

DIAGNOSIS OF NASOPHARYNGEAL CARCINOMA BY DNA AMPLIFICATION OF TISSUE OBTAINED BY FINE-NEEDLE ASPIRATION

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Abstract *Background.* In nasopharyngeal carcinoma the primary lesion is often difficult to find. Metastatic lesions occur frequently but are difficult to distinguish from other head and neck tumors. The viral genome of the Epstein-Barr virus (EBV) can be identified in the cells of this carcinoma.

Methods. We used the polymerase chain reaction (PCR) to test for the presence of EBV genomes in 15 samples of metastatic squamous-cell carcinoma of the neck obtained by fine-needle aspiration and in 26 samples obtained by biopsy of lymph nodes. For controls we used disease-free lymph nodes from 10 patients with various head and neck tumors, tonsillar tissue from 46 subjects, blood from 59 EBV-seropositive blood donors, and mononuclear cells from 8 patients with fatal lymphoproliferative lesions.

ALTHOUGH nasopharyngeal carcinoma accounts for only 3 percent of all head and neck tumors in North America, it is the most common tumor in China and is also frequent in Greenland, Alaska, and Mediterranean countries. Largely unidentified environmental factors are believed to play a part in the development of this cancer and explain its curious global distribution.¹

The diagnosis of nasopharyngeal carcinoma is often difficult, because the nasopharynx is hard to visualize and the primary lesions tend to infiltrate submucosally and are easily missed in superficial biopsies. Many tumors are detected late or remain undiagnosed until they present as squamous-cell metastases of the neck, often in the absence of overt pathologic features at the primary site.^{2,3} Patients with squamous-cell metastases and occult primary tumors are subjected to long and costly investigations that often yield insufficient data for critical therapeutic decisions. The preferred treatment for nasopharyngeal carcinoma is irradiation of both the primary site and neck metastases, whereas other types of head and neck cancers are treated surgically.⁴⁻⁶

Epstein-Barr virus (EBV) has been associated with lymphoid and nonlymphoid tumors but rarely with squamous-cell lesions. A relation between nasopharyngeal carcinoma and EBV was postulated on the basis of the finding of antibodies to EBV in serum and the identification of viral genomes by *in situ* hy-

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Results. Of the 41 malignant lesions examined, only the nine nasopharyngeal carcinomas (one primary lesion and eight metastases) contained EBV genomes. None of the 20 nodes with other types of cancer, the 10 disease-free nodes, or any of the 105 normal control samples contained detectable EBV. In two patients with suspected metastases from occult primary tumors, the presence of EBV was predictive of nasopharyngeal carcinoma; in both cases overt nasopharyngeal carcinoma developed within one year.

Conclusions. In patients with suspected nasopharyngeal carcinoma, fine-needle aspiration can provide tissue for diagnosis by DNA amplification of EBV genomes. The presence of EBV in metastases from an occult primary tumor is predictive of the development of overt nasopharyngeal carcinoma. (*N Engl J Med* 1992;326:17-21.)

bridization of epithelial tumor cells.⁷⁻¹⁰ Although the presence of antibodies to EBV in serum, in particular the IgA isotype, is very common in patients with nasopharyngeal carcinoma, such antibodies are also found in the general population, especially in high-risk regions. Hence, serum levels of anti-EBV antibodies are not reliable indicators of occult nasopharyngeal tumors.^{8,11,12}

EBV may be a prerequisite for or play a facultative role in the development of nasopharyngeal carcinoma, but it is unclear whether metastases carry the virus.¹³ We investigated the relation between EBV and nasopharyngeal carcinoma with metastasis to the neck. We used fine-needle aspiration to obtain specimens for histopathological diagnosis because it is less traumatic than classic biopsy and does not have the attendant risk of tumor dissemination.¹⁴ We employed the polymerase chain reaction (PCR) to detect cells positive for EBV, and we evaluated this approach to the diagnosis of nasopharyngeal carcinoma, especially in patients with metastasis to the neck and an occult primary lesion.

