Functional promoter polymorphisms of *NFKB1* influence susceptibility to the diffuse type of gastric cancer

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Abstract. In the present study, we report an association between gastric cancer and polymorphisms in *NFKB1* (rs28362941 and rs78696119). We employed the PCR-SSCP method to detect gene polymorphisms in 479 gastric cancer cases and 880 controls. The rs28362941 del/del homozygote was significantly associated with gastric cancer development; in particular it was closely associated with diffuse type gastric cancer. The rs78696119 GG homozygote was also associated with the diffuse type of gastric cancer. In young subjects, both polymorphisms were significantly associated with the development of gastric cancer. In addition, both polymorphisms were related to tumor progression such as tumor invasion and lymph node metastasis. The inflammatory cell infiltration into non-cancerous gastric mucosa was greater in the subjects with the rs28362491 del/del or rs78696119 GG genotype when compared to those with the other genotypes. In conclusion, functional polymorphisms of *NFKB1* are associated with an increased risk of gastric cancer; in particular they are closely associated with the development of diffuse type of gastric cancer via severe gastric inflammation. These polymorphisms also appear to be associated with gastric cancer progression.

Introduction

Gastric cancer (GC) is one of the most common and lethal malignancies in Japanese and East Asian populations, and the second most common cause of cancer-related death in the world (1). Although the incidence and mortality rate of GC located outside the cardia have decreased over the last few decades, a considerable percentage of patients still have advanced disease at diagnosis. *Helicobacter pylori* (*H. pylori*) infection is now accepted as a crucial event in the development of atrophic gastritis and is implicated in the development of gastric carcinoma, particularly those not located in the cardia (2-4). However, there is marked variation in the extent of gastric inflammation among *H. pylori*-infected patients, and only a small percentage of them actually develop GC. That is, the occurrence and development of GC is a process involving genetic and environmental factors, for example *H. pylori* infection and other environmental factors. This suggests that genetic factors play an important role in the long-term outcome of *H. pylori* infection (5-9).

Lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria including *H. pylori*, is a signaling molecule for the innate immune system and is one of the main sources of inflammation (10). LPS binding to TLR4 activates signal transduction through MyD88, IRAK and TRAF6 to activate the nuclear factor (NF)-κB (11). Activation of NF-κB by *H. pylori* induces nuclear translocation, which causes an increase in IL-8 messenger RNA and protein levels (12). In addition, the NF-κB pathway is responsible for the generation of several cell adhesion molecules including ICAM-1 (13). Thus, *H. pylori* is a potent activator of NF-κB in gastric epithelial cells, and NF-κB is a major molecule in *H. pylori*-induced inflammation (14). On the other hand, NF-κB activation is known to regulate cellular growth responses, including apoptosis, and is required for the induction of inflammatory and tissue-repair genes (15). These facts suggest that NF-κB plays an important role in inflammation-associated carcinogenesis. *NFKB1* which is a gene encoding 2 subunits (p50 and p105) of NF-κB is located on 4q24 (16). Recently, many studies have reported the association between polymorphism rs28362491 (-94 ins/del ATTG of *NFKB1*) and cancer risk in various organs (17). However, these results do not always lead to the same conclusions, and there has been no report concerning the risk of this polymorphism in the development of GC in Japan. Furthermore, certain genetic variation in rs72696119 (-449 C>G in 5'-UTR of *NFKB1*) has been identified. We previously reported a closely association between *NFKB1* polymorphisms (rs28362491 and rs72696119) and aberrant gene methylation in gastric mucosa, which is considered to be a pre-malignant condition (18).

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In the present study, we attempted to clarify the association between -94 ins/del ATTG polymorphism (rs28362491) of *NFKB1* and GC development in Japanese subjects. In addition, the -449 C>G polymorphism (rs72696119) in 5'-UTR of *NFKB1* was also investigated.

Materials and methods

Clinical samples. Our gastric cancer group included 479 patients (GC cases) enrolled at the Endoscopy Center of Fujita Health University Hospital or Kanazawa Medical University Hospital between July 2006 and August 2012. As a control group, 880 subjects without a malignant neoplasm, confirmed endoscopically and histologically, were selected at random from our DNA biobank, collected over the same period as that defined above (controls). Our final study cohort comprised 1,359 subjects for whom polymorphisms could be clearly analyzed.

