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Gastric adenoma – carcinoma sequence with special reference to p53 and Ki-*ras* gene alterations

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Abstract With the aim of detecting the timing of p53 and Ki-*ras* gene alterations in the gastric adenoma–carcinoma sequence, 19 early gastric adenocarcinomas arising from adenomas were studied. Immunohistochemically, 5 adenocarcinomas were positive for p53; 3 focally and 2 diffusely. The p53 point mutations were detected in a focal area with p53 immunoreactivity in 2 of the 5 p53-positive adenocarcinomas. This indicated that p53 point mutations may play a less crucial part in malignant conversion of adenoma to adenocarcinoma in the stomach than in the colon. No Ki-*ras* gene mutations at codons 12 and 13 were detected in any lesion. These results suggest that the adenoma–carcinoma sequence in the stomach has a different mechanism from that in the colon.

Key words p53 · Ki-*ras* · Gastric carcinoma · Immunohistochemistry · Polymerase chain reaction-single-strand conformational polymorphism

Introduction

In general, gastric cancers are divided histologically into well-differentiated and poorly differentiated categories based on their tendency to form glandular patterns. The site of development and the degree of their malignancy of these categories are usually different, and recent advances in molecular biology have suggested that they are derived from different routes of gastric carcinogenesis [1]. Well-differentiated or intestinal type carcinomas are usually preceded by a gastric adenoma or by dysplasia after intestinal metaplasia [1].

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Gastric adenomas are lesions frequently observed in elderly persons and sometimes coexist with a well-differentiated adenocarcinoma [2, 3]. The lesions are thus thought to be precancerous, leading to the assumption that the adenoma–carcinoma sequence in the stomach is similar to that in the colon. In the colon, the adenoma–carcinoma sequence has been analysed from the viewpoints of both genetics and the resultant morphological changes [4, 5]. In the development of the various stages of colon adenoma, several genetic changes in *APC*, Ki-*ras* and *DCC* genes have important roles in its progression [5–7], and p53 gene alterations are crucial for malignant conversion from adenoma to carcinoma [8–10]. We previously investigated the p53 gene alterations in gastric adenomas and found that they were less frequent than in colon adenomas [11]. However, very few studies have been performed to clarify the genetic changes in gastric adenomas, and the true nature of the gastric adenoma–carcinoma sequence remains uncertain.

In this study, we analysed both p53 and Ki-*ras* genes in 19 cases of early gastric adenocarcinoma arising from adenoma to determine the incidence and timing of p53 and Ki-*ras* gene alterations in the gastric adenoma–carcinoma sequence.

Materials and methods

Gastric carcinoma coexisting with adenoma cases

From the pathology files of Gunma University of its affiliated hospitals, 19 gastric adenocarcinomas coexisting with adenomas were collected from 18 patients. In 1 case, there were two separate lesions in a surgically resected specimen, in both of which adenomatous and adenocarcinomatous components coexisted. In 13 cases open surgical resection was performed, and in 3 resection was accomplished endoscopically. In the other 3 cases, only biopsy specimens were used. Of the 18 patients, 7 were followed up for 3–5 years with repeated endoscopic examinations (Fig. 1).

The tumours were classified by gross appearance according to the early gastric cancer classification elaborated by the Japanese Society of Gastroenterology. For the histopathological study, the diagnosis of adenomas and adenocarcinomas was based on the criteria laid down in "*Histological Typing of Oesophageal and Gastric Tumours*" published by the WHO [12].

