Expression of microRNAs in gastric cancerous tissues and their association with *Helicobacter pylori* and Epstein-Barr virus infections

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Abstract. Gastric cancer (GC) is a serious public health concern, being one of the most frequent types of cancer worldwide, and the most lethal of all gastrointestinal cancers. Among the numerous causes of the occurrence and progression of GC, are infections of the gastric epithelium by microbial agents, such as Epstein-Barr virus and/or *Helicobacter pylori* (*H. pylori*). Recent findings have shed new light on the involvement of a class of non-coding RNAs, known as microRNAs (miRNAs/miRs) that are differentially expressed in GC tissues. Among these miRNAs, miR-21, miR-223 and miR-19, appear to play a crucial role in tumorigenesis. The present study aimed to investigate the differential expression of miR-21, miR-223 and miR-19 in GC tissues and their association with Epstein-Barr virus and *H. pylori* infections. The expression levels of miR-21, miR-223 and miR-19 in 70 samples of cancerous and adjacent normal tissues from patients with GC were detected using reverse transcription-quantitative polymerase chain reaction. The clinical relevance was then statistically analyzed. The results revealed the overexpression of the three miRNAs, miR-21, miR-223 and miR-19, in tumor tissues compared to the corresponding normal tissues. In addition, the upregulation of the mentioned miRNAs in GC tissues was also significantly associated with Epstein-Barr virus and *H. pylori* infections, as well as with aggressive clinicopathological features. On the whole, the findings of the present study indicate the potential of miR-21, miR-223 and miR-19 for use as novel diagnostic, prognostic and predictive biomarkers for gastric tumors. However, further randomized studies involving diverse populations and functional assessments are required to extend and confirm these findings.

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Abbreviations: GC, gastric cancer; miRNA/miR, microRNA; H. pylori, Helicobacter pylori; EBV, Epstein-Barr virus; ROC, receiver operating characteristic; AUC, area under the ROC curve

Key words: Gastric cancer, miR-21, miR-223, miR-19, Epstein-Barr virus, *Helicobacter pylori*

Introduction

Gastric cancer (GC) is a serious public health concern, and is one of the most common malignancies in the world (1). Despite reductions in incidence and mortality rates over the past few decades, it remains the fifth most frequent type of cancer and the fourth leading cause of cancer-related mortality worldwide, with an estimated 1,089,103 new cases per year, and 768,793 GC-related deaths recorded in 2020 (2).

The occurrence and progression of GC have been attributed to a number of factors; these factors may involve an infection of the gastric epithelium by *Helicobacter pylori* (*H. pylori*), a bacteria found in half of the population worldwide (3); or by Epstein-Barr virus (EBV), a double-stranded DNA virus that has recently been associated with GC (4). Researchers investigating this type of tumor believe that these two agents can lead

to prolonged and irreversible infection in the epithelial tissue of the stomach, which eventually leads to stomach cancer (5).

Recently, microRNAs (miRNAs/miRs) have attracted increasing interest in tumor biology due to their differential expression patterns observed in numerous types of cancer (6-8). miRNAs are small non-coding RNAs (21-25 nucleotides in length) that play a critical role in regulating genre expression at the post-transcriptional level by regulating the stability and translation of protein-coding mRNAs, typically through sequence-specific binding to the 3'-untranslated region (3'-UTR) of a target messenger RNAs (mRNAs) (9). The miRNA-mRNA interaction usually causes a translocation and/or cleavage repression of the mRNA, thus reducing the production of the final protein (10,11). Several studies have revealed that various miRNAs play critical roles in cell growth, differentiation, apoptosis and carcinogenesis (12-16). miR-21 has been found to be upregulated in certain types of solid cancers of the breast, colon, lung, pancreas, prostate and stomach (17). In addition, the overexpression of miR-223 and miR-19 has been reported in GC tissues (18). Thus, it can be assumed from previous studies that miR-21, miR-223 and miR-19 may function as oncogenes in GC. However, the targets of these miRNAs have not yet been identified, and their association with the clinicopathological characteristics of GC has also not been illustrated, at least to the best of our knowledge. In recent decades, among the >2,000 mature miRNAs discovered to date, only 120 miRNAs have been studied in GC or GC cell lines (19). Extensive research has been conducted individually on the differential expression of miRNAs and *H. pylori*/EBV infections, and the association of these miRNAs with infections in GC specimens is also being increasingly reported (20-23). Recent studies have suggested that *H. pylori* and EBV infections contribute to the dysregulation of these miRNAs (24-26).