METHODS

Patients

Tissue samples were obtained from 41 patients with head and neck tumors at various stages. Samples were obtained by fine-needle aspiration from 15 consecutive patients. Paraffin blocks of lymph-node metastases were available for 16 patients, 6 of whom had histologically confirmed nasopharyngeal carcinoma, and tissue obtained during cervical-lymph-node resection was available for 10 patients. Mononuclear cells from eight patients without nasopharyngeal carcinoma who had fatal lymphoproliferative lesions after lung (two patients) or kidney (six patients) transplantation served as EBV-positive controls.

Preparation of Fresh Specimens

Standard procedures¹⁵ were used to extract DNA from lymphocytes from tonsillar tissue in 46 normal persons and from peripheral-blood mononuclear cells from 59 additional healthy blood donors

who were seropositive for EBV. Fresh lymph nodes were obtained during prophylactic neck dissection in 10 patients with various head and neck tumors and confirmed to be disease-free by histopathological analysis. Samples were lysed for six hours at 56°C in lysis buffer A (10 mmol of TRIS-hydrochloric acid per liter [pH 8.0], 1 mmol of EDTA per liter, 0.1 percent sodium dodecyl sulfate [vol/vol], and 100 µg of proteinase K per milliliter). The samples were then heated at 90°C for 10 minutes and subjected to phenol-chloroform extraction; 0.05 to 0.5 µg of DNA was used for the PCR.

Preparation of Fixed Tissue

A single thin section (3 µm thick) of each paraffin block of lymph-node metastases was prepared for each amplification reaction. Each section was cut with a fresh blade to avoid cross-contamination, deparaffinized with 400 µl of xylene, rehydrated in decreasing concentrations of alcohol, dried, and dissolved in 500 µl of lysis buffer B, consisting of 1× PCR buffer (Perkin-Elmer Cetus, Mississauga, Ont.) (1× PCR consists of 10 mM TRIS-hydrochloric acid per liter [pH 8.3], 50 mM of potassium chloride per liter, and 1.5 mM of magnesium chloride per liter), 0.5 percent Tween 20, 0.5 percent Nonidet-P40, and 100 µg of proteinase K per milliliter (Pharmacia, Montreal). After an overnight incubation at 56°C, the samples were heated at 90°C for 10 minutes and centrifuged for 5 minutes at 15,000×g, and 10 µl of the lysate (2 percent of the total volume) was amplified by the PCR.

Fine-Needle Aspiration

Tissue samples from 15 patients with squamous-cell metastases to a neck node (from known primary tumors in 13 cases and from unknown primary tumors in 2 cases) were obtained by standard techniques with a 21-gauge needle.¹⁴ Two aspirates were used for histopathological diagnosis, and two were pooled into 500 µl of lysis buffer C (1× PCR buffer, 0.45 percent Nonidet-P40, 0.45 percent Tween 20, and 100 µg of proteinase K per milliliter), digested for 4 hours at 56°C, and boiled for 10 minutes; 2 percent of the total volume was used for the PCR. Similar lysates of 2D5 cells—the EBV-transformed human B-cell line developed in our laboratory—(equivalent to approximately 100 cells) or B95-8 cells were used as EBV-genome-positive templates, and LS180 cells, a human colon-cell line, were used as EBV-negative control templates.¹⁵

PCR

Primer pairs were constructed that amplified the nonpolymorphic EBV nuclear antigen 1 (EBNA-1) gene (5'GTAGAACGCCATT-TTCCAC3' and 5'CTCCATCGTCAAAGCTGCA3'), the polymorphic EBNA-2 gene present only in the A strain of EBV (5'CAGGTACATGCCAACACCTT3' and 5'GGTGGTGGTG-GGGGGTGGT3'), and a human β-actin genomic sequence¹⁶⁻¹⁸ (5'ATCATGTTGAGACCTTCAA3' and 5'CATCTCTTGCT-CGAAGTCCA3'). The appropriate primer pairs (0.1 µg), test or control templates, *Taq* polymerase (2.5 U; Pharmacia, Montreal), and deoxynucleoside triphosphate (200 µmol of each per liter; Perkin-Elmer Cetus) were amplified in 100 µl of standard PCR buffer with an Ericomp Programmable Cyclic Reactor (Ericomp, San Diego, Calif.) for 30 cycles, including denaturation for 45 seconds at 94°C, primer annealing for 30 seconds at 5°C below melting temperature, and chain extension for 45 seconds at 72°C. After amplification, 10 µl of the PCR product was electrophoretically separated on 2 percent agarose gels containing predigested lambda size markers (Pharmacia) containing 0.5 µg of ethidium bromide per milliliter. Gels were blotted onto nylon membranes and probed with the following internal reporter probes¹⁵ that had been end-labeled with phosphorus-32: 5'GCTTTATCTGCCGCCATCCT3' for EBNA-2, 5'TAGCCAGGAGAGCTCTAA3' for EBNA-1, and 5'GACCTGGCTGGCCCGGACCTGACTGACT-AC3' for β-actin.

