Patterns of p21$^{\text{waf1/cip1}}$ expression in non-papillomatous nasal mucosa, endophytic sinonasal papillomas, and associated carcinomas

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Abstract

Aims—To clarify p21$^{\text{waf1/cip1}}$ expression in sinonasal lesions.

Methods—Archived surgical specimens from 38 patients were investigated by means of immunohistochemistry. p21$^{\text{waf1/cip1}}$ staining was evaluated in the different layers of the epithelium. In addition, human papillomavirus (HPV) infection and p53 protein overexpression were assessed and correlated with p21$^{\text{waf1/cip1}}$ expression.

Results—p21$^{\text{waf1/cip1}}$ staining was negative in non-papillomatous nasal mucosa. HPV infection and p53 protein overexpression were not seen. Sixteen of 20 inverted papillomas showed p21$^{\text{waf1/cip1}}$ expression. HPV infection was found in 16 cases and p53 protein overexpression was present in 13 specimens. Expression of p21$^{\text{waf1/cip1}}$ was restricted to surface cells in five cases, but involved basal/parabasal cells in 11 specimens. Immunoreactivity for p21$^{\text{waf1/cip1}}$ in basal/parabasal cells colocalised with p53 protein overexpression. Enhanced expression rates for p21$^{\text{waf1/cip1}}$ were seen in transitional and squamous epithelium compared with columnar epithelium. p21$^{\text{waf1/cip1}}$ expression involved only surface cells in cylindrical cell papillomas. HPV infection and p53 protein overexpression were detected in all specimens. One of five squamous cell carcinomas showed p21$^{\text{waf1/cip1}}$ expression. HPV infection was seen in two cases, and all carcinomas showed p53 protein overexpression.

Conclusions—Expression of p21$^{\text{waf1/cip1}}$ is associated with terminal differentiation in surface cells in inverted papillomas and cylindrical cell papillomas, but not in non-papillomatous nasal mucosa. Overexpression of p53 protein colocalises with p21$^{\text{waf1/cip1}}$ expression in basal/parabasal cells in inverted papillomas but not in cylindrical cell papillomas. Expression of p21$^{\text{waf1/cip1}}$ in squamous cell carcinomas involves a subset of tumours with p53 protein overexpression.

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Keywords: p21$^{\text{waf1/cip1}}$; nasal mucosa; sinonasal papillomas; squamous cell carcinomas

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The waf1/cip1 (wild-type p53 associated fragment/cyclin dependent kinase (cdk) interacting protein) gene is localised on chromosome band 6p21.2 in the human genome.[1 2] It encodes a 21 kDa protein, p21$^{\text{waf1/cip1}}$, which in normal cells exists in quarternary complexes with cyclins, cdks, and proliferating cell nuclear antigen.[3–5] Complexing of p21$^{\text{waf1/cip1}}$ inhibits the activity of cdks and thereby controls the G_1 to S phase transition of the cell cycle.[3–5] The induction of p21$^{\text{waf1/cip1}}$ is associated with terminal differentiation, senescence, and apoptosis in several tissues.[2 3 6] Furthermore, p21$^{\text{waf1/cip1}}$ acts as a downstream mediator of wild-type p53 protein, suppressing DNA replication under genomic stress.[1–4 7] Although p21$^{\text{waf1/cip1}}$ functions as a putative tumour suppressor, mutations of the waf1/cip1 gene have been detected only rarely in human malignancies.[8 9]

Recently, p21$^{\text{waf1/cip1}}$ expression has been demonstrated in several head and neck malignancies—for example, oral squamous cell carcinomas and their precursors.[10 11] However, p21$^{\text{waf1/cip1}}$ expression has not yet been investigated in the nasal mucosa and in sinonasal lesions. Endophytic sinonasal papillomas, comprising inverted papillomas and cylindrical cell papillomas according to the World Health Organisation classification, show malignant progression in up to 25% of cases.[12–14] Squamous cell carcinomas associated with endophytic sinonasal papillomas develop either synchronous or metachronous to the original papilloma.[13 14] However, sinonasal squamous cell carcinomas developing independently from endophytic sinonasal papillomas are a rare type of tumour.[13–15]

We aimed to clarify the patterns of p21$^{\text{waf1/cip1}}$ expression in nasal mucosa, endophytic sinonasal papillomas, and their associated squamous cell carcinomas.

