6/15/98.
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[1] This work was supported by a research grant from the Council of Scientific and Industrial Research India and the Indo-French Centre for the Promotion of Advanced Research.
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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; SCC, squamous cell carcinoma; Ab, antibody; HSP70, heat shock protein 70; EIA, enzyme immunoassay.

Table 1 p53 Abs in the sera of patients with oral lesions and normal subjects

Group	No. of cases	Ab positive		Specific activity (mean ± SD)	P^a
		No.	%		
Cancer	70	24	34%	0.650 ± 0.384	C/N, <0.001
Primary	42	20		0.804 ± 0.352	C/L, <0.05
Recurrent	28	4		0.426 ± 0.311	P/R, <0.01
Premalignant (Leukoplakia)	50	15	30%	0.496 ± 0.283	L/N, <0.01
Hyperplasia	29	4		0.398 ± 0.263	H/D, <0.05
Dysplasia	21	11		0.626 ± 0.288	
Normal	63	4[b]		0.187 ± 0.120	
Nonconsumers	27			0.139 ± 0.070	
Consumers[c]	36			0.209 ± 0.138	

[a] C, cancer; L, leukoplakia; N, normal; P, primary; R, recurrent; H, hyperplasia; D, dysplasia.

[b] Clinical records of these four normal subjects revealed that they are heavy consumers of tobacco (smoking and chewing) and betel quid.

[c] Tobacco use: moderate, <20 bidis/cigarettes per day for 1–10 years or equivalent amount of chewable tobacco; heavy, >20 bidis/cigarettes per day for >10 years. Betel: moderate, 5–10 betel (pan) per day for 2–10 years; heavy, >10 betel (pan) per day for >10 years.

the patients was 51 years (SD = 11.8); 88% were male. Fifty sera were collected from patients with premalignant lesions (leukoplakia) with histological evidence of hyperplasia or dysplasia. The mean age of patients was 43 years (SD = 12.6); 85% were male. Sixty-three sera were obtained from normal healthy individuals. The mean age was 42 years (SD = 14.4); 78% were male. The clinicopathological data recorded at the time of enrollment of the subjects in the cancer clinic included age, sex, tobacco (chewing and smoking), and betel chewing history, site of tumor, and histological grade. The tumor stage was determined following the tumor-node-metastasis classification (International Union Against Cancer, 1987). Sera were obtained from the patients prior to any treatment (surgery, chemotherapy, or radiotherapy). Histopathological examination of the premalignant lesions revealed that 29 of the 50 lesions showed hyperplastic changes, whereas 21 lesions showed evidence of dysplasia. The distribution of oral cancer patients according to their site of primary tumor included buccal mucosa (10 cases), lower alveolus (6 cases), tongue (8 cases), floor of the mouth (9 cases), lip (5 cases), and cheek (4 cases). The site distribution of recurrent oral tumors comprised buccal mucosa (8 cases), lower alveolus (4 cases), tongue (5 cases), floor of the mouth (5 cases), lip (3 cases), and cheek (3 cases). The site distribution of premalignant lesions comprised buccal mucosa (18 cases), lower alveolus (6 cases), tongue (5 cases), floor of the mouth (4 cases), lip (4 cases), and cheek (13 cases). The majority of the patients were heavy consumers. Serum samples obtained from normal individuals, used as controls in this study, were obtained from cancer-free subjects. Of the 63 sera obtained from normal subjects, 36 sera were obtained from habitual tobacco consumers, whereas 27 sera were obtained from individuals who were not habitual consumers of betel and tobacco. All the serum samples were aliquotted and immediately stored at −80°C until analysis.

EIA to Detect p53 Abs in Human Serum. Purified p53 protein was prepared by a modification of a procedure described previously (19). Recombinant baculoviruses that express wild-type human p53 were used to infect Sf9 insect cells growing in culture. The harvested cells were lysed, and the p53 protein was immunoaffinity purified using p53-specific monoclonal Ab PAb421-coupled protein A-Sepharose (Oncogene Science, Uniondale, NY). The bound p53 protein was eluted using PAb421-specific synthetic peptide. The eluant was purified by passing through Mono Q column. The purity of p53 protein was checked by SDS-PAGE, and the concentration was determined by the Bradford protein assay (Bio-Rad). Ab-captured EIA was used to detect the p53 Abs in human sera.

