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RESEARCH ARTICLE

Association of single nucleotide polymorphism rs2065955 of the *filaggrin* gene with susceptibility to Epstein-Barr virus-associated gastric carcinoma and EBV-negative gastric carcinoma

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The relationship between the *Filaggrin* gene (*FLG*) rs2065955 polymorphism and susceptibility to Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) and EBV-negative gastric carcinoma (EBVnGC) was investigated in Shandong Province, China. We detected the *FLG* rs2065955 genotype and allele distribution by using PCR and restriction fragment length polymorphism (RFLP) in 64 EBVaGC, 82 EBVnGC, and 111 normal control samples. Immunohistochemistry was used to detect the level of FLG protein in 35 EBVaGC and 51 EBVnGC tumor tissues. Compared with normal controls, the genotype CC and allele C of *FLG* rs2065955 showed higher frequency in EBVaGC and EBVnGC. There was no significant difference between EBVaGC and EBVnGC in allele distribution of *FLG* rs2065955, but the genotype CC was found more frequently in EBVaGC than in EBVnGC. The risk of developing either EBVaGC or EBVnGC in genotype CC was higher than in other genotypes. Furthermore, genotype CC of *FLG* rs2065955 may contribute more to the risk of developing EBVaGC than EBVnGC. There was no significant difference in the expression level of FLG protein between EBVaGC and EBVnGC. In conclusion, the FLG rs2065955 polymorphism was significantly related to gastric carcinoma. Allele C of *FLG* rs2065955 could be a risk factor for EBVaGC or EBVnGC, while genotype CC of *FLG* rs2065955 was especially associated with EBVaGC.

KEYWORDS ***Filaggrin* gene (*FLG*); rs2065955; gastric carcinoma; Epstein-Barr virus (EBV); gene polymorphism; immunohistochemistry**

INTRODUCTION

Epstein–Barr virus (EBV), a well-known tumor-causing virus, is closely associated with several human lymphoid and epithelial tumors, such as Burkitt's lymphoma, Hodgkin's disease, AIDS-associated lymphoma, nasopharyngeal carcinoma, and gastric cancer (Thompson et al., 2004). In the last 20 years, the relationship between EBV infection and the occurrence of gastric carcinoma has been confirmed. EBV infection is found in 80%–100% of gastric lymphoepithelioma-like carcinoma cases and 2%–16% of ordinary gastric adenocarcinoma cases (Fukayama et al., 1994; Wu et al., 2000; Andal et al., 2003; Lee et al., 2004;). The worldwide occurrence of EBV-associated gastric carcinoma (EBVaGC) is estimated at more than 50, 000 cases per year (Takada, 2000). Therefore, it is also widely accepted that EBVaGC is one of the most common cancers among EBV-related malignancies.

The development of gastric cancer is a complex, multistep process involving multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, and signaling molecules (Nagini, 2012). Recognition of bacteria or viruses

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by the diversification level of the cytokine response and immune system is closely related to polymorphisms in host inflammatory response genes (Garza-gonzalez et al., 2005). Analysis of polymorphisms in genes related to the inflammatory response in the gastric mucosa and the associated risk for malignancy has been the central focus of many studies (Rad et al., 2009; Partida-rodriguez et al., 2010). However, little is known about the association of host gene polymorphisms with EBVaGC development.

Filaggrin (FLG) is an intermediate-filament-associated protein that aggregates keratin intermediate filaments in the epidermis. It plays an important role in the function of skin as a barrier against invasion of harmful factors from the external environmental, including microorganisms (Candi et al., 2005). Mutations in the *FLG* gene are associated with some skin disorders such as atopic dermatitis (Palmer et al., 2006) and ichthyosis vulgaris (Smith et al., 2006). A recent study found that loss-of-function mutations of *FLG* were closely associated with higher incidence of human papillomavirus (HPV)-related cancers and pre-cancers (Skaaby et al., 2014).

In a previous study, we used a high-throughput sequencing method (RNA-Seq) to compare the transcriptomes of the gastric cancer cell lines AGS and AGS-EBV to determine the impact of EBV on gastric cancer and uncover clues to the tumorigenic mechanisms of EBV. The RNA-Seq results showed 929 single nucleotide polymorphisms (SNPs) between the two cell lines (Supplementary Table S1). There were 539 SNPs in intronic

