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Differential gene expression in patients with anal fistula reveals high levels of prolactin receptor

Diferencijacija genske ekspresije kod bolesnika sa analnom fistulom ukazuje na visoki nivo prolaktinskih receptora

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Abstract

Background/Aim. There are limited data examining variations in the local expression of inflammatory mediators in anal fistulas where it is anticipated that an improved understanding of the inflammatory milieu might lead to the potential therapeutic option of instillation therapy in complicated cases. The aim of the present study was to examine prolactin receptors (PRLR) as inflammatory markers and to correlate their expression with both the complexity of anal fistulas and the likelihood of fistula recurrence. **Methods.** Microarray was used to screen the differentially expressed gene profile of anal fistula using anal mucosa samples with hemorrhoids with age- and sex-matched patients as controls and then a prospective analysis of 65 patients was conducted with anal fistulas. PRLR immunohistochemistry was performed to define expression in simple, complex and recurrent anal fistula cases. The quantitative image comparison was performed combining staining intensity with cellular distribution in order to create high and low score PRLR immunohistochemical groupings. **Results.** A differential expression profile of 190 genes was found. PRLR expression was 2.91 times lower in anal fistula compared with control. Sixty-five patients were assessed (35 simple, 30 complex cases). Simple fistulas showed significantly higher PRLR expression than complex cases with recurrent fistulae showing overall lower PRLR expression than *de novo* cases ($p = 0.001$). These findings were reflected in measurable integrated optical density for complex and recurrent cases (complex cases, $8.31 \pm 4.91 \times 10^4$ *vs* simple cases, $12.30 \pm 6.91 \times 10^4$; $p < 0.01$; recurrent cases, $7.21 \pm 3.51 \times 10^4$ *vs* primarily healing cases, $8.31 \pm 4.91 \times 10^4$; $p < 0.05$). In univariate regression analysis, low PRLR expression correlated with fistula complexity; a significant independent effect maintained in multivariate analysis odds ratio [(OR) low to high PRLR expression = 9.52; $p = 0.001$)]. **Conclusion.** PRLR expression inversely correlates with anal fistula complexity. Further work must define the specificity of this finding and its relationship to other conventional mediators of inflammation.

Key words:
rectal fistula; receptors, prolactin; gene expression; immunohistochemistry; biological markers.

Apstrakt

Uvod/Cilj. Ispitivanja varijacija u lokalnoj ekspresiji medijatora inflamacije kod analnih fistula su ograničena, a očekuje se da bolje razumevanje ove oblasti može da doprinese razvoju potencijalnih terapijskih opcija poput uvođenja instilacione terapije kod komplikovanih slučajeva. Cilj ove studije bio je da se ispitaju prolaktinski receptori (PRLR) kao pokazatelji inflamacije i utvrdi korelacija njihove ekspresije sa kompleksnošću analnih fistula i verovatnoćom pojave recidiva. **Metode**. Pomoću metode *microarray* najpre je izvršena diferencijacija gena u uzorcima analne mukoze bolesnika sa hemoroidima (kontrolna grupa), a potom prospektivno i kod bolesnika sa analnim fistulama (n = 65). Obe grupe bolesnika bile su komparabilne u pogledu starosti i pola. Imunohistohemijska analiza PRLR učinjena je sa ciljem definisanja njihove genske ekspresije kod nekomplikovanih, kompleksnih i recidivantnih analnih fistula. Kvantitativno poređenje bojenih preparata postignuto je kombinovanjem intenziteta bojenja sa ćelijskom distribucijom čime je dobijen nizak i visok skor PRLR. **Rezultati**. Diferencijalni profil ekspresije nađen je za 190 gena . Ekspresija PRLR bila je 2,91 puta manja kod analnih fistula u poređenju sa uzorcima kontrolne grupe. U grupi sa analnim fistulama, od 65 bolesnika bilo je 35 sa nekomplikovanim i 30 sa kompleksnim fistulama. Kod nekomplikovanih fistula ekspresija PRLR bila je značajno viša nego kod kompleksnih slučajeva sa recidivantnim fistulama, a ukupno niža u odnosu na *de novo* fistule ($p = 0,001$). Ovi nalazi reflektovali su se u izmerenim vrednostima integrisane optičke gustine za kompleksne i rekurentne fistule (kompleksne fistule, $8,31 \pm 4,91 \times$