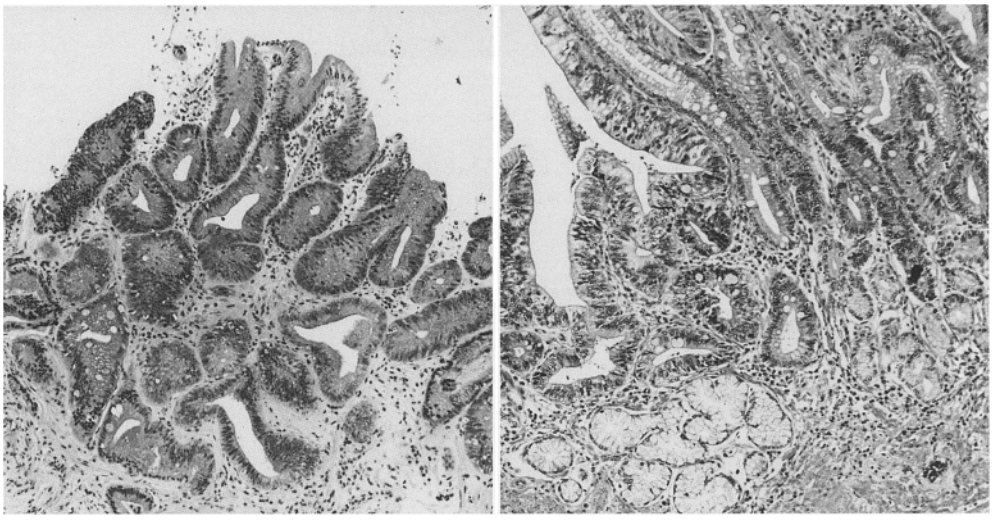

Fig. 1 Adenoma (*left*), which progressed to adenocarcinoma (*right*) during the follow-up period. HE, ×85

Immunohistochemical examination for p53

All tumour specimens were fixed in 15% formalin after resection and embedded in paraffin. For the immunohistochemical study, 3-μm-thick paraffin sections were cut from the paraffin block containing representative histological features of the tumour. The immunohistochemical examination for p53 was carried out by the method described previously, using a microwave oven to retrieve antigenic activity [11]. Monoclonal antibody DO7 (Dako Japan, Kyoto, Japan), which recognizes both wild and mutant forms of p53 protein, was used for the first antibody at a 1:25 dilution.

The results of the immunohistochemical study were evaluated as follows: negative, none of the tumour nuclei were positive for p53; sporadic, a few p53-positive nuclei were scattered in neoplastic glands; focal, a cluster of p53-positive glands was present; diffuse, all the neoplastic glands were positive for p53.

In addition to the 18 cases, already detailed, we also studied 27 early gastric adenocarcinomas without any adenomatous components, taken from 26 patients. These were randomly selected and were examined immunohistochemically for the status of p53 overexpression in comparison with that in early gastric adenocarcinomas arising from adenoma.

Molecular biological analysis

All 19 adenocarcinomas arising from adenomas were examined. Each adenomatous and carcinomatous component or p53-positive and -negative area was separately collected by sectioning after demarcation of each component with a sharp knife on the paraffin block. Several 6-μm-thick paraffin sections containing either adenoma or adenocarcinoma tissue were placed in a 1.5-ml Eppendorf tube and digested in 50 mmol/l TRIS-HCl buffer (pH 8.5) containing 1 mmol/l EDTA, 200 μg/ml proteinase K and 0.5% Tween 20. After incubation for 8 h at 62° C, 30% Chelex-100 (Bio-Rad) was added and samples were boiled for 10 min and then centrifuged for 5 min. Then 1 μl of the liquid phase was used for the polymerase chain reaction (PCR), to amplify the p53 and Ki-*ras* genes.

PCR-single strand conformational polymorphism (SSCP) analyses for the p53 and Ki-*ras* genes were carried out according to the method described previously [13]. Briefly, the oligonucleotide primers amplifying each exon from 5 to 8 of the p53 gene and a sequence across codons 12 and 13 of the Ki-*ras* gene were used for the PCR amplification (Table 1). To increase the sensitivity and specificity of the SSCP, the PCR was conducted twice by a DNA thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer Cetus, Norwalk, Conn.) for 35–40 cycles. DNAs extracted from cell lines containing various mutated p53 genes from codons 5 to 8 (small cell lung cancers, Lu130, Lu135 and Lu139; and a uterine cancer, EM54) or a mutated Ki-*ras* gene in codon 12 (large cell lung cancer, A549) and from a normal foreskin were used as positive and negative controls, respectively. After the PCR, amplified DNA was electrophoresed on 12.5% or 20% acrylamide gel (100×100×1 mm) for 2.5–5 h at 20° C.

For the DNA sequencing, a single mutated band was excised from the dried gel and the DNA was extracted from the gel and used for the DNA sequencing. A dsDNA cycle sequencing system kit (GIBCO, Grand Island, N.Y.) was used according to the manufacture's instruction manual.