RESULTS

Lysates of B95-8 cells (1 to 10 µl, 10³ to 10⁵ cells per milliliter) were amplified with the EBNA-1 or EBNA-2 primer pair, and the PCR product generated was

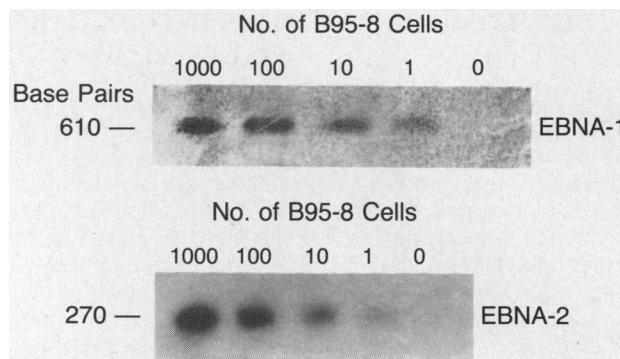


Figure 1. Sensitivity of the EBV Detection System.
Lysates of B95-8 (EBV-antibody-producing) lymphoblasts were amplified by standard PCR with primer pairs specific for EBNA-1 or EBNA-2 virus genes. Aliquots of the amplification product were hybridized with an internal reporter probe; both detect single cells that are positive for the EBV genome.

separated and probed (Fig. 1). PCR-amplification fragments of the correct size hybridized with the reporter probes over a wide range of template concentrations. Lysate from 1 to 10 B95-8 cells consistently generated a detectable hybridization signal. This was deemed adequate for the analysis of specimens obtained by fine-needle aspiration and thin-section material available for this study. No hybridization was observed in reactions that used the β-actin probe, and amplification of lysates of LS180 cells, the negative controls, never generated EBV sequences (data not shown).

The yield of cells from fine-needle aspiration varies markedly from zero—"empty needles"—which occurs in approximately 10 percent of cases, to 5 to 10,000 cells, the yield provided by three-dimensional tissue fragments. It was necessary to include an amplification control for each sample obtained by fine-needle aspiration (i.e., a primer pair that amplifies a human gene sequence, β-actin) to indicate whether there were sufficient numbers of cells for the procedure. Primers for β-actin amplification were used together with EBV primers for coamplification (Fig. 2), or amplification reactions for both genes were performed in separate aliquots of the template, and similar results were obtained.

Of 13 samples obtained by fine-needle aspiration (Fig. 2), 11 had good amplification of β-actin, whereas 2 did not, indicating insufficient amounts of genomic DNA. Of the 11 informative samples (Fig. 2), 1 was obtained from a patient with a diagnosis of primary nasopharyngeal carcinoma. This sample clearly contained EBV genomic sequences. Additional samples were obtained by fine-needle aspiration from two patients with suspected neck metastases from an unknown primary tumor (Fig. 3). Both samples contained EBV sequences. Overt nasopharyngeal carcinoma developed in these patients within one year of the fine-needle aspiration. Amplification of the aspirates obtained from patients with tumors other than nasopharyngeal carcinoma did not generate EBNA-1 or EBNA-2 PCR products.

The above observations suggested that the detection of EBV genomes in a squamous-cell neck metastasis might be diagnostic of nasopharyngeal carcinoma. We therefore analyzed paraffin-embedded samples from 16 patients with squamous-cell neck metastases due to a variety of head and neck tumors; 6 of the patients had been given a histopathological diagnosis of metastatic nasopharyngeal carcinoma. As shown in Figure 4, all 6 metastatic samples from patients with confirmed nasopharyngeal carcinoma contained EBV genomes, whereas none of the other 10 samples did. As was the case for samples obtained by fine-needle aspiration, all these samples contained EBV genomes of the A strain, which were detected with the EBNA-2 as well as the nonpolymorphic EBNA-1 amplification system. Thus, a total of eight samples of metastatic tissue from nasopharyngeal primary tumors and one sample of primary tumor contained detectable EBV genomes; two were tissue samples from patients in whom overt nasopharyngeal carcinoma developed only 7 and 11 months after fine-needle aspiration.