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10^4 *vs* nekomplikovane fistule, 12,30 ± 6,91 × 10^4 ; $p < 0,05$); recidivantne fistule, 7,21 ± 3,51 × 10^4 *vs* primarno izlečene fistule, 8,31 ± 4,91 × 10^4; $p < 0,05$). Univarijatna regresiona analiza pokazala je da je niska ekspresija PRLR bila u korelaciji sa kompleksnošću fistule, a ovo je se pokazalo i kao značajan nezavisan faktor u multivarijantnoj [odds ratio (OR) za nisku prema visokoj ekspresiji PRLR = 9,52; $p = 0,001$]. **Zaključak**. Ekspresija PRLR stoji u inverznoj korelaciji sa kompleksnošću analnih fistula. Dalja istraživanja trebalo bi da definišu specifičnost ovog nalaza i njegovu povezanost sa drugim konvencionalnim medijatorima inflamacije.

Ključne reči:
rektum, fistula; receptori, prolaktinski; geni, ekspresija; imunohistohemija; biološki pokazatelji.

Introduction

The successful management of complex anal fistulas continues to represent a significant surgical challenge [1]. Low and simple fistulas can be safely treated by fistulotomy [2] but complex cases may be managed with a wide variety of alternatives, each balancing long-term cure with reported postoperative continence disturbance [3–5]. Whereas it is believed that most fistulas develop from a cryptoglandular source, it is unclear why some cases become complicated or recurrent [6, 7] with most of these more specialized cases generally being separated in reported analyses from those with an underlying cause such as perianal Crohn's disease, ulcerative colitis, HIV-related sepsis or anorectal malignancy. It is speculated that the development of recalcitrant, recurrent cryptoglandular anal fistulas may be consequent upon a disturbed local immune microenviroment. Concerning this point, there are limited data examining variations in the local expression of inflammatory mediators in anal fistulas where it is anticipated that an improved understanding of the inflammatory milieu might lead to the potential therapeutic option of instillation therapy in complicated cases [8].

Prolactin (PRL) is one of a group of important stress hormones which has been shown to play an important role in local immune regulation where it has a specific immunostimulatory effect [9, 10]. The complex mechanisms by which PRL stimulates the proliferation of immune cells include an enhancement of mitogenic responses, the preservation of B and T lymphocyte function through antagonism of glucocorticoid-induced apoptosis [11, 12], the activation of macrophage phagocytosis and the induction of humoral IgM and IgG responsiveness and cell-mediated immunity [13]. Local regulation of PRL is mediated by specific PRL receptors (PRLR) which are expressed in many cells including those of the mammary gland, prostate, skin, decidua, brain and rectal mucosa [14]. In this regard, PRL and PRLR expression is likely to be upregulated in anal fistula, however, it is unknown if this is the case or whether ligand and receptor expression correlates with fistula chronicity. In our preliminary work, we found 190 genes of significant difference between the anal mucosa derived from patients with anal fistulas when compared with age- and sex-matched non-fistula controls. Among them, we noted differential PRLR expression (FC = 2.91, $p < 0.01$), suggesting a role for this molecular marker in the prediction of fistula complexity.

The aim of this study was to examine immunohistochemical PRLR expression within nearby anal canal mucosa of anal fistula and to correlate this tissue expression with clinical fistula type.

Methods

Patients

The study protocol was approved by the local hospital ethics committee and all patients signed an informed consent for study participation. Patients in this prospective analysis were obtained through the Department of Colorectal Surgery at the Third People's Hospital of Hangzhou China between June 2010 and September 2013. For the purposes of classification for this particular study, simple anal fistulas were defined as those with a single external and internal opening and a single straightforward fistula track. Complex fistulas were defined by two or more external or internal openings, two or more fistula tracks and those accompanied by secondary branching tracks [15]. Patient data were obtained from electronic medical records with a postoperative review at 6 weeks and subsequently at 3 monthly intervals until healing became evident or until the operation was deemed to be unsuccessful. Success was defined as closure of both the internal and external openings without persistent discharge. Time to failure was defined as the time from the fistula operation until clinical examination which confirmed fistula persistence or recurrence requiring further surgical intervention. A recurrent fistula was defined as one which had undergone at least one previous definitive operation. All patients underwent 3D endoanal sonography and appropriate gadolinium-enhanced magnetic resonance imaging. Patients with non-cryptogenic anal fistula (perianal Crohn's disease, ulcerative colitis and gastrointestinal tuberculosis) were excluded from analysis. All operations were performed in the prone jack knife position under spinal anaesthesia. Bowel preparation was used in all cases one day prior to scheduled surgery with all procedures performed by a consultant colorectal surgeon.