Immunopurified human p53 antigen (50 ng/well) or BSA (50 ng/well) was coated in wells of 96-well microtiter plates. The plates were incubated for 16–18 h. Nonspecific binding was blocked by incubation with 3% (w/v) BSA for 1 h, followed by incubation for 1 h with patient serum/normal serum (1:500 dilution in PBS). Goat antihuman IgG conjugated to horseradish peroxidase (1:1000 dilution in PBS) was subsequently added to the wells and allowed to incubate for 45 min. The color was developed using the substrate *ortho*-phenylenediamine (0.5 mg/ml) in citrate phosphate buffer (0.1 M, pH 5.0). The reaction was stopped after 15 min with 5 N H_2SO_4, and absorbance was measured at 492 nm. All incubations were carried out at 37°C in a moist chamber. After each step, the wells were washed several times with PBS-Tween 20 (0.05%, v/v). Appropriate controls for antigen, Ab, and horseradish peroxidase conjugate were used to exclude any nonspecific background in patient samples. A known serum sample was used as an internal control in each batch of assays to take into account interassay variations. Each serum sample was assayed twice in triplicates using p53 antigen, as well as BSA. Mean absorbance of all six observations (wells) was calculated. BSA was used as the irrelevant antigen to obtain a clear distinction between p53-specific binding and possible background binding of each of the tested serum samples.

Criteria for EIA-positive Assays and Samples. Sera were assayed a minimum of twice. A positive assay required a mean p53:BSA ratio of ≥1.5 and a difference between the two means ≥2 SDs (20). A positive sample required at least two positive assays. To corroborate the data obtained by EIA, matched tissue specimens of oral lesions obtained from a subset of EIA-positive and EIA-negative patients were coded and used for analysis of p53.

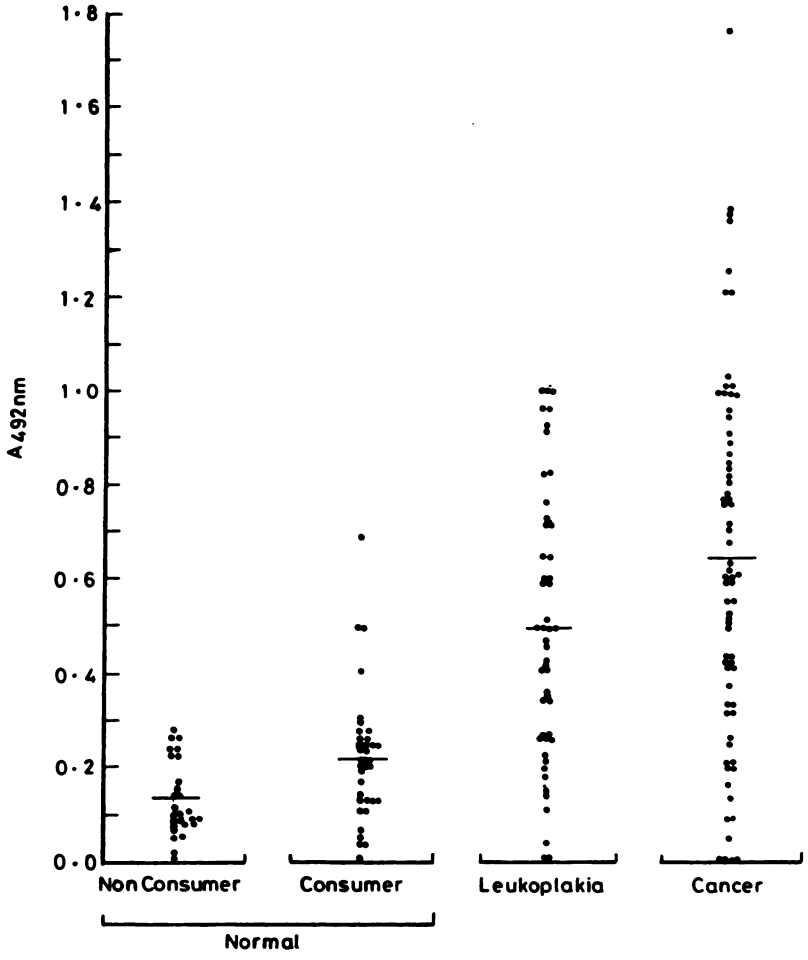

Fig. 1 EIA results, shown as specific activities (mean p53 $A_{492\ nm}$ − mean BSA $A_{492\ nm}$) of individual sera in groups of patients screened for p53 Abs (see "Materials and Methods"). Sera from 4 of the 36 normal subjects (who were heavy consumers of tobacco and betel) were seropositive for p53 Abs. EIA results in patients with cancer were significantly higher than those of patients with leukoplakia ($P < 0.05$) and normal subjects ($P < 0.001$).

