Squamous Cell Carcinoma of the Esophagus Can Be Detected by Microsatellite Analysis in Tumor and Serum[1]

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ABSTRACT

Purpose: **Esophageal squamous cell cancer can be treated effectively by potentially curative surgery if diagnosed at an early stage. Our aim was to develop a novel molecular approach as a noninvasive test for squamous cell cancer detection and as an indicator for the prognosis of the patients.**

Experimental Design: **Matched normal, tumor, and serum samples were obtained from 28 patients with squamous cell carcinoma (SCC) of the esophagus. DNA was extracted, and the samples were subjected to microsatellite analysis using 12 markers. Serum and normal DNA from 10 healthy individuals served as controls.**

Results: **Twenty-six of the 28 patients (92.9%) with SCC were found to have one or more microsatellite DNA alterations in their primary tumor. Twenty-seven of the 28 patients (96.4%) had at least one alteration in the serum by microsatellite analysis. Mean age was 61.5 years. Microsatellite alterations were not identified in the serum DNA of samples from normal control subjects. Median follow-up was 13 months. Survival and recurrence were not significantly correlated with either loss of heterozygosity in the tumor or in the serum.**

Conclusions: **Microsatellite DNA analysis of tumor and serum specimen is a potentially valuable tool for detection and for the evaluation of the prognosis of SCC of the esophagus. The follow-up in our study is still too short to draw final conclusions on the correlation of disease-specific survival and disease recurrence with microsatellite alterations. The evidence of circulating tumor DNA in almost all of our patients underlines a systemic component of the disease that is not surgically amenable.**

INTRODUCTION

SCC[3] and adenocarcinomas comprise the two most common cell types of esophageal cancer (1). Despite recent advances in treatment modalities, esophageal cancer is still associated with a high mortality rate. Although organ-confined esophageal cancer can be cured by radical esophagectomy, carcinoma that extends beyond the muscular layer of the wall and that has already spread to the lymphatic system is associated with a high rate of recurrence of the disease. Most of the patients presenting to the physician are not curable by surgical resection because of advanced disease. The specific clinical signs and symptoms are not usually helpful in making an early diagnosis, but are usually signs of an advanced stage of the disease. Diagnostic examinations and imaging studies are not reliable; widespread screening is usually not possible in our population and only results in incidental discovery of small tumors (2). A noninvasive method for the early detection of esophageal cancer and to advance the indication for additional therapy could represent an important clinical advancement in the management of the patients. Furthermore, the presence of microsatellite alterations in the tumor and/or in the serum could represent a prognostic indicator. Microsatellite analysis of circulating nucleic acids represents an emerging class of molecular tumor markers. Their widespread application and clinical relationship with the malignant phenotype will likely give them an increasing clinical importance in the future (3).

In recent studies microsatellite instability was a favorable prognostic indicator for patients with renal cancer and head and neck cancer (4, 5). This has not been studied in SCC of the esophagus.

The precise mechanisms underlying the pathogenesis of esophageal cancer are still unclear, although both environmental and genetic factors are suspected to play roles. Tobacco and alcohol consumption represent major environmetal risk factors (6).

Microsatellite analysis is a PCR-based technique that permits the detection of cancer-specific DNA alterations, LOH, and microsatellite instability (SHIFT, new allele) in neoplastic tissue (7, 8). We created a panel of 12 microsatellite markers commonly altered in SCC of the esophagus and commonly altered in human malignancies. The panel included 4 markers on chromosome *9p* (*p16*; Refs. 9–13), 3 markers on chromosome *17p* (*p53*; Refs. 12–15), 2 markers on chromosome *18q* (*DPC4-gene*; Refs. 16–18), 1 marker on chromosome *18p* (18), and 2 on chromosomes *5 p* and *q* (*APC* gene; Refs. 19–21).

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[3] The abbreviations used are: SCC, squamous cell carcinoma; LOH, loss of heterozygosity; TNM, Tumor-Node-Metastasis.

The *p16* gene and *p53* play key roles in the cell cycle regulation and in cancer progression (9, 12, 14, 15, 22). In a study by Pearlstein *et al.* (5) LOH at chromosome *18q* predicted poor survival, and the region of *18p*/*18q* showed high frequency of LOH in SCC in a study by Karkera *et al.* (18).