Gene expression microarray

Total RNA was isolated and purified from anal mucosa samples of three sex- and age-matched anal fistulas and hemorrhoids patients respectively, using QiagenRNeasy Mini Kit, QIAshredder kit and RNase-Free DNase Set kit (Qiagen, Valencia, CA) following manufacturer's recommendations. A total of 100 ng of each RNA was used to perform reverse transcription and one-color labeling steps. Amplified and labeled samples were purified using the RNeasy Mini Kit from Qiagen. Gene expression profiling was done with human whole-genome Agilent 28004, 8 × 60 K microarray chips following standard operating procedures from Agilent Tec-

hnologies. The labeled RNA was hybridized at 65°C for 17 hours at 10 rpm. Raw data files from Feature Extraction were imported into R with LIMMA, an R package from the Bioconductor project, and processed as follows: gMedianSignal data were imported, control probes were systematically removed, and flagged probes were set to Non Available. Interarray normalization was performed by quantile normalization. A single value was obtained for each transcript, taking the mean of each replicated probes summarized data. Missing values were inferred using k-Nearest Neighbors (KNN) algorithm from the package "impute" from R bioconductor. Normalized data were then analyzed with GeneSpring GX. To assess differentially expressed genes between two groups, we started by fitting a linear model to the data. Then we used an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. The top-ranked genes were selected with the following criteria: an absolute fold change > 2 and an adjusted p value < 0.05.

Immunohistochemistry

At the time of surgery, biopsies were obtained for PRLR expression immunohistochemistry from the rectal mucosa above the dentate line and at least 5 mm away from the internal opening. All samples were analyzed by a single pathologist (Dr Lin) experienced in the assessment of anorectal specimens and blinded to the clinical and operative findings. Formalin-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated, following which they were retrieved for heat-induced epitope retrieval. Endogenous peroxidase was inhibited with 3% hydrogen peroxide (H_2O_2) and non-specific antigen was blocked with 5% bovine serum albumin (BSA; Amresco, Solon, OH, USA). Slides were then incubated overnight with the primary antibody (Rabbit Anti-PRLR, Boster, China) at 4°C and then rinsed 3 times in phosphate-buffered saline (PBS) for 5 minutes at room temperature. Following this they were incubated with a biotinylated secondary antibody (diluted 1:100) and then incubated with a streptavidin–biotin peroxidase complex (diluted 1:100). Immunohistochemical detection was performed with 3,3′-diaminobenzidinetetrahydrochloride (DAB) in accordance with the manufacturer's instructions. Tissue sections were examined with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) equipped with a camera. Images were captured using the NIS-Element S.F. 2.30 software at × 40, × 200, and × 400 magnification. Two techniques were used to analyze the immunohistochemical section. In the first method, 5 non-overlapping images were captured from each slide at × 400 magnification. Quantitative image analysis was performed using the Image-Pro Plus 6.0 software (Media Cybernetics, USA) and data were expressed as an integrated optical density (IOD) equal to the area × the average density of PRLR immunoreactivity identified, represented as the mean expression ± SD. In the second method, immunoreactivity was evaluated based upon the percentage of positively-stained cells combined with an estimate of staining intensity. For recording purposes, the percentage of positive cells was scored as 0 (< 10 %), 1 (10–40 %), 2 (40–70 %), or 3 (≥ 70 %) with the staining intensity being scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The sum of the intensity and distribution score was used as the final staining score. Pathological sections with a final score of ≥ 3 were considered to have high PRLR expression and those < 3 as having low PRLR expression.