All subjects underwent upper gastrointestinal endoscopy, and patients with severe systemic diseases, malignancies in other organs, and who had previously received non-steroidal anti-inflammatory drugs, antibiotics, and *H. pylori* eradication treatment were excluded. *H. pylori* infection status was assessed by serology, histological examination, or the urea breath test. Patients were diagnosed as having infection when at least one of the diagnostic tests was positive.

The Ethics Committees of Fujita Health University and Kanazawa Medical University approved the protocol, and written informed consent was obtained from all of the participating subjects.

Histological evaluation. In 778 of the 1,359 subjects (592 controls and 186 GC cases), the severity of chronic gastritis in non-cancerous mucosa was classified according to the updated Sydney system (19) by a pathologist who had no access to any clinical information.

Genotyping of polymorphisms. DNA was isolated from biopsy specimens or peripheral blood and genotyped using the PCR-SSCP method as reported previously (18,20). We had previously confirmed that each genotype was clearly determined by this method. To detect *NFKB1* rs28362491 (-94 ins/del ATTG) using the primer pairs (94F, 5'-gctatggaccgcatgactctatcag-3' and 94R, 5'-ggggctctggcttcctagcag-3'), PCR was carried out in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 96˚C for 15 sec, 58˚C for 40 sec, and 72˚C for 30 sec, with final extension at 72˚C for 5 min. Thereafter, 2 μl of the PCR product was denatured with 10 μl of formamide (Sigma-Aldrich Co., St. Louis, MO, USA) at 90˚C for 5 min. SSCP was carried out at 6˚C using a GenePhor DNA separation system with GeneGel Excel 12.5/24 (Amersham Biosciences Corp., USA), after which the denatured single-strand DNA bands were detected using a DNA Silver Staining kit (Amersham Biosciences Corp.).

To detect *NFKB1* -449 C>G, using the primer pairs (449F, 5'-cgtgtgtccgtctgtctgtatgctc-3' and 449R, 5'-cgctggtgcacttctctctctttct-3'), PCR was carried out in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 95˚C for 30 sec, 57˚C for 40 sec, and 72˚C for 45 sec, with a final extension at 72˚C for 5 min. Thereafter, SSCP was carried out in the same manner as described above.

Table I. Subject characteristics and genotype frequencies.

Characteristics	Controls	GC cases	p-value
No. of subjects	880	479	
Mean age ± SD	61.4±13.5	65.2±11.6	<0.0001[a]
Male:female	506:373	336:143	<0.0001[b]
HP-positive rate	61.8%	86.0%	<0.0001[b]
NFKB1 rs28362491			
ins/ins	342	172	
ins/del	435	239	
del/del	103	68	
del. allele frequency	36.4%	39.1%	NS
NFKB1 rs72696119			
CC	352	189	
CG	428	226	
GG	100	64	
G allele frequency	35.7%	37.0%	NS

[a]Student's t-test; [b]Fisher's exact test. NS, not significant. GC, gastric cancer.

Statistical analysis. Data are expressed as means ± SD. Mean ages between GC cases and controls were compared using the Student's t-test. The ratios of male/female and *H. pylori* infection were compared between 2 groups using a 2x2 table and the Fisher's exact test. Allele and genotype frequencies were calculated by direct counting. The allele counts and genotype distribution were also compared by the Fisher's exact test. Furthermore, the strength of association between genotype frequencies and the disease was assessed by calculating the odds ratio (OR) at 95% confidence intervals (CI). Adjusted ORs were calculated with the use of logistical multivariate regression analysis. The association of genotypes with the progression of gastric cancer was assessed by ANCOVA using the number of alleles as a covariate. Each updated Sydney system was compared using Mann-Whitney U test. For all analyses, the level of significance was set at $p<0.05$.

Results

Subject characteristics and genotype frequencies. In the controls, rs72696119 was in Hardy-Weinberg equilibrium (p=0.091), whereas rs28362491 was not (p=0.0495). The mean age, male/female ratio and frequency of *H. pylori* positivity of the controls were significantly lower than these value in the GC cases (Table I). The minor allele frequency and genotype distribution were not significantly different between the controls and GC cases.