p53 Immunohistochemistry. Cryosections of oral tissue specimens (premalignant and malignant lesions) were used for immunohistochemical analysis of p53 protein as described previously (8). Monoclonal p53 Ab DO-1 (Oncogene Science) was used as the primary Ab. It was detected using avidin-biotin complex and diaminobenzidine tetrachloride as the chromogen. Positive and negative controls were used in each experiment (8).

Statistical Evaluations. Statistical analyses of the data were performed using Microstat software. A χ^2 test was performed to determine the association between the presence of p53 autoantibodies and clinicopathological features of the patients. Kruskal-Wallis (non-parametric) one-way ANOVA were used to compare the significance in the subgroups; a P of <0.05 was considered significant.

Table 2 Correlation of p53 Abs with clinicopathological features in cancer patients

Clinical features	Total no. of cases	Specific activity (mean ± SD)	P^a
Histological grading			
Well	17	0.339 ± 0.233	
Moderate	27	0.707 ± 0.307	W/M, <0.05
Poor	26	0.817 ± 0.413	W/P, <0.001
Stage			
T_1	9	0.271 ± 0.141	T_1/T_3, <0.01
T_2	18	0.365 ± 0.260	T_2/T_3, <0.001
T_3	22	0.772 ± 0.336	T_1/T_4, <0.001
T_4	21	0.880 ± 0.352	T_2/T_4, <0.001

[a] Statistical analysis done by Kruskal-Wallis test. W, well; M, moderate; P, poor.

RESULTS

Circulating p53 Abs and p53 protein accumulation were examined in patients with premalignant and malignant oral lesions. Of the 183 sera tested, circulating p53 Abs were detected in 24 of 70 (34%) oral cancer patients, 15 of 50 (30%) patients with premalignant lesions, and 4 of 63 (6%) normal subjects by the positivity criteria described above (Table 1). The circulating levels of p53 Abs in the premalignant group [specific activity, (mean ± SD) 0.496 ± 0.283] and the cancer group (specific activity, 0.650 ± 0.384) were significantly higher than that of the normal subjects (specific activity, 0.187 ± 0.120; $P < 0.01$). A significant increase in p53 Abs was observed in sera of cancer patients, as compared to those having premalignant lesions ($P < 0.05$). The specific activity (mean p53 − mean BSA $A_{492\ nm}$) of individual serum samples in groups of

Table 3 Correlation of p53 Abs with p53 protein expression in premalignant and malignant oral lesions