Statistics

Statistical analyses were performed with the SPSS v11.5 software (SPSS Inc.,Chicago, USA). Comparisons between groups were performed with the χ^2 test and the Fisher's exact method where appropriate. Assessment of immunoreactivity and comparisons of fistula complexity (as defined) was performed by a multiple logistic regression with values for two-sided significance < 0.05 considered as significant.

Results

We compared 36,788 genes between patients with anal fistulas and the control group, finding a differential gene expression profile of 190 genes (Figures 1 and 2). Among them we noted differential PRLR expression (FC = 2.91, p <

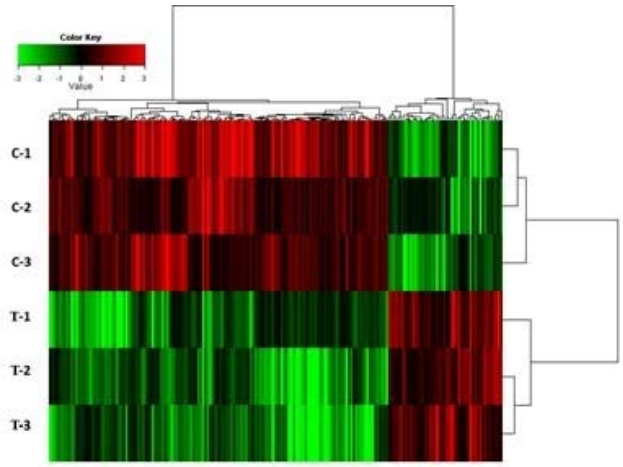

Fig. 1 – Cluster result of differentially expressed genes between anal fistula and control patients with Agilent human mRNA microarray. T1-3 – anal mucosa tissue of anal fistula patients; C1–3-control patients.

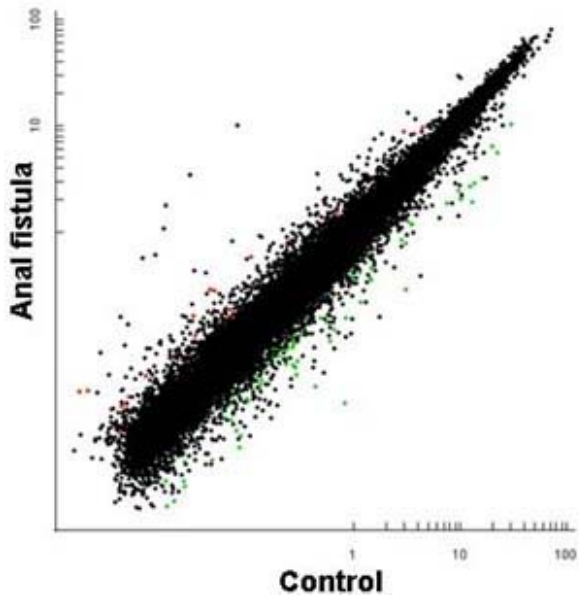

Fig. 2 – Scatter plot of differentially expressed genes between anal fistula and control patients with Agilent human mRNA microarray, (red dot – more than two folds up-regulation in anal fistula; green dot – more than two folds down-regulation in anal fistula; black dot – no significant difference).

0.01). Further analysis revealed the top three biological processes whose genes involved were keratinization, immune response and cell signaling.

Table 1 shows the clinicopathologic demographics for 65 patients assessed in the analysis including 35 simple (33 primary) and 30 complex (27 primary) cases (as defined). No differences were noted in the mean age of the patient groups, the mean body mass index (BMI) or in the time between diagnosis and surgery. Figure 3 shows examples of high and low PRLR expression in 2 rectal mucosal samples. Table 2 shows the separation into high and low PRLR expression groups where simple fistulas had significantly higher expression than complex cases ($p = 0.001$) and where low PRLR expression was more common in recurrent fistulas ($p = 0.001$). These findings were also reflected in the mean IOD measures where the recorded IOD for complex anal fistulas was significantly lower than that of simple cases ($8.31 \pm 4.91 \times 10^4$ *vs* $12.30 \pm 6.91 \times 10^4$, respectively; $p < 0.01$). The mean IOD measures of non-healing cases was lower than that in primarily healing fistulas ($7.21 \pm 3.51 \times 10^4$ *vs*. $8.31 \pm 4.91 \times 10^4$, respectively; $p < 0.05$). Univariate analysis showed that low PRLR expression was associated with fistula complexity but not with age, gender or the time between fistula diagnosis and surgery. The complexity of the fistula (as defined) was maintained in a multivariate analysis as an independent predictor for low fistula PRLR expression (odds ratio low to high PRLR score = 9.52; $p = 0.001$) (Table 3).