Recent applications of this approach to the evaluation of body fluids and serum have shown that multiple malignant diseases can be detected through the analysis of serum, urine, bronchial lavage fluid, peritoneal fluid, and saliva (19, 23–37). Because many of the above-mentioned tumor entities have similar clinical characters and risk factors as esophageal cancer, we hypothesized that DNA alterations, characteristic of malignant tumors, could be identified in the serum specimen obtained from patients with esophageal cancer as well. Furthermore, we tested whether microsatellite alterations in the primary tumor have an impact on the staging as a potential prognostic marker.

MATERIALS AND METHODS

Matched normal, tumor, and serum samples were obtained from 28 patients with SCC of the esophagus. The tumor samples were taken intraoperatively and were stored as fresh-frozen samples in liquid nitrogen, followed by storage at −80°C. Blood samples were drawn preoperatively before manipulation at the site of the tumor. The serum was stored at −80°C after immediate preparation. Normal DNA was obtained from histologically tumor-free esophageal tissue away from the primary tumor. Before extracting the DNA, H&E stains were done to assure a tumor cell content of ≥70%. To achieve 70% pure tumor for DNA extraction, microdissection was done after H&E stain of the fresh-frozen section whenever necessary. The DNA was extracted from serum (200 μl) and tumor tissue using a QIAamp blood kit and a QIAamp tissue kit, respectively, from Quiagen (Hilden, Germany; Ref. 38). Mean DNA concentration after isolation from the serum was 8.9 μg/ml. Microsatellite analysis was done using 12 microsatellite markers. Microsatellite markers (and their chromosomal location) are as follows: *D17S520* (*17p*), *D17S796* (*17p*), *D17S 804* (*17p*), *D9S162* (*9p*), *D9S171* (*9p*), *D9S1748* (*9p*), *D9S126* (*9p*), *D18S51* (*18q*), *D18S70 (18q*), *ACTBP2* (*5p*), *CSF1R* (*5q*), and *D18S479* (*18p*). Primer sequences and locations were obtained from the Genome Database (The Johns Hopkins University, Baltimore, MD). The primers were ordered as labeled primers with fluorescent dye (fluorescein/carboxyfluorescein, rhodamin/6-carboxytetramethylrhodamine, and HEX) at the 5′ terminus from MWG Biotech (Ebersberg, Germany). PCR was done using paired tumor, normal, and serum DNA. Genomic DNA (2 μl) was subjected to 35 PCR cycles at a denaturing temperature of 95°C for 1 min, followed by various annealing temperatures (52–60°C, depending on the primer sequence) for 1 min, an extension step at 72°C for 1 min, and a final extension step at 72°C for 5 min using a thermocycler (Biometra, Göttingen, Germany). Three amplified markers from each sample were pooled together and analyzed under denaturing conditions using an ABI Prism genetic analyzer 310c (ABI, Foster City, CA). Analysis was done using the 310 gene scan software (3.1.2).

Serum and normal samples (lymphocyte DNA) from 14 healthy persons served as normal controls. LOH was determined by comparison of the intensity of the allelic bands of the normal DNA with the allelic bands of the target samples (tumor and serum). Shift was determined by the appearance of new alleles in the tumor and/or serum.

Table 1 Age, pTNM stage, grading, number of LOH/SHIFT of all patients

No.	Age (years)	pTNM[a]	Grade[a]	LOH (No.) Tumor/Serum[b]
3018	66	T2 N0	3	3/0
3084	67	T3 N1	2	6/6
3190	70	T2 N0	3	0/3
3177	71	T3 N1	3	3/1
3049	61	T2 N0	3	5/6
2902	74	T4 N1 M1	3	4/5
3261	61	T3 N0	2	3/4
2663	74	T2 N1	2	1/4
2448	59	T2 N1 M1	2	6/3
2447	72	T3 N0	2	2/1
2364	46	T3 N0	2	3/2
2451	60	T3 N0	2	3/4
2758	52	T3 N0	3	4/3
2747	71	T1 N1	3	5/2
2445	37	T3 N1	3	4/4
2821	60	T3 N1	2	6/3
3270	52	T3 N1	3	7/3
3263	62	T1 N0	2	0/3
3323	63	T2 N0	2	3/2
3332	73	T3 N1	3	8/5
3373	70	T3 N1	2	1/1
3404	53	T2 N1	2	6/3
3417	53	T2 N1	2	2/2
2268	55	T3 N1	2	3/2
2752	52	T2 N1	3	3/3
2822	60	T2 N1	2	8/5
2638	65	T2 N1	2	3/3
3378	63	T3 N1	2	7/3

[a] pTNM and grade according to the American Joint Committee on Cancer staging.