Association between gene polymorphisms and gastric carcinogenesis. The rs28362491 del/del homozygote was significantly but weakly associated with susceptibility to gastric carcinogenesis (OR, 1.43; 95% CI, 1.01-2.02; p=0.045).

Table II. Association between genetic polymorphisms and gastric cancer.

NFKB1 rs28362491	ins/ins	ins/del	del/del	del/del vs. others; OR (95% CI)	p-value
Controls (880)[a]	342	435	103	Reference	
Overall GC cases (479)[b]	172	239	68	1.43 (1.01-2.02)	0.045
Intestinal (283)	101	150	32	1.14 (0.724-1.78)	0.58
Diffuse (191)	69	86	36	1.85 (1.21-2.84)	0.0049
NFKB1 rs72696119	CC	CG	GG	GG vs. others; OR (95% CI)	p-value
Controls (880)	352	428	100	Reference	
Overall GC cases (479)[b]	189	226	64	1.36 (0.955-1.94)	0.089
Intestinal (283)	116	138	29	1.05 (0.656-1.67)	0.85
Diffuse (191)	71	85	35	1.81 (1.17-2.78)	0.0073

By logistical regression analysis after adjustment for age, gender and *H. pylori* infection status. [a](Number) of subjects. [b]The type of GC in 5 cases was unknown. GC, gastric cancer. OR, odds ratio; CI, confidence intervals.

Table III. Risk of gastric carcinogenesis with genotype in young and old subjects.

NFKB1 rs28362491	ins/ins	ins/del	del/del	del/del vs. others; OR (95% CI)	p-value
≤60 years of age					
Controls (359)[a]	144	171	44	Reference	
GC cases (167)	57	73	37	2.24 (1.33-3.75)	0.0023
NFKB1 rs72696119	CC	CG	GG	GG vs. others; OR (95% CI)	p-value
≤60 years of age					
Controls (359)	142	172	45	Reference	
GC cases (167)	60	72	35	1.95 (1.16-3.28)	0.012
NFKB1 rs28362491	ins/ins	ins/del	del/del	del/del vs. others; OR (95% CI)	p-value
>60 years of age					
Controls (521)	198	264	59	Reference	
GC cases (312)	115	166	31	0.938 (0.583-1.51)	0.79
NFKB1 rs72696119	CC	CG	GG	GG vs. others; OR (95% CI)	p-value
>60 years of age					
Controls (521)	210	256	55	Reference	
GC cases (312)	129	154	29	0.913 (0.559-1.49)	0.71

By logistical regression analysis after adjustment for gender and *H. pylori* infection status. [a](Number) of subjects. GC, gastric cancer; OR, odds ratio; CI, confidence intervals.

This homozygote was strongly associated with diffuse type of GC (OR, 1.85; 95% CI, 1.21-2.84; p=0.0049), whereas it was not significantly associated with intestinal type of GC (Table II). The rs72696119 GG homozygote appeared to be associated with gastric carcinogenesis (p=0.089). This homozygote was also strongly associated with diffuse type of GC (OR, 1.81; 95% CI, 1.17-2.78; p=0.0073).

In subjects younger than or 60 years of age, both rs28362491 del/del and rs72696119 GG homozygotes conferred an increased risk for the development of gastric cancer (OR, 2.24; 95% CI, 1.33-3.75; p=0.0023 and OR, 1.95; 95% CI, 1.16-3.28; p=0.012, respectively) (Table III). In subjects older than 60 years of age, however, no significant association was found between polymorphisms and gastric carcinogenesis.