Table 4 presents list of highest rank of over-expressed and under-expressed genes in anal mucosa in patients with anal fistula.

Table 1

Demographic data for 65 patient with anal fistulas

Patient characteristics	Anal fistula		*p*
	simple	complex	
Number (n)	35	30	
Age (years), $\bar{x} \pm$ SD (range)	34.95 ± 12.18 (18–45)	32.45 ± 14.58 (21–54)	0.560
Gender (n)			
males	30	27	0.325
females	5	3	
Type of fistula (n)			
primary fistula	33	27	0.302
recurrent fistula	2	3	
BMI (kg/m^2), $\bar{x} \pm$ SD	24.32 ± 4.22	23.18 ± 5.38	0.452
Time between diagnosis and surgery (weeks), median (interquartile range)	14 (4–38)	20 (6–50)	0.152

$\bar{x}$ – mean; SD – standard deviation.

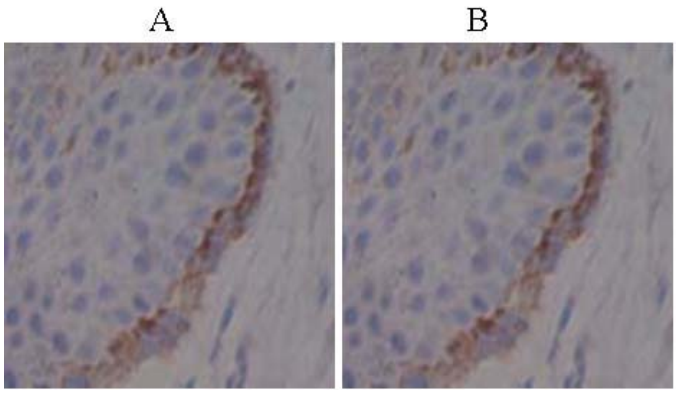

Fig. 3 – Representative microscopic appearance of the rectal mucosa in anal fistula biopsy specimens stained for prolactin receptor (PRLR): A) shows high PRLR expression, and B) demonstrates low PRLR expression (magnification ×200)

Table 2

Comparison of clinicopathological and fistula features with high or low prolactin receptors (PRLR) expression

Clinicopathological features		Score of PRLR expression		*p*
		high[a]	low[b]	
Number of patients (n)	65			
Age (years)				
< 40	41	17	24	0.913
≥40	24	13	11	
Gender (n)				
males	57	28	29	0.826
females	8	2	6	
Fistula classification (n)				
simple	35	30	5	0.001
complex	30	6	24	
Fistula recurrence (n)				
yes	5	1	4	0.001
no	60	35	15	

See Methods for definition of the intensity of PRLR immunoreactivity; [a] Score ≥ 3, [b] Score < 3.

Table 3

Univariate and multivariate analysis assessing the association between clinical features and fistula complexity

Clinicopathological features	Univariate analysis			Multivariate analysis	*p*
	n (%)	n (%)	*p*	OR	
Age, (years)					
< 40 *vs* ≥ 40	21 (32.3)	9 (13.8)	0.564		ns
Gender					
males *vs* females	27 (41.5)	3 (4.6)	0.236		ns
Time between diagnosis and surgery (weeks)					
< 24 *vs* ≥ 24	13 (20.0)	17 (26.1)	0.385		ns
PRLR expression groups					
low *vs* high	24 (36.9)	4 (15.4)	0.0001	9.520	0.001

ns – not significant; Low prolactin receptor (PRLR) expression – < 3; High PRLR expression – ≥ 3 (see Methods).