[b] Numbers under LOH columns indicate positive microsatellite markers in tumor and serum DNA. In total 12 markers were tested by microsatellite analysis in each clinical sample.

A reduction in the intensity of one allele in the target sample of >50% (30% in serum as a result of a greater dilution with normal DNA) was considered to represent LOH or the presence of new shifted alleles (appearance of new bands), as described previously (25). Alterations were confirmed by reamplification.

Clinical data were obtained from the patient charts. The patients were followed for disease recurrence and mortality. Additional evaluations were done using the Fisher's exact test. *P*s are two-sided.

RESULTS

Twenty-eight consecutive patients with SCC of the esophagus were recruited for this study. We tested 12 microsatellite markers from 4 chromosomal regions in paired tumor, normal mucosa, and serum samples ($n = 28$ patients; mean age, 61.5 years; range, 37–74 years; Table 1). Samples from lymphocytes and serum from 10 healthy individuals served as normal controls. Three patients (10.7%) had pT1 tumors, 12 patients (42.9%) had pT2, and 13 patients (46.4%) had pT3 or pT4 tumors, respectively (Table 2). Sixteen patients (57.1%) had

Table 2 Microsatellite analysis of clinical samples by T-stage of the tumor at the time of surgery[a]

Sample (n = 28)	Stage of tumor: All T-Stages (n = 28)	pT1 (n = 3)	pT2 (n = 12)	pT3 & pT4 (1) (n = 13)	P^b
Tumor LOH/SHIFT	26 (92.9)	2 (66.7)	11 (91.7)	13 (100)	0.12 n.s.
Tumor 1–2	5 (17.9)	0 (0)	2 (16.7)	2 (15.4)	0.22 n.s.
Tumor > 2	21 (75)	2 (66.7)	10 (83.4)	11 (84.6)	0.16 n.s.
Serum LOH/SHIFT	27 (96.4)	3 (100)	11 (91.7)	13 (100)	0.50 n.s.
Serum 1–2	8 (28.6)	1 (33.3)	2 (16.7)	5 (38.5)	0.32 n.s.
Serum > 2	20 (71.4)	2 (66.7)	10 (83.4)	8 (61.5)	0.62 n.s.

[a] Number (%) of LOH or SHIFT in the sample.
[b] χ^2-test.

Table 3 Microsatellite analysis of clinical samples by N-stage of the tumor at the time of surgery[a]

Sample (n = 28)	pN-Stage: pN0 (n = 12)	pN1 (n = 16)	P^b
Tumor LOH/SHIFT	10 (83.3)	16 (100)	0.17 n.s
Tumor 1–2	2 (16.7)	3 (18.8)	0.28 n.s
Tumor > 2	8 (66.7)	13 (81.3)	0.17 n.s
Serum LOH/SHIFT	11 (91.7)	16 (100)	0.42 n.s
Serum 1–2	3 (25)	5 (31.4)	0.44 n.s
Serum > 2	8 (66.7)	11 (68.8)	0.45 n.s

[a] Number (%) of LOH or SHIFT in the sample according to N-stage.
[b] Two-sided Fisher's exact test.

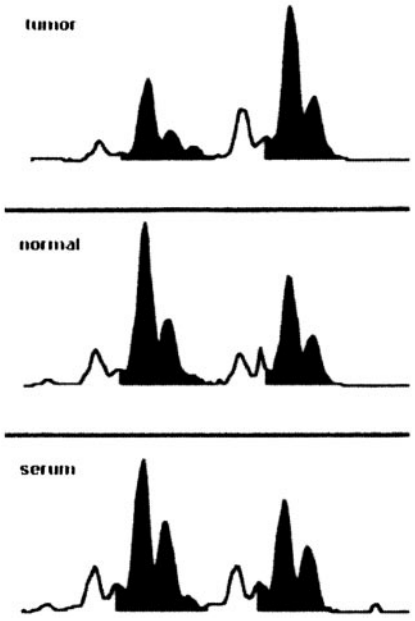

Fig. 1 LOH in the tumor on D17S804 in patient 3822

lymph node involvement (Table 3). Twenty-six (92.9%) of 28 patients with malignant tumors of the esophagus were found to have one or more microsatellite alterations (SHIFT or LOH) in the tumor and 27 (96.4%) in the serum. The microsatellite alterations were not significantly correlated with the T stage of the patients.