Methods

TISSUE SELECTION AND STANDARD HISTOLOGY

A highly selective cohort of 38 patients (30 men, eight women; age range, 17–76 years; median, 49) was investigated. Archived surgical specimens were retrieved from the files of the department of pathology, Military Hospital Ulm, Ulm/Donau, Germany. Material with exact clinical documentation of the origin and extension of the lesions was used exclusively. Furthermore, only specimens with an adequate amount of lesional material were investigated. Cases showing insufficient fixation, necrosis, haemorrhage, and/or atrophy were excluded. Histological examination for adequacy of lesional tissue and classification of specimens was carried out by two independent observers using haematoxylin and eosin stained slides. Differences between their individual reports were resolved by re-examination and consensus. Non-papillomatous nasal mucosa was

Table 1 Expression of p21$^{waf1/cip1}$ in 20 inverted papillomas

	Basal/parabasal cells		Median cell layer		Surface cells	
	p21 negative	p21 positive	p21 negative	p21 positive	p21 negative	p21 positive
Columnar epithelium						
p53 negative	5	0	5	0	1	4
p53 positive	3	3	6	0	0	6
Transitional epithelium						
p53 negative	7	0	7	0	7	0
p53 positive	3	9	11	1	5	7
Squamous epithelium						
p53 negative	5	0	5	0	5	0
p53 positive	2	9	11	0	7	4

Columnar epithelium was present in 11 cases, transitional epithelium in 20 cases, and squamous differentiation in 16 specimens.

studied in seven cases with either normal nasal mucosa or minimal chronic inflammation. Specimens with more than minimal chronic inflammation, active inflammation, dysplasia, or carcinoma were excluded. Twenty six endophytic sinonasal papillomas were studied, comprising 20 inverted papillomas and six cylindrical cell papillomas. Specimens with dysplasia or associated malignancies were excluded from this group. Five squamous cell carcinomas associated with endophytic sinonasal papillomas were investigated.
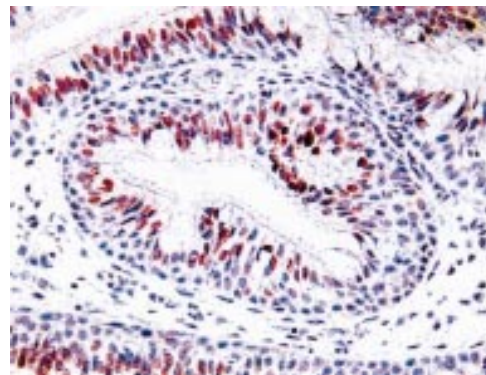

Figure 1 Columnar epithelium in the invaginating portion of an inverted papilloma from a 45 year old man. Positive staining for p21$^{waf1/cip1}$ is restricted to surface cells. Negative staining for p21$^{waf1/cip1}$ in basal/parabasal cells, although human papillomavirus infection and p53 protein overexpression were present in this specimen.
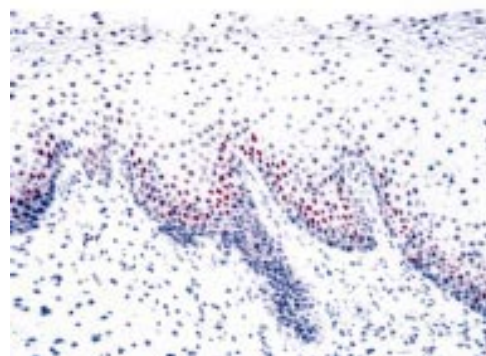

Figure 2 Squamous epithelium in an inverted papilloma (same specimen as shown in fig 1). Note abundant immunoreactivity for p21$^{waf1/cip1}$ in basal/parabasal cells. In this case, positive staining in surface cells only (fig 1) together with positive staining in basal/parabasal cells only (fig 4) were found in different regions within the same specimen.