Results

Clinicopathological observations

Of the 18 patients in this series, 14 were men and 4 were women. The age distribution was from 56 to 85 years (average 71 years) at the time of the initial biopsy.

Gross examination revealed 9 slightly elevated tumours, 4 protruding tumours, and 2 flat ones; the remaining 4 were depressed. During the follow-up period, 1 tumour changed in endoscopic gross appearance from flat-type to protruding-type. The size of the tumours at resection ranged from 4 to 45 mm in diameter (average 18.6 mm).

In 16 of the 19 lesions, the adenomatous components were histologically classified as intestinal-type growing

Table 1 List of primers used for p53 gene (exon 5–8) and Ki-*ras* gene

p53 Gene	
Exon 5	5′-TTCCTCTTCCTGCAGTAC-3′
	5′-GCCCCAGCTGCTCACGATCG-3′
Exon 6	5′-CACTGATTGCTCTTAGGTCTGGC-3′
	5′-AGTTGCAAACCAGACCTCAGGCG-3′
Exon 7	5′-CTCCTAGGTTGGCTCTGACTGT-3′
	5′-CAAGTGGCTCCTGACCTGGA-3′
Exon 8	5′-CCTATCCTGAGTAGTGGTAATC-3′
	5′-ACTTAGACTCCGTATGACG-3′
Ki-*ras* gene	5′-GGCCTGCTGAAAATGACTGA-3′
	5′-GTCCTGCACCAGTAATATGC-3′

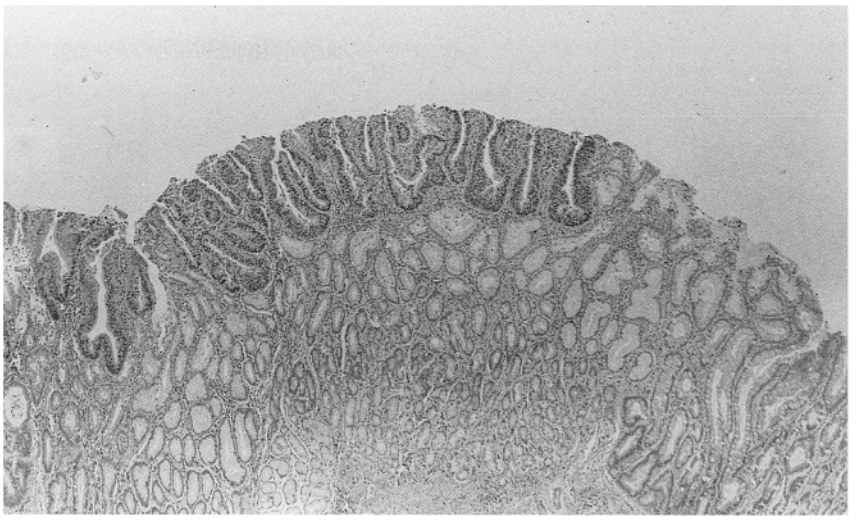

Fig. 2 Adenocarcinoma without adenoma component showing diffuse positive staining for p53. Immunoperoxidase, counterstained with haematoxylin, ×16

in tubules and often containing goblet and Panet's cells. The other three adenoma components were mixed-type adenomas, consisting of cells similar to gastric foveolar epithelium and intestinal absorptive cells without any differentiation toward goblet or Panet's cells. The ratio of carcinomatous to adenomatous components varied from case to case, and adenocarcinomatous components infiltrated the upper submucosal layer in 2 of 16 16 surgically resected lesions. The others were located within the lamina propria.

In contrast to these adenocarcinomas arising from adenoma, clinicopathological details of 27 early gastric adenocarcinomas without any adenomatous components were as follows: 22 were in men and 4 in women, and the patients' age distribution was from 46 to 74 years (average 62 years). Grossly, most tumours were seen to be depressed in form (21/27, 78%). The size of tumours ranged from 4 to 132 mm in diameter (average 26 mm). Histologically, 14 of 27 adenocarcinoma were classified as well-differentiated adenocarcinoma and the remaining ones, as moderately differentiated. Of 27 tumours, 14 adenocarcinomas were localized within the lamina propria and the rest infiltrated the upper submucosal layer.