Thus, the detection of miRNAs will not only provide potential biomarkers for the early detection and evaluation of GC, but may also guide future functional analyses of miRNAs in order to better understand their role in gastric carcinogenesis. The present study aimed to investigate the differential expression of miR-21, miR-223 and miR-19 in tissues from patients with GC and their association with *H. pylori* and EBV infections.

Patients and methods

Study population and samples. Clinical data and 70 gastric tissue specimens (of which 35 were tumor tissues and 35 were corresponding adjacent normal tissues) were collected during the period June 2020, to March, 2021 from patients, who found to have gastric adenocarcinoma by an endoscopic biopsy, and who underwent gastric resection at the Department of Surgery of Ibn Rochd University Hospital Center, Casablanca, Morocco. Patients who simultaneously had another type of cancer were excluded from the study.

The Biomedical Research Ethics Committee of the Faculty of Medicine and Pharmacy of Casablanca, Morocco (Reference no. 13/19 and committee reference no. 02/DRC/00) approved the study, and each patient signed an informed consent form.

DNA extraction of H. pylori and EBV. The collected tissue samples (both tumor and adjacent normal) were then immediately frozen and stored at -80˚C until use. DNA extraction from the biopsies was performed using the pure link Invitrogen® Genomic DNA mini kit (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. The quality and quantity of the DNA extracted were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.).

Detection of H. pylori and cytotoxin-associated gene A (cagA). H. pylori was detected in biopsies using PCR with glmM primers as previously described (27). To enhance the detection sensitivity, cagA was examined using the primers indicated in Table SI and as previously described (28). For each reaction, DNA from *H. pylori* CagA+ strain and a tube containing water in place of DNA were assayed as positive and negative controls used for comparisons.

EBV detection was performed using nested PCR, which consists of two successive PCRs in such a way that the product of the first PCR serves as a matrix for the second, and the sequence amplified by the second PCR is located inside the first fragment. The primers for the EBV (Table SI) were as previously described (29).

Briefly, the PCR reaction was carried out in a 25 μl reaction mixture containing genomic DNA (8 ng), 2X Taq PCR Master Mix (Qiagen, Inc.), 10 μmol forward and reverse primers. PCR amplification was performed using a PerkinElmer 2400 GeneAmp PCR System 2400 Thermal Cycler® (Thermo Fisher Scientific, Inc.). The primers used are presented in Table SI. The cycling conditions were as follows: Denaturation at 94˚C for 3 min, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at the specific temperature for 1 min, extension at 72˚C for 1 min, and the reaction was terminated by a 10-min extension at 72˚C.

miRNA extraction and the detection of mature miRNAs using reverse transcription-quantitative PCR (RT-qPCR). miRNAs were extracted and purified from cancerous tissues and adjacent normal tissues using the mirVanaTM miRNA isolation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The reverse transcription process (RT) of the miRNA samples into cDNA was performed using a TaqMan™ MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.; details are presented in Table SII), and reagents from the TaqMan™ MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). For the qPCR process, the PCR amplification was performed using TaqMan microRNA assays, and TaqMan™ Universal Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.).

The expression level of the three candidate miRNAs relative to the internal control, small nuclear U6 RNA, was determined using the following equation: $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq$=(Cq target-Cq U6 RNA)cancer-(Cq target-Cq U6 RNA)$_{\text{normal adjacent}}$ (30). The quantification cycle (Cq) is defined as the intersection between the amplification plot of the PCR product and the threshold line. The Cq value is relative to the PCR product concentration.

Statistical analysis. The statistical analysis of the results was performed using SPSS 23.0 statistical software (IBM Corp.),

Table I. Association of miRNA expression with various clinicopathological features of patients with gastric cancer.