Human B cells are the main natural target of EBV, and latently infected cells derived from B lymphocytes can be expected in an EBV-infected host.¹⁹ Since lymph nodes contain many such cells, we examined three sets of control samples (Table 1): disease-free lymph nodes obtained during prophylactic neck dissection in 10 patients with head and neck tumors, DNA from tonsillar tissue from 46 normal persons, and peripheral-blood mononuclear cells from 59 EBV-seropositive blood donors. None of these tissues contained EBV genomes. Our system was able to detect *in vivo* EBV-infected B cells, since EBV genomes were clearly detected in peripheral-blood DNA from eight organ-transplant recipients with fatal B-cell lymphoma that developed during treatment for rejection with OKT3, prednisone, and cyclosporine (Table 1).

DISCUSSION

The phrase "neck metastasis of unknown primary" refers to a diagnosis of squamous-cell carcinoma in a cervical lymph node in which efforts to find the primary tumor have failed.³ These neck metastases usually originate from primary tumors of the head and neck, mainly the nasopharynx.²⁰ The submucosal and infiltrative characteristics of nasopharyngeal carcinoma, as well as its submucosal location, make this type of cancer difficult to diagnose. In many patients the first evidence of disease is cervical-lymph-node metastases, often with little overt evidence of disease at the primary site.³ Therapeutic decisions for patients with primary tumors at unknown sites are often based on marginal clinical and pathological data and thus may result in suboptimal therapy.^{6,21} We searched for an alternative diagnostic strategy that was based on fine-needle aspiration of cervical nodes, since the squamous origin of these tumors is typically not clearly identifiable and since therapeutic decisions are based mainly on the histopathological diagnosis.

The number of patients undergoing surgery for na-

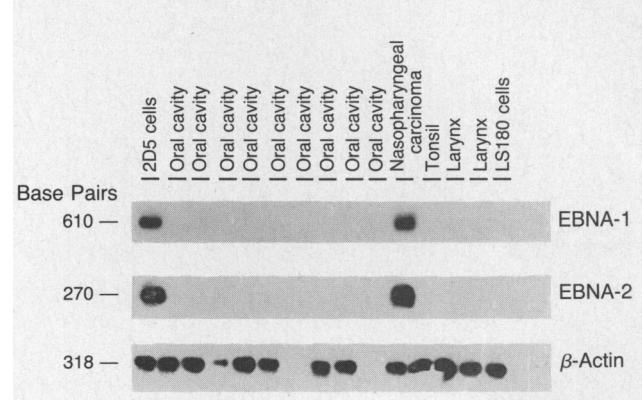


Figure 2. Coamplification of EBNA (Virus) and β -Actin (Human) Gene Regions in 13 Samples of Squamous-Cell Neck Metastases Obtained by Fine-Needle Aspiration, Lysates of the EBV-Transformed 2D5 Cell Line, and the EBV-Negative LS180 Cell Line.

Amplification products were hybridized successively with the EBNA and β -actin reporter probes indicated. Two samples contained insufficient DNA for analysis (i.e., there was no amplification of β -actin); only the sample of nasopharyngeal carcinoma from a primary site contained EBV genomes.

sopharyngeal carcinoma is small, so the number of fresh tissue samples from neck metastases is limited. We therefore investigated archival material stored in paraffin blocks. To approximate the quantities of tissue expected with fine-needle aspiration, we extracted and amplified DNA from a single section that was 3 μ m thick. The results of these studies — EBV genomes were detected in metastatic nasopharyngeal carcinoma but not in neck metastases of other head and neck tumors — are in full agreement with our findings in fresh tissue from neck metastases.