Association between genetic polymorphisms and the progression of gastric cancer. We further investigated the influence of genetic polymorphisms on the progression of GC. According

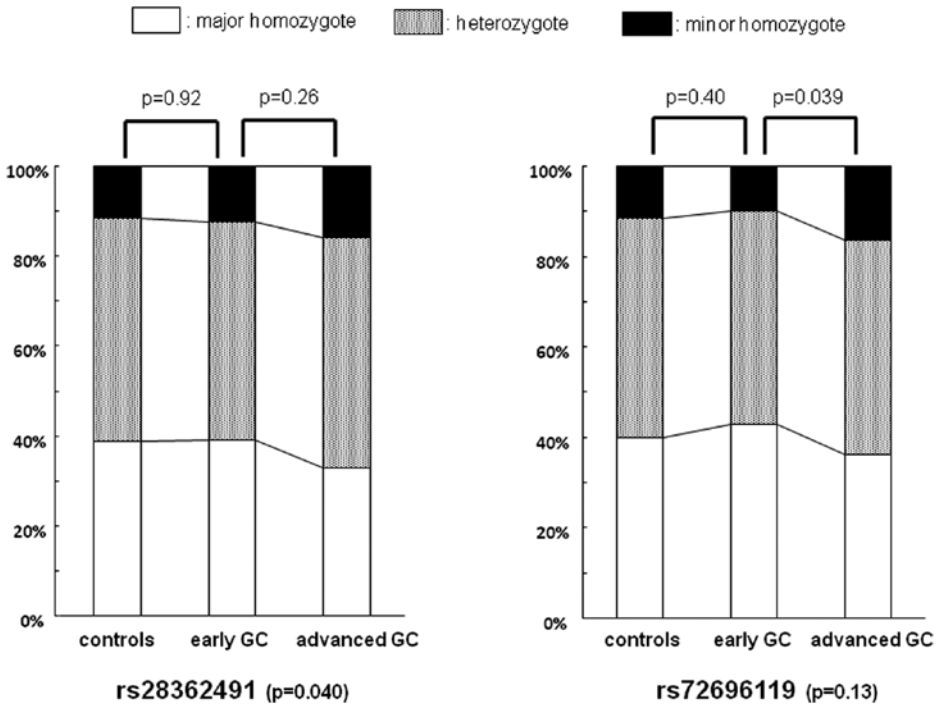

Figure 1. Relationship between genotype frequency and clinical stage of gastric cancer (GC). When considering that the controls, early GC, and advanced GC progress continuously, the correlation of genotype frequency to clinical stage was estimated by ANCOVA. Early GC, stage 0-I; advanced GC, stage II-IV.

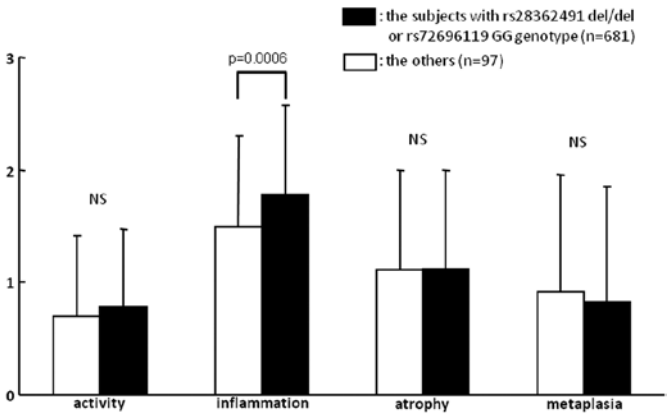

Figure 2. Relationship between the *NFKB1* genotype and updated Sydney system scores. The inflammation score of the subjects with rs28362941 del/del or rs78696119 GG genotype was significantly higher than the score in subjects with the other genotypes, whereas the other updated Sydney system scores did not differ between the two groups. NS, not significant.

to the UICC (Unio Internationalis Contra Cancrum) classification v. 7, gastric cancer cases at stage 0-I are classified as early GC cases and those at stage II-IV are classified as advanced GC cases. Thus, in this study, early GC and advanced GC cases consisted of 210 and 262 cases, respectively (unknown, 7 cases). The minor allele rs28362491 was significantly correlated to the progression of GC by ANCOVA (p=0.040) (Fig. 1), whereas that of rs72696119 was not. However, the distribution of the rs73696119 genotype was significantly different between advanced GC cases and early GC cases (p=0.039). The risk of the rs73696119 GG homozygote for advanced GC compared with early GC was OR, 1.78; 95% CI, 1.01-3.13 (p=0.046) (Table IV). In addition, the rs28362491 del/del homozygote conferred an increased risk for both tumor invasion over the muscle layer and lymph node metastasis (OR, 1.75; 95% CI, 1.02-3.00; p=0.041 and OR, 1.71; 95% CI, 1.01-2.89; p=0.047, respectively). The rs78366119 GG homozygote was also associated with an increased risk for both factors (OR, 2.33; 95% CI,1.32-4.11; p=0.0036 and OR, 1.83; 95% CI, 1.07-3.15; p=0.028, respectively).