Clinicopathological features	No. of cases	miR-21 expression			miR-223 expression			miR-19 expression		
		Low	High	P-value	Low	High	P-value	Low	High	P-value
Age group										
<59 years	17	6	11	0.38	9	8	0.86	7	10	0.84
≥59 years	18	9	9		9	9		8	10	
Sex										
Male	20	9	11	0.77	12	8	0.24	10	10	0.32
Female	15	6	9		6	9		5	10	
Smoking status										
Smoker	17	7	10	0.85	9	8	0.86	7	10	0.84
Non-smoker	18	8	10		9	9		8	10	
Alcoholic status										
Alcoholic	6	1	5	0.15	3	3	0.93	1	5	0.20
Non-alcoholic	29	14	15		15	14		14	15	
Tumor size										
<5 cm	7	6	1	0.027[a]	6	1	0.042[a]	6	1	0.02[a]
≥5 cm	28	9	19		12	16		9	19	
Histopathological differentiation										
Well	8	7	1	0.011[a]	7	1	0.041[a]	7	1	0.01[a]
Moderate/poor	27	8	19		11	16		8	19	
Lauren's classification										
Intestinal type	16	11	5	0.005[a]	11	5	0.02[a]	11	5	0.005[a]
Diffuse type	19	4	15		6	13		4	15	
Lymph node metastasis										
Negative	10	8	2	0.008[a]	8	2	0.032[a]	8	2	0.008[a]
Positive	25	7	18		10	15		7	18	
Lymphatic duct vessel invasion										
Negative	11	9	2	0.003[a]	9	2	0.015[a]	9	2	0.003[a]
Positive	24	6	18		9	15		6	18	
Tumor stage										
Low (I and II)	11	10	1	0.0001[a]	10	1	0.002[a]	10	1	0.0001[a]
High (III and IV)	24	5	19		8	16		5	19	

[a]Indicates a statistically significant difference (P<0.05).

and the association between the miRNA expression levels, and the various clinicopathological parameters were analyzed using a Student's t-test (paired t-test), or a Chi-squared test or Fisher exact test. A P-value <0.05 was considered to indicate a statistically significant difference.

Receiver operating characteristic (ROC) curve analysis was performed on the three miRNAs examined to investigate their performance as a discriminatory tool for classifying tumor and normal tissues. It is a plot of the true positive rate (sensitivity) in function of the false positive rate (100-specificity) for different cut-off points of miRNA expression. Cut-off values were determined using the Youden index, and P-values <0.05 for any parameters were considered to indicate statistically significant differences. Sensitivity indicates the ability of a ROC curve to correctly identify cancerous tissues (true positive). Specificity is the ability of ROC curve to correctly identify non-cancerous tissues (true negative). The area under the ROC curve (AUC) indicates the effectiveness of a miRNA to distinguish between cancerous tissues and non-cancerous tissues.

Results

Detection of H. pylori and EBV. Overall, the results indicated the presence of *H. pylori* DNA in 31.4% of the GC samples. Additionally, all *H. pylori*-positive tissues were cagA-positive.