The frequencies of LOH (tumor/serum) were as follows: *D17S520* (32%/21%), *D17S796* (32%/32%), *D17S 804* (43%/39%), *D9S162* (32%/32%), *D9S171* (35%/32%), *D9S1748* (11%/14%), *D9S126* (43%/25%), *D18S51* (50%/32%), *D18S70* (32%/18%), *ACTBP2* (32%/18%, one shift), *CSF1R* (32%/11%), and *D18S479* (14%/29%).

Twenty-seven of these 28 patients were found to have at least one alteration in the serum DNA (Table 2; Figs. 1 and 2). No statistical significant correlation of the tumor stage with the serum alterations was found (Tables 1 and 2; Figs. 1–3). There was no tendency toward more microsatellite alterations in the serum in higher T stages (pT1 100%, pT2 91.7%, and pT3/4 100%). The distribution was equally high. The results according to the pT stages are listed in Table 2.

Sixteen patients in our study had lymph node involvement (57.1%). Lymph node involvement was not significantly associated with microsatellite alterations in the serum or the tumor. But there was a tendency toward more microsatellite alterations in the tumor in higher pN stages. The results according to the pN stages are listed in Table 3. Sixteen of 28 patients had G_2 (60.7%), and 10 patients had G3 tumors (39.3%; Table 4). Microsatellite alteration in the tumor and the serum were not associated with tumor grading. The results according to the G stages are listed in Table 4.

Some patients showed alterations in the serum without showing an identical alteration in the tumor. None of the 10 healthy controls displayed alteration in the serum.

There was no significant association between the evidence of either microsatellite instability or LOH in the tumor/serum and the TNM stage. The number of LOH in our panel of markers in every single tumor had also no significant correlation to the tumor staging and grading.

Median follow-up was 13 months. Follow-up was available for 100% of our patients. Patients had recurrent disease totaled 38.5%. Tumor-related mortality was 30.8%. In the patients with recurrent disease, it occurred after a median time of >12 months after surgery. LOH in the tumor and in the serum did not correlate significantly with survival and recurrence of our patients. There was a trend toward shorter survival for patients with LOH in the tumor (Fig. 4) and LOH in the serum (Fig. 5).

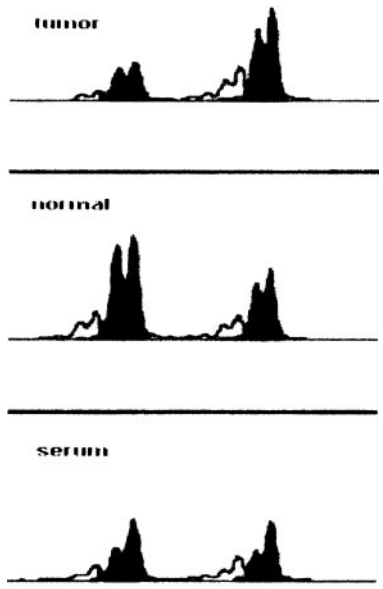

Fig. 2 LOH in tumor and serum on D18S51 in patient 3332.

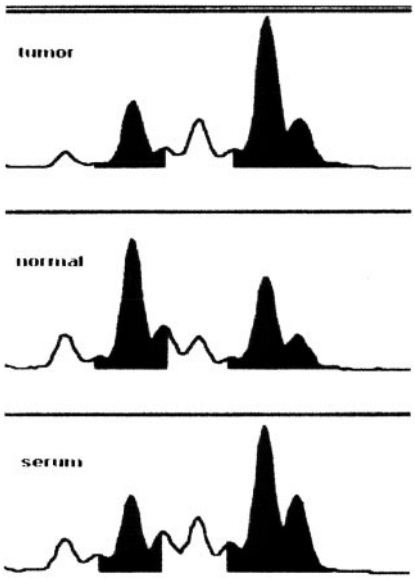

Fig. 3 LOH in tumor and serum on D17S804 in patient 3332.