Risk of the subjects with the rs28362491 del/del or rs72696119 GG genotype for gastric inflammation and carcinogenesis. When assessing the risk of the rs28362491 del/del or rs72696119 GG genotype (del/G group) for gastric carcinogenesis, a stronger association with GC was found (OR, 1.44; 95% CI, 1.02-2.03; p=0.037), particularly with the diffuse type of GC (OR, 1.88; 95% CI, 1.23-2.85; p=0.0033) (Table V). In addition, the inflammation score of the del/G group was significantly higher than that of the non-del/G group (p=0.0006), whereas the other updated Sydney system scores did not differ between the two groups (Fig. 2).

Discussion

In the present study, we demonstrated the association between *NFKB1* polymorphisms and gastric cancer risk. We revealed that the rs28362941 del/del genotype was significantly associated with an increased risk for the development of GC, and that both rs28362941 del/del and rs72696119 GG genotypes were closely associated with development of the diffuse type of GC. In addition, in the subjects with the rs28362941 del/del or rs73696119 GG genotype, the frequency of inflammatory cell

Table IV. Association between cancer progression and genotype.

NFKB1 rs28362491	ins/ins	ins/del	del/del	del/del vs. others; OR (95% CI)	p-value
Early GC cases (210)[a]	82	102	26	Reference	-
Advanced GC cases (262)	86	134	42	1.34 (0.783-2.29)	0.28
≤ T1 (234)	92	116	26	Reference	-
≥ T2 (238)	76	120	42	1.75 (1.02-3.00)	0.041
N(-) (266)	91	144	31	Reference	-
N(+) (206)	77	92	37	1.71 (1.01-2.89)	0.047
NFKB1 rs72696119	CC	CG	GG	GG vs. others; OR (95% CI)	p-value
Early GC cases (210)	90	99	21	Reference	-
Advanced GC cases (262)	95	124	43	1.78 (1.01-3.13)	0.046
≤ T1 (234)	100	113	21	Reference	-
≥ T2 (238)	85	110	43	2.33 (1.32-4.11)	0.0036
N(-) (266)	103	135	28	Reference	-
N(+) (206)	82	88	36	1.83 (1.07-3.15)	0.028

By logistical regression analysis after adjustment for gender, age and *H. pylori* infection status. [a](Number) of subjects. The cancer stage of 7 cases was unknown. GC, gastric cancer; OR, odds ratio; CI, confidence intervals.

Table V. Association of del/del or GG genotype with gastric cancer.

	del/del or GG	Others	del/del or GG vs. others; OR (95% CI)	p-value
Controls (880)[a]	108	772	Reference	
Overall GC cases (479)[b]	71	408	1.44 (1.02-2.03)	0.037
Intestinal (283)	33	250	1.13 (0.726-1.77)	0.58
Diffuse (191)	38	153	1.88 (1.23-2.85)	0.0033

By logistical regression analysis after adjustment for age, gender and *H. pylori* infection status. [a](Number) of subjects. [b]The type of GC in 5 cases was unknown. GC, gastric cancer; OR, odds ratio; CI, confidence intervals.

infiltration into non-cancerous gastric mucosa was higher than this frequency in the other genotypes. Based on the fact that rs28362941 and rs73696119 are in linkage disequilibrium (18), these results suggest that more severe gastric inflammation may be induced in the homozygote of *NFKB1* minor allele, subsequently developing diffuse type of GC.

According to the Lauren classification (21), there are two histologically distinct types of GC, which is still widely accepted. The intestinal type consists of gland-like structures that mimic the intestinal glands, and a series of precancerous lesions are recognized. The diffuse type of gastric cancer lacks any glandular structures and arises closer to the advancing edge of gastric mucosal inflammation without any identifiable histological precursor lesion (22). The former develops in stomachs affected by chronic inflammation by passing through the intermediate steps of atrophic gastritis or intestinal metaplasia (23). On the other hand, the severity of mucosal inflammation and various host features may directly induce mutagenetic events that ultimately lead to the onset of the latter. Moreover, the diffuse type of GC develops in comparatively younger subjects (24). Therefore, we suspect that *NFKB1* polymorphisms were significantly associated with susceptibility to GC in young subjects, not elder subjects, in the present study.