Table 2 Immunohistochemical p53 overexpression in adenocarcinoma with or without adenoma components

	Negative/ sporadic	Focal	Diffuse'
Carcinomas with adenomatous components (n=19)	14 (74%)	3 (16%)	2 (10%)
Carcinomas without adenomtous components (n=27)	14 (52%)	3 (11%)	10 (37%)

Table 3 Immunoreactivity for p53 in each adenoma and adenocarcinoma component

	p53 Immunohistochemistry			
Adenomatous component	(−)	(+)	(−)	(+)
Adenocarcinoma component	(−)	(−)	(+)	(+)
Number of cases (n=19)	12	2	3	2

Immunohistochemical and molecular biological studies

The results of immunohistochemical and molecular biological studies are summarized in Tables 2–4. In immunohistochemical examination for p53, both focal and dif-

Table 4 Immunohistochemical and molecular biological analysis of seven cases with p53 positivity in the adenomatous and adenocarcinomatous components (*n.d.* not detected)

Case	Pattern of P53 staining		Point mutation detected in this study
	Adenoma	Carcinoma	
1	Negative	Focal	Carcinomatous component; codon 282: CGG (Arg)→TGG (Trp) →TAG (Stop)
2	Negative[a]	Diffuse[a]	n.d.
3	Focal[a]	Focal	adenoma component; codon 300: CCC (Pro)→CTC (Leu) Carcinoma component; codon 175: CGC (Arg)→TGC (Cys)
4	Negative	Focal	n.d.
5	Diffuse	Diffuse	n.d.
6	Focal[a]	Negative	n.d.
7	Focal	Negative	n.d.

[a] Biopsy specimen

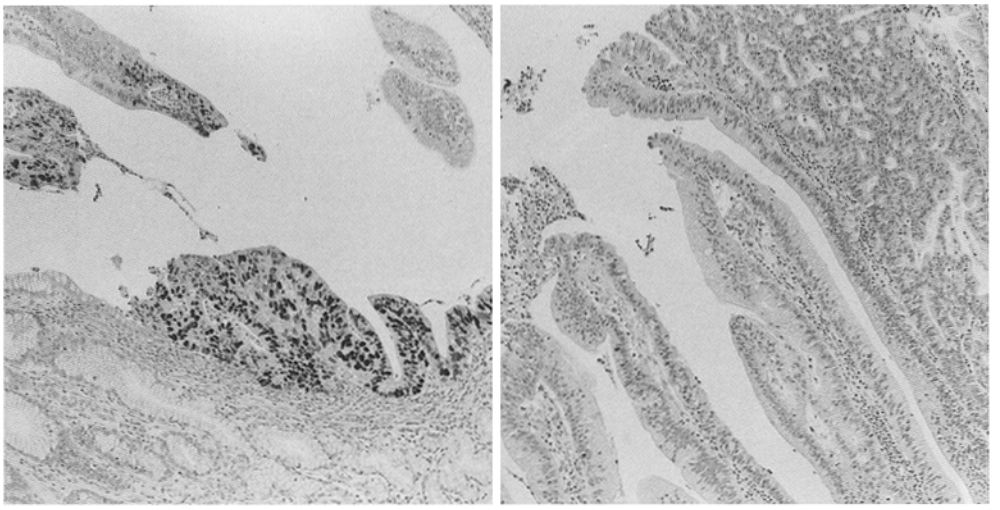

Fig. 3 Immunohistochemical study for p53 (case 1). Positive staining was found in a focal area of the adenocarcinoma component (*left*). Adenoma and adenocarcinoma in other areas were negative for p53 (*right*). Immunoperoxidase, counterstained with haematoxylin, ×85