IMMUNOHISTOCHEMISTRY

Additional 5 μm paraffin wax embedded sections were prepared and immunohistochemistry was carried out using the streptavidin–biotin–peroxidase technique, as reported previously.[16] For all antibodies, a microwave based antigen retrieval protocol was used for a total of 30 minutes in citrate buffer (pH 6.0).[17] The NCL-Waf 1 antibody (Novocastra, Newcastle upon Tyne, UK) was used for p21$^{waf1/cip1}$ staining, at a dilution of 1/20. Gastric body mucosa samples provided positive controls. Distinct nuclear staining was found in foveolar cells on the gastric tips, in addition to terminally differentiated cells in the glands. Furthermore, reproducible strong staining in vascular smooth muscle cells was seen in all specimens, which served as an internal positive
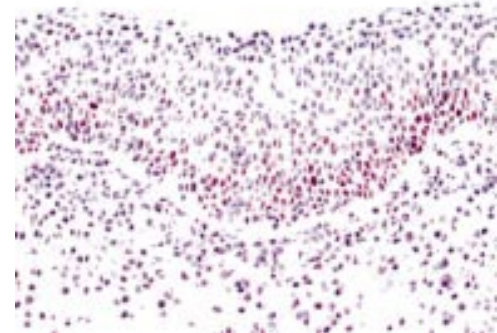

Figure 3 Transitional epithelium in an inverted papilloma from a 25 year old man. Abundant immunoreactivity for p53 protein is present in the lower half of the epithelium. Human papillomavirus infection was demonstrated in this specimen.
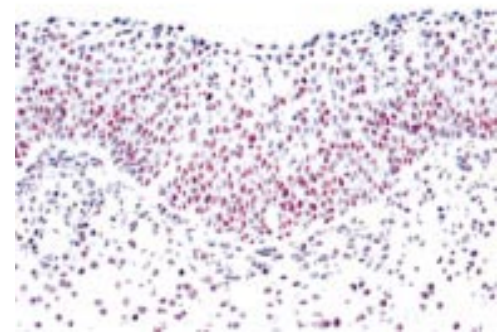

Figure 4 Immunoreactivity for p21$^{waf1/cip1}$ in a serial slide from the same lesion shown in fig 3. Note colocalisation of positive staining results for p53 protein and p21$^{waf1/cip1}$ between figs 2 and 3.

Table 2   Expression of p21^{waf1/cip1} in cylindrical cell papillomas (n = 6)

	Basal/parabasal cells		Median cell layer		Surface cells	
	p21 negative	p21 positive	p21 negative	p21 positive	p21 negative	p21 positive
p53 negative	0	0	0	0	0	0
p53 positive	6	0	4	2	0	6

control. The presence of human papillomavirus (HPV) infection was investigated using the NCL-PVp antibody (Novocastra), at a 1/100 dilution. This antibody detects all HPV types in paraffin wax embedded tissues.[18] Positive controls were provided by a carcinoma in situ from the ectocervix. Strong nuclear and perinuclear staining was seen in most of the tumour cells. Furthermore, the expression of the p53 protein was studied using the antibody DO-7 (Dako, Hamburg, Germany), at a 1/50 dilution. Positive controls were carried out using a moderately differentiated gastric adenocarcinoma previously shown to be p53 positive. Intensive nuclear staining was found in more than 90% of cells on the invasion front. For all antibodies, negative controls were provided using normal horse serum with the same dilution.

EVALUATION OF STAINING RESULTS
Assessment of immunohistochemistry was done independent from classification of specimens. The following semiquantitative p21^{waf1/cip1} expression scores were used: negative staining, less than 5% positive nuclei, 5–15% positive nuclei, 16–25% positive nuclei, and more than 25% positive nuclei. Basal/parabasal cells, medial cell layers, and surface cells of the epithelium were evaluated. In non-papillomatous nasal mucosa and cylindrical cell papillomas columnar epithelium were assessed. In inverted papillomas, columnar, transitional, and squamous epithelium were evaluated. In squamous cell carcinomas, representative regions on the invasion front of the tumour were investigated. Staining results were compared between these groups. In addition, HPV infection and p53 protein overexpression were studied and correlated with p21^{waf1/cip1} expression. Physiological expression of the p53 protein was defined as less than 15% immunoreactive basal/parabasal cells. Overexpression of the p53 protein was defined as positive staining of more than 15% basal/parabasal cells, and/or immunoreactivity more superficial than the parabasal cell layer. Furthermore, overexpression of the p53 protein was subgraded in cases showing more or less than 40% immunoreactive nuclei.[19 20]

Results

NON-PAPILLOMATOUS NASAL MUCOSA
All specimens were negative for p21^{waf1/cip1} staining. HPV infection and p53 protein overexpression were not found.