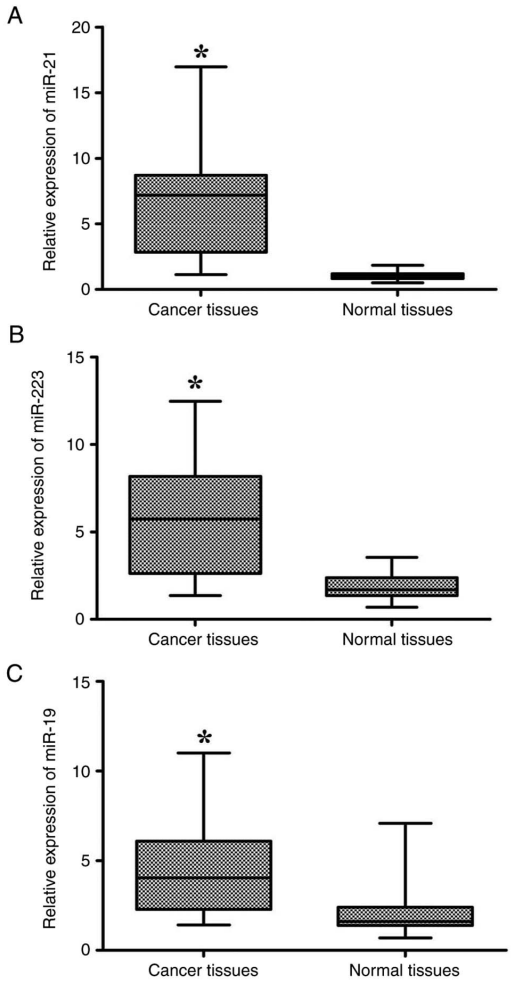

Figure 1. Expression levels of (A) miR-21, (B) miR-223, and (C) miR-19 in 35 gastric carcinomas specimens and adjacent normal tissues detected using reverse transcription-quantitative polymerase chain reaction and analyzed using a paired Student's t-test. The expression of the three miRNAs was clearly increased in cancer tissue compared to normal tissue (mean ± SD: 6.5±3.7 vs. 1.5±0.4, *P<0.0001; 5.73±3.09 vs. 1.84±0.75, *P<0.0001; 4.73±2.55 vs. 1.93±1.12, *P<0.0001, respectively).

EBV DNA found was in 40% of the GC tissues, and co-infection was found in 11.4% of the GC samples.

miRNA expression. RT-qPCR detection revealed that the miR-21, miR-223 and miR-19 expression levels, normalized to U6 RNA, were significantly increased in cancerous tissues compared with normal tissues (mean ± SD: 6.5±3.7 vs. 1.5±0.4, P<0.0001; 5.73±3.09 vs. 1.84±0.75, P<0.0001; 4.37±2.55 vs. 1.93±1.12, P<0.0001, respectively; Fig. 1). Using the average miRNA expression levels ($2^{-\Delta\Delta Cq}$) in GC tissues as a maximum, all patients were divided into two groups as follows: A group with a high miRNA expression, and a group with a low miRNA expression. The analysis of the association between the clinicopathological parameters of the patients, and the miRNA expression level revealed that miR-21, miR-223 and miR-19 expression levels were not associated with age, sex, or toxic habits (e.g., smoking or alcohol consumption; P>0.05); however, they were associated with histological type, tumor size, lymphatic vessel invasion, the presence of lymph node metastasis and tumor stage (P<0.05; Table I).