Table 4

List of highest ranked over-expressed and under-expressed genes in anal mucosa of anal fistula patients

Gene symbol	Fc (abs)	*p*
TREML1	6.41038	0.03559
UTY	9.945895	0.036397
CAMK1G	11.80925	0.000414
LOC643923	-9.164125	0.001235
LRCH1	-7.205685	0.039337
GPR116	5.369098	0.02471
DOK7	5.901879	0.04199
ELMOD1	5.434485	0.04398
IL37	10.87358	0.025695
LCE2D	7.009463	0.027215
PLB1	6.446507	0.014236
LCE5A	34.11338	0.00434

Fc (abs) – fold change, absolute.

Disscusion

As far as we are aware, this is the first report on the differentially expression profile of anal fistula and also the first recorded association between PRLR expression and anal fistula. This preliminary study shows that immunohistochemical PRLR expression is reduced in complex anal fistula cases when compared with simple fistulas. Low PRLR expression is also shown in non-healing cases confirming the qualitative findings with quantitative IOD measurements of the receptor. On multivariate analysis, fistula complexity was an independent predictor for low PRLR expression.

Currently there are limited available data concerning disturbances in the local peri-fistular inflammatory milieu of cryptogenic cases where it is anticipated that an improved understanding of the role of inflammatory mediators may explain why some fistulas become chronic, complex or fail to heal. PRLR which was originally identified as a lactotrophic hormone secreted by the pituitary has been implicated as an important immunomodulator under conditions of stress [16]. In this setting PRLR opposes the effects of glucocorticoids and other inflammatory mediators attenuating the acute phase response [17], with a regulatory effect on the generation of other inflammatory mediators [18]. During the acute phase response, PRL activity is typically suppressed [19] although the effects on the adaptive immune response during chronic inflammation on both PRL and its receptor have only been poorly characterized. The autocrine and paracrine interaction between the lactotrophes and other pituitary hormones, cytokines, inflammatory mediators and growth factors or with the other pituitary endocrine cell types (somatotropes, corticotropes, thyrotropes and gonadotropes) in simple or persistent surgical inflammatory conditions, remains to be elucidated [20]. Our study has shown that receptor expression is much higher in simple, healing anal fistula types probably consequent upon cytokine-mediated inhibition with coincident inhibition of its ligand. We believe a personalized surgery is very important when we try to address a fistula. Especially when it is a complex fistula, we have to weigh between the recurrence rate and potential chance of anal incontinence. Our study suggests that the molecular milieu of anal fistula might help us to evaluate the complexity and recurrence, which is useful for surgeons to make a reasonable decision about what operation to do for a specific anal fistula. Our study can also serve as a basis for a future attempt of pharmacological treatment of anal fistula by *ie* prolactin inhibitors or prolactin analogs.

This preliminary study has a number of significant limitations, however, the early findings are of sufficient interest as to demand further study. The small number of patients and the somewhat unexpected finding of PRL and PRLR association with anal fistula should be currently viewed with caution until larger number of patients is accumulated. The fistula classification system used in our study is unorthodox and more prolonged analysis along with magnetic resonance (MR) imaging may better define the association between PRLR expression and fistula outcome. It is accepted at this early stage that the findings may reflect the intensity of inflammation rather than show a specificity for high-risk anal fistula. It is currently unknown how the chronicity of inflammation in any part of the body influences local PRL and PRLR expression or the effect of glucocorticoids and catecholamines on PRL/PRLR dynamics within this orchestrated host response [21]. Planned future work will use an animal anal fistula model [22] so as to examine both the total PRLR mRNA expression and that of any different PRLR isoform transcripts which have previously been shown to differentially affect PRL signaling [23]. Comparison of anal fistula expression with other inflammatory tissues will be needed since stress responses to exogenously administered immunostimulants show that PRLR regulation is relatively tissue-specific [17]. Moreover, differences in PRLR expression will also reflect the differential activation of multiple promoters in the PRLR gene which will require molecular analysis [24].

Conclusion

Attenuation of the PRL/PRLR system appears to correlate with anal fistula complexity. It promotes our understanding of the occurrence of anal fistula, might help us to tailor the operation approach during preoperative evaluation of anal fistula and even provide rationale for a future attempt of pharmacological treatment of anal fistula. Further study is needed to examine and dissect the molecular genetics of the PRL/PRLR system in anal fistula.

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