The value of fine-needle aspiration for histopathological diagnoses is well established. It is practical and rapid and has minimal side effects.^{14,22} The ability of fine-needle aspiration to provide sufficient material for

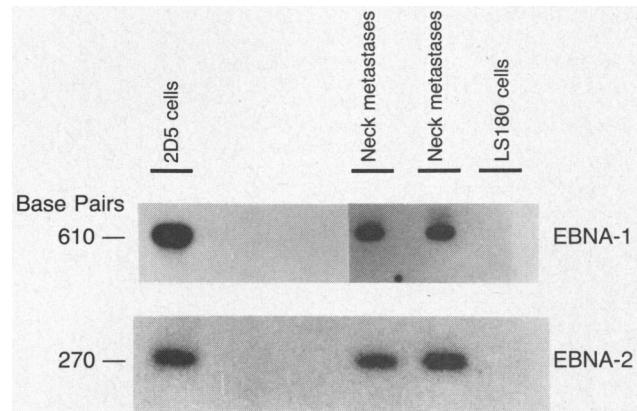


Figure 3. Amplification of the EBV-Gene Fragments in 2D5 Cells and in Samples Obtained by Fine-Needle Aspiration from Two Patients with Squamous-Cell Neck Metastases from Unknown Primary Tumors.

Both samples of metastatic tissue contained EBV genomes. Overt nasopharyngeal carcinoma developed in these two patients 7 and 11 months after fine-needle aspiration. Lysates of LS180 cells were used as an EBV-negative control.

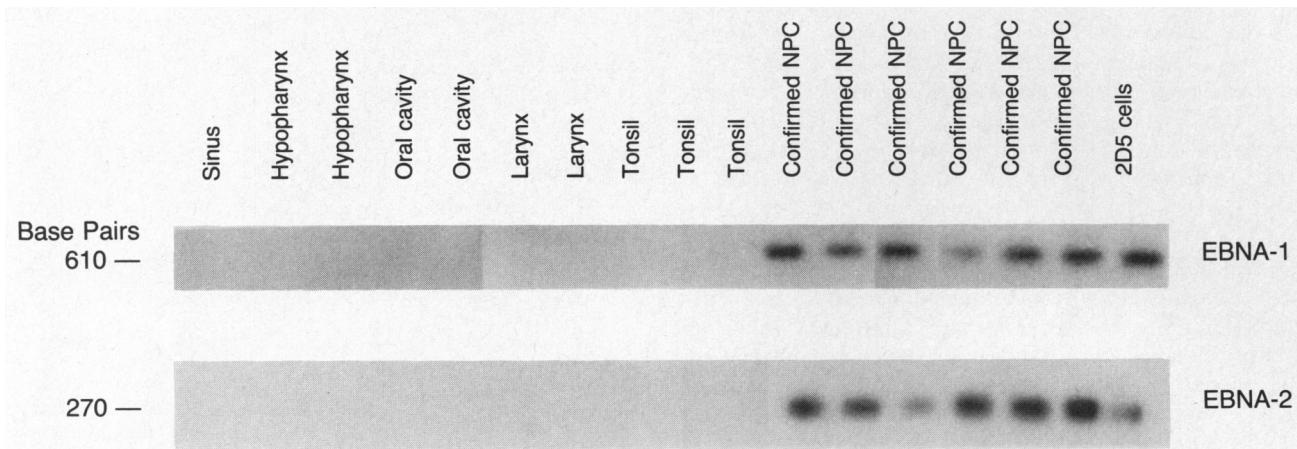


Figure 4. Amplification of EBNA-1 and EBNA-2 Gene Fragments in DNA Extracted from Paraffin-Embedded Specimens Obtained from 16 Patients with Head and Neck Metastases at Various Stages.

Only the six samples of metastatic tissue from patients with confirmed nasopharyngeal carcinoma (NPC) contained EBV genomes. Lysates of the EBV-transformed 2D5 cell line were used as a positive control.

DNA amplification by PCR adds to its usefulness. In our experience, at most 10 percent of lymph-node aspirates yield few or no cells. This figure is lower than that for thyroid and breast tissue, probably because of the highly cellular content of lymph-node tissue and the sparse vascularity. Nevertheless, given the variable cell yields, it was critical to develop an internal PCR control that would indicate the presence of sufficient genomic DNA in the sample. When amplified with a given virus sequence (Fig. 2), this internal standard also controls for the integrity of the reaction and for such variables as uneven temperature control across thermocycler blocks.²³

The system of β -actin amplification that we used

consistently detects between 10 and 100 human cells (data not shown), and the EBNA system of amplification detects 1 to 10 cells. Since each EBV-infected cell may contain several copies of the virus, the sensitivities of the two systems are probably comparable. We have not encountered samples positive for EBV but negative for β -actin.