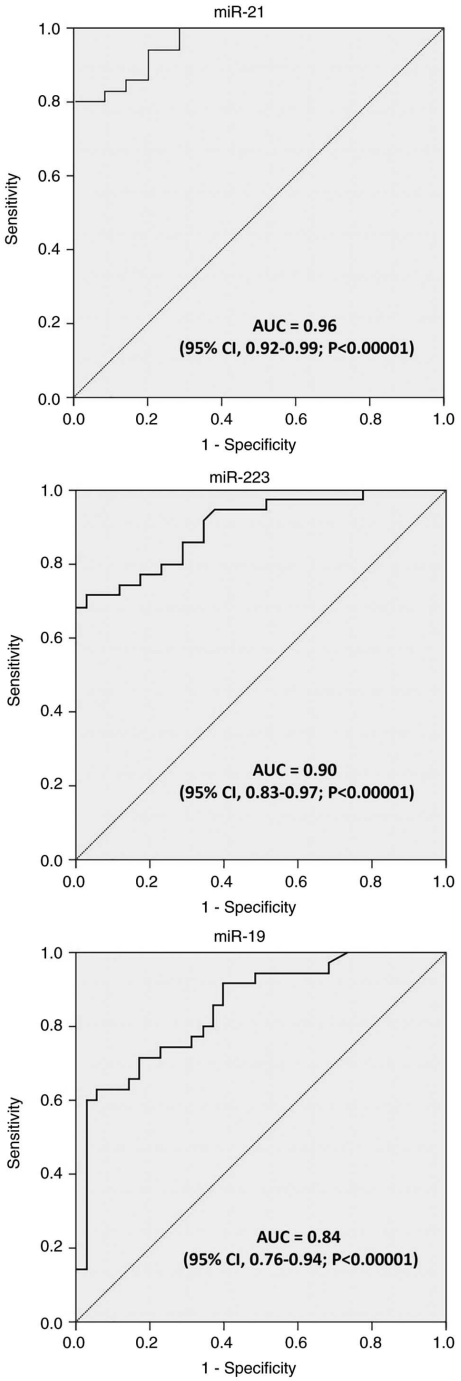

Figure 2. ROC curve analysis of three candidate miRNAs to distinguish cancerous tissues from normal tissues. The three ROC curves were generated to show the specificity and the sensitivity of miR-21, miR-223 and miR-19. ROC, receiver operating characteristic.

Moreover, a significant association between infections and miRNA overexpression was observed. It was found that patients who had one infection or co-infection with *H. pylori*/EBV had a higher expression level of all three miRNAs compared to those with no infections (P<0.05; Table II).

miRNA expression profiles for differentiating between GC and normal tissues. ROC curve analysis was performed on the three miRNAs to investigate their performance as discriminatory tools for tumor and normal tissue classification. As demonstrated in Fig. 2 and Table III, the ROC curves

Table II. Association between miRNA expression and H. pylori and EBV infections in patients with gastric cancer.

Infections status	No. of cases	miR-21 expression			miR-223 expression			miR-19 expression		
		Low	High	P-value	Low	High	P-value	Low	High	P-value
H. pylori status										
Infected	11 (31.4%)	0	11	0.001[a]	1	10	0.001[a]	0	11	0.001[a]
Uninfected	24 (68.6%)	15	9		17	7		15	9	
EBV status										
Infected	14 (40%)	2	12	0.005[a]	4	10	0.027[a]	1	13	0.001[a]
Uninfected	21 (60%)	13	8		14	7		14	7	
Co-infection										
Co-infected	4 (11.4%)	0	4	0.002[a]	1	3	0.005[a]	0	4	0.0003[a]
Uninfected	14 (40%)	13	1		14	0		14	0	

[a]Indicates a statistically significant difference (P<0.05). *H. pylori*, *Helicobacter pylori*; EBV, Epstein-Barr virus.

Table III. ROC curves tested for specificity and sensitivity and the AUC of miR-21, miR-223 and miR-19.

miRNA	AUC	Sensitivity	Specificity	P-value	Youden index	Cut-off (ΔΔCq)
miR-21	0.96	82.9	85.7	0.0001	0.69	1.46
miR-223	0.90	80	74.3	0.0001	0.54	2.35
miR-19	0.84	71.4	82.9	0.0001	0.54	2.50

ROC, receiver operating characteristics; AUC, area under the ROC curve.

for the three miRNAs indicated that these miRNAs were able to discriminate between cancerous and non-cancerous tissues with a relatively high sensitivity and specificity. Moreover, the sensitivity and specificity of miR-21 were detected at 82.9 and 85.7%, respectively, with an AU of 0.96 (95% CI, 0.92-0.99; P<0.00001), based on the reported data (Fig. 2). As regards miR-223, it achieved a suitable diagnostic accuracy for distinguishing GC tissues from non-cancerous tissues, with a sensitivity of 80%, a specificity of 74.3%, and an AUC of 0.90 (95% CI, 0.83-0.97; P<0.00001). In addition, it was found that miR-19 expression profiles had a sensitivity of 71.4% and a specificity of 82.9% in distinguishing GC tissues from normal non-cancerous, with an AUC of 0.84 (95% CI, 0.76-0.94; P<0.00001) (Fig. 2).

Discussion

Despite advancements made in the early diagnosis and surgical techniques, the majority of patients with GC continue to have a relatively poor prognosis. The molecular biomarkers for both early diagnosis and prognosis prediction are urgently required. Research has been conducted in an attempt to identify biomarkers with diagnostic and prognostic implications for GC. miRNAs can be critical regulators of carcinogenesis and tumor progression (31,32). The present study evaluated three miRNAs, including miR-21, miR-223 and miR-19 using RT-qPCR.