DISCUSSION

Microsatellite analysis of serum and tumor can detect the presence of a malignant tumor in patients with suspected SCC of the esophagus, whereas control subjects had no serum alterations.

It is evident that DNA circulates freely in the blood of patients with malignant disease, but the source remains unclear (39). The presence of tumor DNA in the blood stream could be because of lysis of circulating cancer cells or micrometastatic cells, because of DNA leakage resulting from tumor necrosis or because of apoptosis (25, 27, 32, 40, 41).

Table 4 Microsatellite analysis of clinical samples by G-stage of the tumor at the time of surgery[a]

Sample (n = 28)	Stage of tumor G2 (n = 17)	G3 (n = 11)	P^b
Tumor LOH/SHIFT	16 (94.1)	10 (90.9)	0.66 n.s
Tumor 1–2	5 (29.4)	2 (18.2)	0.58 n.s
Tumor > 2	11 (64.7)	8 (72.7)	0.68 n.s
Serum LOH/SHIFT	17 (100)	10 (90.9)	0.39 n.s
Serum 1–2	7 (41.1)	2 (18.2)	0.10 n.s
Serum > 2	10 (58.8)	8 (72.7)	0.27 n.s

[a] Number (%) of LOH or SHIFT in the sample according to G-stage.
[b] Two-sided Fisher's exact test.

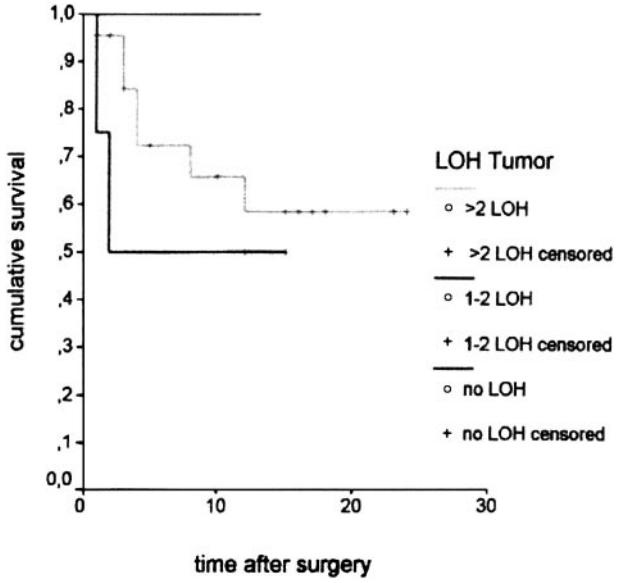

Fig. 4 Cumulative survival according to LOH in the tumor.

Pathological stage of disease predicts the clinical outcome of an individual patient more profoundly than any other currently available marker after surgical treatment for esophageal cancer. Radical esophagectomy remains the primary mode of therapy for patients with carcinoma of the esophagus, although advanced disease with lesions extending beyond the esophagus and lesions with lymph node metastasis are substantially more difficult to cure. Noncurative chemotherapy is the treatment of choice for patients with nonresectable or metastatic disease, although there are some patients with locally advanced disease that profit from surgery with long-time survival. Looking at the stage-specific survival, early detection is essential to increase the disease-specific survival in those patients. Microsatellite analysis in the tumor and serum might be able to identify those patients in the future. Serum microsatellite analysis made cancer consuspicuous in >90% of the patients in our study. It could consequently contribute to an early detection of esophageal cancer for patients at risk. The results of our study do not show any statistical correlation of the findings in microsatellite analysis and the TNM findings, although a tendency toward more