EXPRESSION OF p21^{WAF1/CIP1} IN INVERTED PAPILLOMAS
p21^{waf1/cip1} was expressed in 16 of 20 cases (table 1). Immunoreactivity for p21^{waf1/cip1} was restricted to surface cells in five specimens (fig 1), but involved basal/parabasal cells in 11 specimens. Notably, in all cases immunoreactivity in basal/parabasal cells was separated from positive surface cells by negative medial cell layers of the epithelium. In some cases, positive staining in surface cells only along with positive staining in basal/parabasal cells only was detected in different regions within the same tissue section (fig 2). Frequently, the proportions of p21^{waf1/cip1} positive basal/parabasal cells were enhanced in transitional and squamous epithelium compared with columnar epithelium. Positive staining for p21^{waf1/cip1} in basal/parabasal cells colocalised with p53 protein overexpression (figs 3 and 4). The expression of p53 protein predominantly involved basal/parabasal cells of the epithelium. In all inverted papillomas, less than 40% of the cells were immunoreactive for p53 protein.
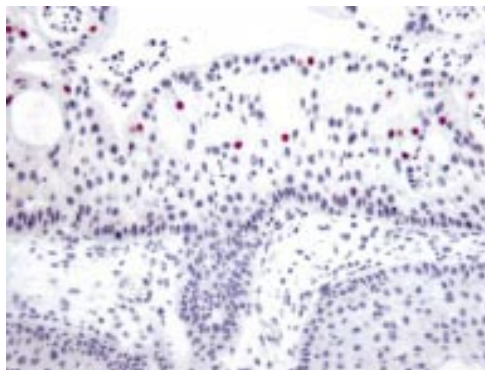

Figure 5   Cylindrical cell papilloma from a 54 year old man. Positive staining for p21^{waf1/cip1} is seen only in suprabasal and surface cells of the epithelium.
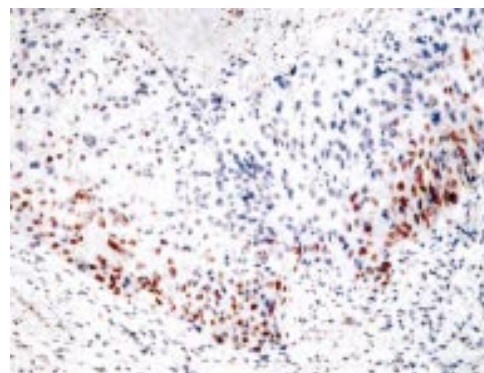

Figure 6   Squamous cell carcinoma associated with an inverted papilloma from a 61 year old man. Note immunoreactivity for p21^{waf1/cip1} on the invasion front of the tumour. Necrotic areas of the tumour were negative for p21^{waf1/cip1} expression (upper part of the picture). Human papillomavirus infection was not detected in this specimen. Abundant overexpression of the p53 protein was seen in this tumour.

EXPRESSION OF p21^WAF1/CIP1 IN CYLINDRICAL CELL PAPILLOMAS

All specimens showed p21^waf1/cip1 expression only in surface cells (table 2; fig 5). HPV infection and p53 protein overexpression were present in all cases.

EXPRESSION OF p21^WAF1/CIP1 IN SQUAMOUS CELL CARCINOMAS

One of five squamous cell carcinomas showed p21^waf1/cip1 expression (fig 6). HPV infection was found in two cases and p53 protein overexpression was detected in all tumours.