The results of the present study revealed that all three miRNAs were overexpressed in tumor tissues relative to their corresponding normal tissues, indicating that their levels of expression may be used as suitable potential biomarkers for detecting and distinguishing cancer from non-cancerous tissues. Previous research has highlighted the potential role of miR-21 as an appropriate biomarker for the early diagnosis of GC. Wang *et al* (33) suggested miR-21 to be a potential biomarker, and significant differences in expression values were found in GC tissues. Emami *et al* (34) found that the miR-21 expression levels in patients with GC were considerably higher than those in normal subjects (P<0.001).

Previous research has also demonstrated that the upregulation of miR-21 can alter the biological process of cancer cells, including proliferation, apoptosis and cell invasion, possibly by targeting reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and phosphatase and tensin homolog (PTEN) as major tumor suppressor genes. For example, the study by Zhang *et al* (35) found an inverse association between the levels of RECK expression and miR-21, where the increased levels of expression in miR-21 were indicative of a decrease in the expression of RECK. It has been suggested that the downregulation of RECK plays an essential role in GC progression (36,37).

Other studies have revealed that F-box and WD repeat domain containing 7 (FBXW7, also known as hCdc4), one

of the major target genes of miR-223 functions as a general tumor suppressor in human cancer. Li *et al* (38) demonstrated that miR-223 functions as an oncogene in GC by mediating the downregulation of FBXW7/hCdc4. Another study demonstrated that miR-19 was upregulated in GC (39). The function of miR-19 in GC has not yet been fully defined; however, it has been suggested that miR-19 upregulation may play a significant role in the progression of GC (40). The present study confirmed these previous findings, demonstrating that miR-19 expression was upregulated in GC tissues. Notably, the results of the present study indicated that the overexpression of miR-21, miR-223 and miR-19 was associated with histological type, tumor size, lymphatic invasion, the presence of lymph node metastasis and tumor stage ($P<0.05$), which are the main prognostic factors for GC. Their expression was also associated with *H. pylori* and EBV infection ($P<0.05$).

In the study by Chang *et al* (41), miRNAs exhibited a differential expression signature between *H. pylori*-infected and non-*H. pylori*-infected gastric tissues. Riley *et al* (42) found that EBV infection affected cellular miRNA expression. Given the role of *H. pylori* and EBV in the development of GC, this difference in miRNA expression signatures may be used as a prognostic factor. According to recent studies, miRNA expression has a specific association with lymph node metastasis, and is a prognostic factor for patients with GC (43,44).

In conclusion, considering the significant differences in the expression of the three tested miRNAs (miR-21, miR-223 and miR-19) in cancer tissues compared with normal tissues, as well as their association with *H. pylori* and EBV infections and patient clinicopathological parameters, it is suggested that they play a crucial role in GC initiation and progression. These miRNAs may thus be considered as effective biomarkers for the accurate early diagnosis of GC. However, these results are only at a preliminary stage; therefore, further studies are warranted to extend these findings and identify their specific roles in GC diagnosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FER was involved in the conceptualization and methodology of the study, as well as in the formal analysis and investigation, and in the writing, review and editing of the manuscript. DE was involved in the conceptualization of the study, as well as in the provision of resources and in the reviewing of the manuscript. BA was involved in the conceptualization and methodology of the study, as well as in the reviewing and editing of the manuscript. HC was involved in the formal analysis and investigative aspects of the study, and in the reviewing and editing of the manuscript. FC was involved in the conceptualization of the study, as well as in the provision of resources and in the reviewing of the manuscript. MME was involved in the conceptualization and methodology of the study, as well as in the reviewing and editing of the manuscript, and in study supervision. FER and FC confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Biomedical Research Ethics Committee of the Faculty of Medicine and Pharmacy of Casablanca, Morocco (Reference no. 13/19 and committee reference no. 02/DRC/00) approved the study, and each patient signed an informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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