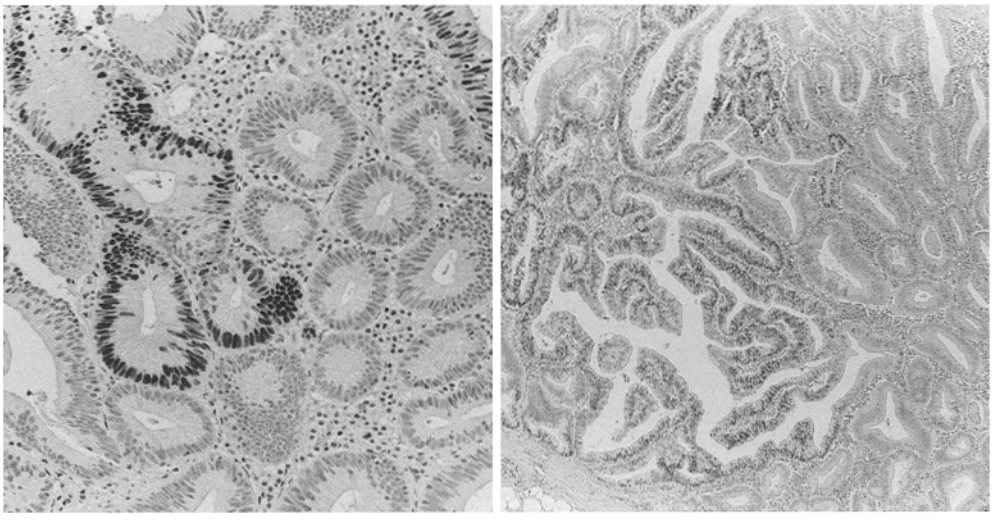

Fig. 4 Immunohistochemical study for p53 (case 3). Both adenoma (*left*, ×170) and adenocarcinoma (*right*, ×17) components showed focal positive staining. Immunoperoxidase, counterstained with haematoxylin. A different kind of mutation was found in each component

fuse staining patterns were regarded as p53 positive. In 27 adenocarcinoma cases without adenomatous components, 13 adenocarcinomas (48%) were immunohistochemically positive for p53, and 10 of these were diffusely stained for p53 (Fig. 2). However, of the 19 lesions with adenomatous components, the adenocarcinomatous components were immunohistochemically positive for p53 in 5 (26%; Figs. 3, 4), but 3 these showed focal staining and the remaining 2 were diffusely positive for p53. Two of these five positive adenocarcinomas were shown to have a point mutation of the p53 gene (Fig. 5). In case 1, two different kinds of point mutation were found only in the immunohistochemically p53-positive carcinoma component (Fig. 5B). In case 3, there was a difference in the site of the p53 point mutation between the adenomatous and adenocarcinomatous components. In cases 2, 4, 5, 6 and 7, the PCR-SSCP method failed to detect any point mutations of the p53 gene, in spite of positive immunostaining for p53. Apart from these 7 cases, only 1 adenocarcinoma biopsy specimen, taken during the follow-up period, showed p53 overexpression and contained a p53 point mutation

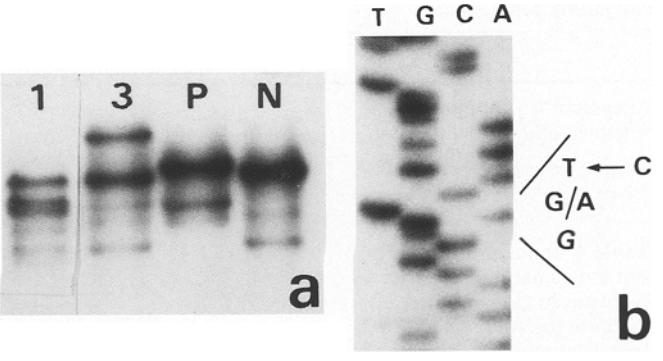

Fig. 5 **a** SSCP analysis of exon 8 of the p53 gene shows abnormally shifted bands in cases 1 and 3. In case 1, four abnormally shifted bands are seen (*P* positive control, *N*, negative control). **b** DNA sequence analysis of the abnormal bands seen in case 1 revealed CGG→TGG and TAG changes at codon 282

(CGC→CAC transition at codon 283: Arg→His); none of the p53 abnormalities were found in the adenocarcinoma component at tumour resection, however. This case was excluded from the p53-positive carcinoma cases in

Tables 2–4. In 11 other p53-negative cases, p53 point mutations were not detected.

No point mutation at codons 12 and 13 of the Ki-*ras* gene was found in any tumour investigated in this study.