Our choice of invariant EBNA-1 and polymorphic EBNA-2 sequences for amplification was based on the critical part these genes play in the function of the virus.¹⁵ The EBNA-2 PCR amplifies only the A strain of EBV.¹⁶ It is intriguing that all tumors due to nasopharyngeal carcinoma carried this strain; a larger study is under way to determine whether this observation was due to chance.

Up to 90 percent of adults carry latent EBV. Although the nasopharynx has been suggested as the usual site of primary infection, hematopoietic tissue in bone marrow (perhaps of B-cell lineage) represents a likely viral reservoir.^{24,25} Although B lymphocytes express EBV receptors and are the main natural targets of EBV,^{15,19,26} we found no virus in disease-free samples of cervical lymph node from 10 patients with head and neck tumors. Furthermore, 105 samples of tonsil and blood were free of EBV, even though all 59 blood donors were seropositive for EBV. Similar observations have been reported by others.^{27,28} Infected B cells are transformed by the virus and efficiently eliminated in immunocompetent hosts, mainly by cytotoxic T lymphocytes recognizing virus-coded surface determinants, such as latent membrane protein.^{29,30} The detection of EBV in eight donors with fatal B-cell lymphoma indicates that our detection system can detect wild-type virus in B cells that were transformed *in vivo*.

In the primary lesions of nasopharyngeal carcinoma the epithelial tumor cell itself is infected with EBV.¹³ Our data indicate that the virus is also present in metastatic lesions. The maintenance of episomal

Table 1. EBV Genomes in Head and Neck Tumors.

HISTOLOGIC DIAGNOSIS	SOURCE	NO. OF SAMPLES	NO. ANALYZABLE*	NO. WITH EBV GENOME
Head and neck lesions				
Primary lesion due to nasopharyngeal carcinoma	Fine-needle aspiration	1	1	1
Metastases from nasopharyngeal carcinoma	Fine-needle aspiration	2	2	2
	Paraffin blocks	6	6	6
Metastases from other head and neck tumors	Fine-needle aspiration	12	10	0
	Paraffin blocks	10	10	0
Disease-free neck nodes†	Purified DNA	10	10	0
Total		41	39	9
Control tissue				
Lymphoproliferative lesions‡	Purified DNA	8	8	8
Normal tonsillar lymphocytes		46	46	0
Normal leukocytes§		59	59	0
Total		113	113	8

*Sufficient DNA in the sample to perform the analysis.

†Samples obtained during prophylactic neck dissection in patients with head and neck tumors.

‡Samples obtained from organ-transplant recipients who died of B-cell lymphoma while receiving intensive immunosuppressive therapy.

§Samples obtained from EBV-seropositive blood donors.

viral genomes in these cells suggests that EBV has a critical role in the oncogenic transformation and growth of infected nasopharyngeal epithelium. Since patients with nasopharyngeal carcinoma maintain effective surveillance against transformed cells of B-cell origin that is chiefly directed against the latent membrane proteins,^{29,30} these virus-coded determinants are probably not accessible in the tumor cells. If correct, this assumption suggests that the transformation pathway of nasopharyngeal epithelium may differ from that of cells derived from B lymphocytes, whose successful transformation depends on the expression of latent membrane proteins.³¹

We conclude from our study of nasopharyngeal carcinoma that fine-needle aspiration is practical and provides sufficient material for diagnostic PCR amplification and that the detection of PCR-amplified EBV sequences in squamous-cell neck metastases is a reliable method of diagnosing this type of cancer, including cases in which the primary lesion is occult. The identification of nasopharyngeal carcinoma is important, since it influences the choice of therapy. The clinical problems of diagnosis are considerable, and in countries with a high incidence of this disease they are of major concern. Although we have initiated a large, prospective study, we believe that the available evidence justifies consideration of the use of EBV-genome detection for the diagnosis of metastases of the neck. Fine-needle aspiration is a routine and nontraumatic procedure, and the use of the PCR is now commonplace in basic and clinical investigations.³²

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