Discussion

p21^waf1/cip1 was not expressed in non-papillomatous nasal mucosa. Currently, it is thought that tissue specific p21^waf1/cip1 expression occurs in terminally differentiated epithelial cells.[6] [21] [22] For instance, normal breast epithelium shows p21^waf1/cip1 expression in less than 1% of cells.[23] In contrast, normal colonic epithelium shows strong p21^waf1/cip1 expression in the upper third of the crypts.[24]

We found p21^waf1/cip1 expression in 16 of 20 inverted papillomas. Positive staining was seen in surface cells and basal/parabasal cells of the epithelium. Immunoreactivity for p21^waf1/cip1 in surface cells was independent of p53 protein overexpression. Hence, p53 independent activation of p21^waf1/cip1 expression along with terminal differentiation of surface cells in inverted papillomas can be postulated. A similar concept has recently been reported from oral squamous epithelium, in which p53 independent p21^waf1/cip1 expression involves the spinous cell layer undergoing terminal differentiation.[10] [11] [25]

The expression of p21^waf1/cip1 in basal/parabasal cells in inverted papillomas colocalised with p53 protein overexpression. At least two wild-type p53 protein responsive elements exist in the promoter region of the waf1/cip1 gene.[26] Expression of p21^waf1/cip1 mediates the antiproliferative effect of p53 protein by the suppression of DNA replication and G_1 cell cycle arrest.[3] [4] [7] [26–28] Hence, it can be hypothesised that wild-type p53 dependent p21^waf1/cip1 expression occurs in basal/parabasal cells in inverted papillomas. However, the antibody DO-7 used in our study recognises both wild-type and mutant p53 protein.[29] The expression of p53 protein in less than 40% of cells was consistently found in our inverted papillomas. Recent studies have associated p53 protein expression in less than 40% of nuclei with the accumulation of wild-type rather than mutant p53 protein.[30–32] Furthermore, a strong association between wild-type p53 protein and non-dysplastic inverted papillomas, in contrast to mutant p53 protein expression found in dysplastic specimens, has recently been reported.[33] Dysplastic inverted papillomas were excluded from investigation in this study. Hence, it is probable that wild-type p53 protein was expressed in our exclusively non-dysplastic inverted papillomas, although further investigations involving molecular analysis of the p53 gene status are necessary to confirm these observations.

A causative role for HPV infection in the wild-type p53 protein overexpression seen in inverted papillomas is well recognised: complexing of the HPV oncoprotein E6 to wild-type p53 protein is followed by stabilisation and overexpression of the p53 protein in benign inverted papillomas infected with HPV types 6/11.[34] [35] In contrast, degradation of these complexes followed by negative immunostaining for p53 protein is present in malignant sinonasal lesions infected with HPV types 16/18.[35] [36] In agreement with previous reports, we found HPV infection in inverted papillomas but not non-papillomatous nasal mucosal specimens.[35] [36] However, the individual HPV types were not subclassified in our study: the antibody NCL-PVp detects all human HPV types.[18] Molecular techniques, such as the polymerase chain reaction, are required for reliable HPV subclassification.[35] [37] However, these techniques require fresh tissue,[35] [37] which was not available from the cases used in our study. Hence, further investigation will be necessary to clarify the relation between distinct HPV types and p21^waf1/cip1 expression in inverted papillomas.

More p21^waf1/cip1 positive nuclei were found in transitional and squamous epithelium than in columnar epithelium in inverted papillomas. A consecutive series of histological changes has been demonstrated in inverted papillomas: columnar epithelium is gradually transformed to transitional epithelium and finally squamous epithelium.[14] [38] Our findings indicate increasing p21^waf1/cip1 expression along this metaplastic process. We have recently identified increasing expression rates of p53 protein along this pathway, when investigating another cohort of inverted papillomas (manuscript in press). It is thought that p21^waf1/cip1 plays a fundamental role in the suppression of papillomatous and metaplastic progression in epithelial tissues (such as the epidermis).[39] [40] Hence, p21^waf1/cip1 may be involved in the regulation of metaplasia in inverted papillomas. Current studies suggest that p21^waf1/cip1 has an inhibitory effect on viral DNA replication in HPV infected tissues, such as oral or genital papillomas.[41] Further investigation is required to specify the detailed function of p21^waf1/cip1 in inverted papillomas.