Discussion

In human cancer development, colon cancer is a representative model of multi-step carcinogenesis or the adenoma–carcinoma sequence, in which the genetic and morphological changes in each step of tumorigenesis have been elucidated [5]. Among various genetic changes in colon tumorigenesis, p53 gene alteration, point mutation and the following loss of hterozygosity (LOH) of 17p, play crucial parts in malignant conversion from adenoma to adenocarcinoma [8–10]. Therefore, the frequency of p53 alterations, such as 17p LOH, point mutation and immunoreactivity, reaches a maximum of about 70% in colon adenocarcinomas [14]. Few studies have been performed to reveal the p53 alteration in the gastric adenoma–carcinima sequence, and these few have led to the conclusion that the frequency of p53 alteration gradually increases from 10–37.5% in intestinal metaplasia through 30–58.3% in gastric adenomas or dysplasia to 43–66.7% in gastric carcinomas [1, 15, 16], indicating that p53 alteration is an early event in gastric carcinogenesis, as in colon carcinogenesis. In our previous report, the maximum frequency of immunohistochemical p53 overexpression in gastric adenomas reached 37.5% (12/32) during endoscopic follow up. However, only one of four gastric adenocarcinomas arising from adenomas during follow up showed p53 abnormality on immunohistochemical study. The result indicate that p53 alterations in the gastric adenoma–carcinoma sequence may not play an essential part in malignant conversion from adenoma to adenocarcinoma.

To determine the exact timing of p53 alteration in the gastric adenoma–carcinoma sequence, we used 19 adenocarcinomas coexisting with adenoma, in which the adenocarcinomas were thought to arise from pre-existing adenomas. Immunohistochemically, p53 accumulation was observed in the adenocarcinoma component in 5 lesions (26%). In early gastric adenocarcinomas without any adenoma components, however, about half of the tumour cells showed immunohistochemical p53 overexpression. Nonetheless, in contrast to adenocarcinomas without adenoma, most of which showed diffuse staining patterns in p53-positive cases, three of these five lesions showed only focal immunostaining for p53. The frequency of immunohistochemical p53 positivity in gastric adenocarcinomas arising from adenomas is lower than that in adenocarcinoma without any adenomatous components or in colon adenocarcinomas. This suggests that p53 alteration may play a less crucial part in malignant conversion in the course of gastric multi-step carcinogenesis than in the cases of colon carcinogenesis.

Of the seven adenocarcinoma or adenoma components that stained positive for p53, two showed point mutations in the p53 gene. The failure to detect p53 mutation in the other five lesions is probably due either to inadequate sensitivity of the PCR-SSCP method or to location of the mutation outside exons 5–8. We used several small biopsy specimen containing only a small focus of glands positive for p53 protein, which might influence the sensitivity of PCR-SSCP method for the detection of p53 point mutation. No p53 point mutation was detected in the other cancer glands, which showed no immunoreactivity for p53 (data not shown). It is therefore reasonable to conclude that p53 point mutation in these two cancers, which showed focal positive staining pattern for p53, may not have a crucial role in malignant conversion from adenoma to carcinoma and probably occurs after malignant conversion. This is the first direct evidence that p53 point mutation may be unrelated to malignant conversion in the gastric adenoma–carcinoma sequence. In case 3, a point mutation was also found in the adenoma component during the follow-up period, but differed from that seen in the adenocarcinoma component. The appearance of p53 alteration in intestinal metaplasia and adenoma has been reported previously [1, 15, 16], but a clone with p53 mutation might not always progress to adenocarcinoma. It is thought that the p53 point mutation appears randomly because of the degree of genetic instability during gastric multi-step tumorigenesis. As our findings are limited, further information about gastric tumorigenesis is necessary before a conclusion can be drawn.

Ki-*ras* gene point mutation is one of the important genetic changes in the development of colon adenomas, and its frequency has been reported as up to 67% [5, 17]; it is suggested that the Ki-*ras* gene mutation is one of the important genetic changes in the gastric adenoma-carcinoma sequence [18]. However, several recent studies revealed infrequent Ki-*ras* gene mutation in gastric adenoma and adenocarcinoma [19]. In our study, no Ki-*ras* gene mutation was observed in either the adenomatous or the carcinomatous component.

In conclusion, p53 gene alteration may not be related to malignant conversion in the gastric adenoma–carcinoma sequence, which this may follow a different pathway in the stomach from that in the colon.

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