It has been reported that the *NFKB1* -94 ATTG deletion mutant in the promoter region destroys a transcription factor binding site, resulting in lower expression of NF-κB (25). One study reported that the *NFKB1* -94 deletion mutant had a reduced risk for auto-immune disorders in China (26). In the stomach, Lo *et al* (27) showed that the -94 deletion mutant had a significantly reduced risk for gastric carcinogenesis in China. Contrary to these results, several studies in Caucasians have shown that the -94 deletion mutant is associated with an increased risk for the development of inflammatory or auto-immune diseases (25,28). In colorectal carcinogenesis, Andersen *et al* (29) demonstrated that carriers of the *NFKB1* -94 deletion were at a 1.45-fold higher risk than homozygous carriers of the insertion allele. On the other hand, other studies found no association of *NFKB1* -94 ins/del polymorphism with inflammatory or auto-immune diseases (30-32). These contrasting observations may be explained by differences in the genotypic composition of populations in different countries

with different racial groups. In fact, the frequency of the -94 deletion allele appears to be rather higher in Chinese healthy subjects (45-55%). However, in our Japanese subjects, it was approximately 35-36%, similar to the value in Caucasians. Our Japanese study indicates, as well as the Caucasian study, that the -94 deletion allele may be an inflammation promoting allele.

NF-κB regulates a number of different transcription factors that are homodimers or heterodimers of p65, p50, p105, C-rel and relB (33). NF-κB is involved in both the inflammatory and the anti-inflammatory process (34). The role of NF-κB in inflammation is determined by subunit type. *NFKB1* encodes both subunits p105 and p50 of the transcription factor NF-κB by alternative splicing (35). As part of the p65/p50 NF-κB transcription factor complex, it is pro-inflammatory, controlling transcription of pro-inflammatory cytokines (36). Conversely, since p50 lacks this COOH-terminal transactivation domain which is necessary for the positive regulation of gene expression, p50 has anti-inflammatory properties in the p50 homodimer by repressing transcription (37). The relative abundance of p65/p50 heterodimers and p50 homodimers may determine the magnitude of inflammation by balancing the pro-inflammatory and anti-inflammatory response (33). In fact, p50-deficient mice have an increased sensitivity to LPS and have increased LPS-induced inflammation (38,39). In subjects with the del/del genotype, decreased p50 synthesis may lead to decreased repressive homodimers and increased active heterodimers of the NF-κB complex. This balance may promote gastric inflammation, resulting in cancer development.

In the present study, *NFKB1* polymorphisms appeared to be associated with GC progression. Sasaki *et al* (40) reported that NF-κB activation was correlated with gastric cancer invasion and lymphatic invasion. It has been shown that the NF-κB pathway has an important role in GC cell growth and metastatic function *in vitro* (41,42). In a study in a Korean population by Kim *et al* (43), however, no correlation was observed between the genotype or allelic frequency of rs28362941 and the T, N or M stage of gastric cancer. The distribution of genotype in their study was 107 ins/ins, 80 ins/del and 274 del/del, which was entirely different from that in the present study. The minor allele frequency in our controls was almost equal to that in JSA426 (426 anonymous unrelated Japanese individuals, from NCBI dbSNP). It is unclear why there is such a discrepancy between the two Asian studies. Further large scale study is needed, since the association observed in this study was significant but weak.

The present study was a hospital-based case-control study. Therefore, sample selection may have affected the outcome as our controls included patients who came to the hospital in order to seek treatment for various complaints and were not completely healthy subjects. Another limitation of this study was that the effect of type II error cannot be excluded in relatively small sample sizes.

In conclusion, the functional promoter polymorphism of *NFKB1* is associated with an increased risk of gastric cancer, in particular, with the development of diffuse type of gastric cancer via severe gastric inflammation. In addition, this polymorphism appears to be associated with gastric cancer progression.

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