In cylindrical cell papillomas, p21^waf1/cip1 expression was found only in surface cells of the epithelium. Thus, p21^waf1/cip1 expression probably occurs along with terminal differentiation. HPV infection and p53 protein overexpression were present in all specimens. However, p21^waf1/cip1 did not colocalise with p53 protein expression in basal/parabasal cells in cylindrical cell papillomas. Hence, activation of the waf1/cip1 gene is probably p53 independent in this group of endophytic sinonasal papillomas. The different behaviour of inverted papillomas and cylindrical cell papillomas in p21^waf1/cip1 expression cannot be explained from our data. A few previous studies have investigated cell cycle control in cylindrical cell papillomas, but none of those reports identified significant differences between inverted papillomas and cylindrical cell papillomas.[37] [38] Further investigations are needed to determine

the basic mechanisms of cell cycle control in cylindrical cell papillomas.

In squamous cell carcinomas associated with endophytic sinonasal papillomas, $p21^{waf1/cip1}$ expression was seen in one of five specimens, although p53 protein overexpression was found in all cases. Several concepts for heterogeneity in $p21^{waf1/cip1}$ expression between individual tumours have recently been presented.[42–45] For instance, mutant p53 protein is often unable to activate the transcription of the waf1/cip1 gene in breast and gastric carcinomas with p53 gene mutations.[24 41 42] Low $p21^{waf1/cip1}$ expression is associated with poor prognosis in breast carcinomas.[41 42] Sinonasal carcinomas almost exclusively express mutant p53 protein, whereas wild-type p53 protein predominates in benign sinonasal lesions.[33] Hence, the absence of $p21^{waf1/cip1}$ expression in sinonasal carcinomas probably results from mutant p53 protein expression. However, in other neoplasms such as malignant melanomas or pancreatic carcinomas $p21^{waf1/cip1}$ expression does not correlate with p53 functional status.[44 45] Transcription of the waf1/cip1 gene independent of p53 controlled pathways and post-transcriptional regulation of $p21^{waf1/cip1}$ accumulation have been demonstrated in these lesions.[44–47] Furthermore, recent studies have reported that cyclin D1 overexpression can regulate $p21^{waf1/cip1}$ expression in several human malignancies, including head and neck squamous cell carcinomas.[48] Hence, the exact regulatory mechanisms for $p21^{waf1/cip1}$ expression in sinonasal squamous cell carcinomas cannot be determined from our data. Additional investigations involving p53 gene and protein alterations, waf1/cip1 gene transcription, post-transcriptional $p21^{waf1/cip1}$ modification, and cyclin D1 overexpression are necessary to shed light on $p21^{waf1/cip1}$ expression and its prognostic value in sinonasal carcinomas.

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A demon in the bathroom

Bathroom demons, an unusual theme for modern man, but in ancient Mesopotamia a daily hazard. Although certain aspects of ancient Mesopotamia, like the law code laid down by Hammurabi, have become ingrained in modern civilisation, others such as the colourful demonology have almost completely vanished in the limbos of time. Only certain members of the demonic family have in a way managed to survive. For instance, Incubus and its female counterpart Succubus have survived into modern psychiatry as the Incubus syndrome, in which there is the delusion of being sexually approached at night by an unseen lover.[1]

Other demons manifested themselves as diseases, the symptoms of which have in some cases been recorded in astonishing detail. Epilepsy, or disorders involving the patient turning "his neck to the left [. . .], while his hands and feet are stretched, his eyes are wide open, [. . .] saliva flows in/from his mouth, he makes . . . sounds; he does not know himself; [. . .] it overwhelms him time and again",[2] could be caused by many different demons or deities, such as Antašubba, Bennu, or Lugal Urra. Another was Šulak, one of many names of the "Lurker" (Rābiṣu), a demon which "lies in wait for its victims in lonely places".[2] In contrast to the other epilepsy or epilepsy-like inflicting demons, Šulak was an entity with a highly restricted territory, namely, the bathroom.

Demons lurking in bathrooms were also notorious among Jews, Arabs, and Europeans until the Middle Ages.[2] In modern times, drowning in the bathtub of patients suffering from an epileptic insult is well known and described as a serious threat.[3] Aside from epilepsy, cerebral haemorrhages ("if the right side of his body is in its entirety let down: stroke (inflicted by) a Lurker . . ."[2]) may also frequently occur in the lavatory or bathroom, as recently pointed out in a Japanese study.[4]

Although ancient and modern aetiology may differ, it is interesting to see that the possible dangers surrounding a patient prone to epilepsy or strokes, while taking a bath, were already noticed over 2000 years ago.

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