Patient no.	Age (yr)	Sex	Site	Stage	Histopathology	Habit[a]	p53 protein[b]	p53 Ab[b]
1	36	M	BM[c]	L	H	Heavy	−	−
2	32	M	BM	L	D	Heavy	+	+
3	32	M	BM	L	H	Moderate	+	+
4	34	M	T	L	H	Moderate	+	+
5	30	M	BM	L	D	Heavy	−	−
6	49	M	BM	L	D	Moderate	−	−
7	36	F	MF	L	D	Moderate	−	−
8	46	M	Lip	L	H	Heavy	++	+
9	48	M	T	L	D	Heavy	+	+
10	45	M	MF	L	D	Heavy	−	−
11	48	F	BM	L	H	Heavy	+++	+
12	60	F	BM	L	H	Heavy	+	−
13	26	M	T	L	H	Moderate	+++	−
14	65	M	MF	L	H	Heavy	+	−
15	27	M	T	L	D	Heavy	+	−
16	54	M	L	L	H	Heavy	+++	−
17	48	M	BM	L	H	Heavy	+	−
18	45	M	BM	L	D	Heavy	++	+
19	54	M	BM	$T_4N_0M_0$	W	Heavy	++	+
20	45	M	T	$T_2N_1M_0$	P	Heavy	+++	+
21	70	M	BM	$T_2N_0M_0$	W	Heavy	+	+
22	60	M	BM	$T_2N_0M_0$	M	Heavy	−	−
23	52	M	BM	$T_3N_0M_0$	M	Heavy	−	−
24	55	M	T	$T_4N_2M_0$	W	Heavy	−	−
25	50	M	BM	$T_2N_2M_0$	W	Moderate	++	+
26	51	M	BM	$T_3N_2M_0$	W	Moderate	−	−
27	40	M	LA	$T_4N_2M_0$	W	Heavy	−	−
28	60	M	MF	$T_3N_1M_1$	W	Heavy	+	+
29	52	M	LA	$T_3N_2M_0$	M	Moderate	+++	+
30	42	M	T	$T_4N_0M_0$	W	Moderate	+++	+
31	50	M	MF	$T_1N_1M_1$	W	Heavy	++	+
32	46	M	T	$T_3N_0M_0$	W	Heavy	−	−
33	38	M	Lip	$T_3N_0M_0$	M	Heavy	++	+
34	57	M	BM	$T_3N_0M_0$	M	Moderate	++	+
35	56	M	MF	$T_2N_0M_0$	W	Moderate	+	+
36	55	M	BM	$T_4N_1M_0$	W	Heavy	++	−
37	50	M	MF	$T_4N_0M_0$	W	Heavy	++	−
38	72	M	BM	$T_2N_1M_0$	P	Heavy	++	−
39	80	M	Lip	$T_2N_0M_0$	W	Moderate	++	−
40	55	M	BM	$T_3N_0M_0$	W	Moderate	+	−
41	62	M	MF	$T_2N_0M_0$	W	Heavy	++	−
42	60	F	MF	$T_4N_1M_0$	P	Heavy	++	−
43	32	M	BM	$T_4N_0M_0$	M	Heavy	+	−

[a] Tobacco use: moderate, <20 bidis/cigarettes per day for 1–10 years or equivalent amount of chewable tobacco; heavy, >20 bidis/cigarettes per day for >10 years. Betel: moderate, 5–10 betel [pan] per day for 2–10 years; heavy, >10 betel [pan] per day for >10 years.

[b] Percentage of positively stained tumor cells: +1, 10–30%; +2, 30–50%; +3, >50%.

[c] BM, buccal mucosa; T, tongue; MF, mouth floor; LA, lower alveolus; L, leukoplakia; H, hyperplasia; D, dysplasia; M, moderately differentiated tumors; P, poorly differentiated tumors; W, well-differentiated tumors.

patients with either premalignant or malignant oral lesions or normal subjects are presented in Fig. 1.

Correlation of p53 Autoantibodies with Clinicopathological Features. Among the cancer patients, there was a significant increase in the level of circulating p53 Abs in patients with poorly differentiated or moderately differentiated tumors, as compared to the well-differentiated cases ($P < 0.05$). There was a significant increase in the level of p53 Abs from the less progressive T_1 stage (specific activity, 0.271 ± 0.141) to the more progressive T_4 stage tumors (specific activity, 0.880 ± 0.352; $P < 0.001$; Table 2).

The expression of p53 protein was analyzed in 43 matched tissue specimens (18 premalignant lesions and 25 oral SCCs; Table 3). All 18 p53 Abs-seropositive patients (7 premalignant and 11 cancer cases) showed p53 protein accumulation in their oral lesions. Eleven p53 Ab-seronegative cases (5 premalignant and 6 cancer) did not show detectable level of p53 protein in their oral lesions. However, 14 p53 Ab-seronegative cases (6 premalignant and 8 malignant) showed detectable levels of p53 protein in their lesions.

DISCUSSION

We previously reported accumulation of p53 and HSP70 proteins not only in malignant but also in premalignant oral

lesions in the tobacco-abusing Indian population, suggesting that alterations in the expression of these proteins are early events in oral tumorigenesis (8, 21). On the basis of regular follow-up studies, we have recently reported the prognostic significance of accumulation of p53 and HSP70 proteins during oral tumorigenesis (22, 23). Furthermore, we showed formation of p53-HSP70 complexes during oral tumorigenesis (24). The functional significance of overexpression of these proteins and formation of p53-HSP70 complexes in eliciting p53-specific humoral immune response in oral cancer patients was determined. Circulating anti-p53 Abs detected by immunoblotting analysis in 7 of 30 oral cancer patients were correlated with the levels of p53 and HSP70 complexes in matched tumor tissues and also with patients' survival. Anti-p53 Ab-seropositive cases showed poor prognosis and significantly decreased overall disease-free survival in comparison with the seronegative cases suggesting that detection of circulating anti-p53 Abs may serve as a useful noninvasive marker for identifying oral tumors having poor prognosis.