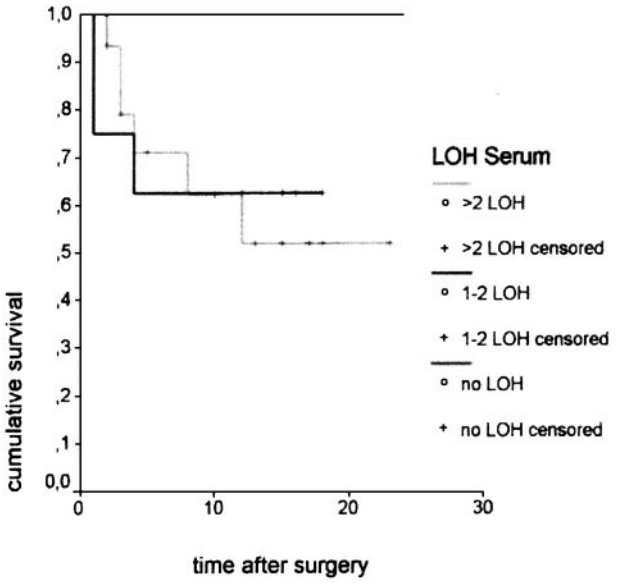

Fig. 5 Cumulative survival according to LOH in the serum.

alterations in the tumor and serum in higher stages was found. Because the number of patients in our study is still limited we must assume at present that LOH and SHIFT are independent from grading and staging.

The central clinical problem facing surgeons and oncologists who care for patients with esophageal carcinoma is that this cancer is unresponsive to systemic adjuvant therapies. Furthermore, looking at the stage-specific survival, the early detection of cancer could be essential for an increase of disease-specific survival in the patients. The serum microsatellite analysis could contribute to early detection. Thus far, because of our limited follow-up, we were not able to finally study the impact of the microsatellite alterations on survival and recurrence of the disease. But in our analysis there was a trend toward longer survival for patients with less microsatellite alterations in the tumor and in the serum (Figs. 4 and 5). This finding reached no statistical significance. However, the follow-up time is still rather short to draw final conclusions on the prognostic significance.

The finding of microsatellite alterations in the serum in 96.4% of the patients in our study reflects the hematogenous mechanism of tumor spread in esophageal cancer or is a sign for high tumor DNA content in the serum, indicating a higher "tumor load." Our results might represent a novel indication for neoadjuvant or adjuvant therapeutic regimens. It is not yet possible to say to what extent patterns of microsatellite alterations are related to the malignant and metastatic potential of individual tumors and the survival of the patients.

Advances in basic research have shed some light on events that putatively contribute to the development of esophageal neoplasia. Alterations at p16 and p53 are the most common tumor suppressor loci in this group of patients (9, 12, 14, 15, 18, 22).

The ease with which microsatellite analysis can be performed on a variety of DNA sources continues to increase. The molecular findings in the serum of the patients are remarkable, particularly because we did not find any alterations in healthy patients. A variety of other studies found similar results in other tumor entities (3, 21, 23, 30, 32, 35–37, 40). Evolving knowledge of the genetic changes that drive the progression of esophageal carcinogenesis will lead to identification of additional diagnostic molecular markers. We have demonstrated the ability to perform microsatellite analysis using a high throughput microcapillary array (ABI Prism) that can markedly enhance the clinical availability of this diagnostic approach.

In our study in some patients we found alterations in the serum, which did not match the alterations found in the tumor of the patients. This finding was reproducible in all of the cases. Artificial alterations because of low DNA content in the specimen need to be excluded, but the measurement in those patients gave a reasonable DNA content for microsatellite analysis in the specimens. More likely those findings are because of the heterogeneity of the tumor. Most importantly, no alterations were found in our healthy controls. This finding correlates with data of the study by Gonzalgo *et al.* (4). With longer follow-up, it might be possible to identify patients at risk for disease progression as demonstrated in the study by Nawroz *et al.* (32) in head and neck cancer. This group of patients might benefit from adjuvant therapeutic regimens and, therefore, this information could be used for decision-making for additional therapeutic options.

In conclusion, we have demonstrated that microsatellite analyses can frequently detect DNA alterations in patients with esophageal cancer. As the alterations in tumor and serum do not necessarily correlate, our approach seems to not be specific enough to use microsatellite analysis as an exclusive detection test for esophageal SCC. The high incidence of these alterations in serum might be an explanation for the poor prognosis in this tumor entity. In addition, microsatellite analysis was not able to define the individual prognosis of patients with esophageal SCC. This finding might be because of our short follow-up and because of our still low number of patients. Additional trials with longer follow-up are necessary to estimate the diagnostic and prognostic potential of this technique for staging of esophageal cancer patients.

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