Detection of serum p53 Abs by immunoblotting procedure may be of limited value for routine use in most pathological/diagnostic laboratories. The simple and rapid ELISA procedure, using the recombinant p53 protein as antigen, may be advantageous for the screening of normal individuals (who are heavy consumers of tobacco and betel) and leukoplakic patients to ascertain the stage of appearance of p53 Abs during oral tumorigenesis. Furthermore, it can be used for retrospective or prospective studies as well as for the follow-up of the patients.

Herein, we report the prevalence of p53 Abs not only in oral SCC patients (24 of 70; 34%) but also in patients with premalignant oral lesions (15 of 50; 30%) in the tobacco-abused Indian population. In HNSCC patients, the prevalence of p53 Abs reported by previous workers ranged from 17 to 44% (25–27). Expression of p53 protein was observed in 13 of 18 premalignant and 19 of 25 (76%) oral SCC cases. The p53 Ab-seropositive patients showed detectable levels of p53 protein in the matched premalignant and malignant lesions. Patients lacking detectable levels of p53 protein in premalignant as well as malignant oral lesions were seronegative for p53 Abs. Thus, a clear dose-response relationship was observed between p53 Abs and p53 protein accumulation in oral lesions and is a determinant of the immune response to p53 protein in these tobacco-abused patients. A similar correlation has been observed between circulating p53 Abs in lung cancer patients and the accumulation of p53 protein in their matched tumor cell lines (12).

The impact of genotoxic environmental factors in producing p53 alterations in tobacco-associated cancers such as lung and esophagus have been discussed (28). In India, oral SCC is causally associated with the habit of chewing betel quid and tobacco (betel quid consists of a betel leaf rolled with lime, areca catechu, areca nut, and tobacco, and it is kept at a particular site in the oral cavity for a long duration). In our series, the majority of the patients (95%) were heavy addicts of betel/tobacco. The constituents of betel quid and tobacco contain a plethora of tumor initiators, promoters, carcinogens, and cocarcinogens. These include, nitrosamines, benzo(*x*)pyrenes, polycyclic aromatic hydrocarbons present in tobacco, and other mutagenic alkaloids in areca nut. It is likely that prolonged exposure to these carcinogeneic constituents of betel quid contributes significantly to genetic insults to the oral mucosa, resulting in accumulation of p53 protein in early stages of oral cancer, thereby accounting for the presence of circulating p53 Abs in patients with premalignant lesions.

Here, the presence of p53 Abs in a few normal subjects (4 of 63 normal subjects) draws attention. The dietary habits of these four normal subjects revealed that they were habitual heavy consumers of tobacco (20 cigarettes/bidis and 10–15 tobacco-containing betel quid for a period of >10 years). The whole oral epithelium of these subjects may accumulate genetic damage over a prolonged period of time; therefore, it is at an increased risk of developing malignancy (29) and may be responsible for the presence of circulating p53 Abs. In several anecdotal cases, the p53 Abs have been detected in serum samples in high-risk patients several years before the clinical detection of cancer (28). Two of the five workers exposed to vinyl chloride in a plastic factory showed the presence of serum p53 Abs before the diagnosis of liver angiosarcoma (30). p53 Abs have also been reported in 4 of 36 women with a positive family history of breast cancer (31). Recently, p53 Abs in sera from patients with chronic obstructive pulmonary disease have been shown to predate cancer (20).

Here, a number of salient and interesting observations have been made that enable us to dissect further at the molecular level the importance of p53 in tobacco related premalignant and malignant oral lesions in patients of the same ethnic origin. The relationship between the p53 protein accumulation and p53 Abs observed in this study suggest the potential usefulness of p53-Abs in tobacco- and betel-abused populations at high risk as a surrogate marker for early p53 alteration and as potential aids in the early detection of some cancers.

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