Table 1. SNPs associate with tumors

No.	Position	SNP ID	Tumor gene	Associated Cancer	Reference
1	Chr1, 147094204	no rs id	BCL9	Renal cell carcinoma	Wang et al., 2016
				Prostate cancer	Ling et al., 2016
2	Chr1, 152193052	rs6587650	HRNR	Breast cancer	Fleming et al., 2012
3	Chr1, 152277055	rs2065955	FLG	Cervical cancer	Bager et al., 2015
				EBV-associated gastric carcinoma	This study
4	Chr1, 70904800	rs1021737	CTH	Gastric cancer	Xie et al., 2015
5	Chr10, 79566632	rs1058198	DLG5	Human Pancreatic Adenocarcinoma	Chang et al., 2016
6	Chr10, 94714427	rs11187225	EXOC6	Breast cancer	Chang et al., 2011
7	Chr10, 97804167	no rs id	CCNJ	Gastric cancer	Venturutti et al., 2016
8	Chr12, 124887096	rs28690647	NCOR2	Cutaneous Melanoma	Zhang et al., 2015
9	Chr12, 48458987	no rs id	SENP1	Prostate cancer	Wu et al., 2016
10	Chr13, 20763642	rs2274084	GJB2	Melanoma	Rangel et al., 2015
11	Chr15, 40855210	rs10152546	C15orf57	Gastric cardia adenocarcinoma	Xu et al., 2014
12	Chr15, 69747829	no rs id	RPLP1	Gynecologic tumors	Artero-castro et al., 2011
13	Chr16, 87788864	rs2303771	KLHDC4	Human Nasopharyngeal Carcinoma	Lian et al., 2016
14	Chr17, 27065211	rs3809797	NEK8	Breast cancer	Bowers and Boylan, 2004
15	Chr17, 8046772	rs2585405	PER1	Human Oral Squamous Carcinoma	Zhao et al., 2013
16	Chr19, 10679240	rs1968445	CDKN2D	Esophageal carcinoma cell	Zang et al., 2015
17	Chr19, 36595436	rs1008328	WDR62	Human ovarian cancer	Zhang et al., 2013
18	Chr19, 43013363	rs11666350	CEACAM1	Melanoma	Ashkenazi et al., 2016
19	Chr19, 49377086	rs611251	PPP1R15A	Metastatic colorectal cancer	Roh et al, 2016
20	Chr22, 31667125	no rs id	LIMK2	Panc-1 Tumor Growth	Rak et al., 2014
21	Chr3, 150262250	no rs id	SERP1	Prostate cancer	Zheng et al., 2015
22	Chr7, 4801973	rs15715	FOXK1	Colorectal carcinoma	Wu et al., 2016
23	ChrX, 132437337	rs1048369	GPC4	EBV-associated gastric carcinoma	Zhao et al., 2016
24	ChrX, 150840895	no rs id	PASD1	Lymphoma immunotherapy	Cooper et al., 2006
25	Chr9, 99278021	no rs id	CDC14B	Renal cell carcinoma	Kim et al., 2014

regions and 162 in exonic regions. Among those SNPs in exonic regions, 110 were synonymous mutations while 72 were nonsynonymous mutations. Of the 72 nonsy nonymous mutations, 25 SNPs were associated with tumors, and *FLG* rs2065955 was among them (Table 1). As considerable interest has arisen in the possible relationship between EBVaGC development and *FLG* gene polymorphisms, we looked for the *FLG* rs2065955 polymorphism in EBVaGC and EBV-negative gastric carcinoma (EBVnGC) tissues and tried to provide experimental evidence for genetic susceptibility to development of gastric carcinoma, especially EBVaGC.

MATERIALS AND METHODS

Specimens

One hundred and forty-six paraffin-embedded gastric carcinoma tissues, including 64 EBVaGC and 82 EBVnGC, were collected from the Department of Pathology of the Affiliated Hospital of Qingdao University, which is located in Shandong Province, northern China (Table 2; please check Supplementary Table S2 for more details). EBV-positive samples were determined by in situ hybridization for EBV-encoded small RNA 1 as described previously (Khan et al., 1992). One hundred and one healthy physical examinees from the Affiliated Hospital of Qingdao University were used as normal controls; 5 mL of fasting venous blood was collected using EDTA as an anticoagulant, and peripheral blood mononuclear cells were separated for DNA extraction. This study was approved by the Medical Ethics Committee of Qingdao University Medical College, and informed consent was obtained from all the study participants.

DNA extraction

DNA of venous blood specimens was extracted by a standard method using proteinase K digestion and phenol–chloroform purification. A QIAamp DNA FFPE tissue kit (QIAGEN GmbH, Hilden, Germany) was used to extract the DNA from paraffin-embedded gastric carcinoma tissues.

Table 2. Clinical characterizations of EBVaGC/EBVnGC patients and healthy controls

		Healthy control	EBVaGC	EBVnGC
Gender	Male (%)	82.88	81.25	73.17
	Female (%)	17.12	18.75	26.83
Age		26–80	27–82	26–78
Lymph node metastasis (%)		0	30.50	45.20

FLG gene polymorphism detection

PCR and restriction fragment length polymorphism (RFLP) were used to detect *FLG* rs2065955 located in the 25265th nucleotide of the *FLG* gene. Nucleotide substitution of G to C leads to an amino acid change from Gly to Ala in this mutation. Primers Forward (5′-CCAGACACTCAGGCATTC-3′) and Reverse (5′-CAATCGGTAGAATAGGTGG-3′) produced a PCR product of 436 bp.

PCR reactions were performed with 2 μL of DNA extracts (100 ng/μL) in a 25 μL reaction mixture containing standard PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1.25 μmol/L of each primer, and 0.2 U *Taq* DNA polymerase (TaKaRa Biotechnology Co, Kyoto, Japan). Cycle conditions were pre-denaturation at 94 °C for 5 min, then 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and final elongation at 72 °C for 10 min. The PCR products were separated on a 1.5% agarose gel. After electrophoresis, the gels were stained with ethidium bromide and photographed under a UV light transilluminator. Sterile double-distilled water was used as a negative control in each PCR reaction. PCR products were purified using a gel extraction kit (Qiaex II; Qiagen) according to the manufacturer's instructions. Restriction enzymatic reactions were performed in a 25 μL mixture including 2.5 μL 10× buffer, 0.5 μL 10 U/μL *Apa*LI, 5 μL PCR product, and 17 μL enzyme-free double-distilled water. After incubation at 37 °C for 15 min, the DNA products were analyzed on a 2% agarose gel and then visualized by ethidium bromide staining.

FLG protein expression detection

Formalin-fixed, paraffin-embedded tissue slides (4 μm thick) were deparaffinized and rehydrated, and subjected to antigen retrieval, followed by blocking of endogenous peroxidase and treatment with a blocking reagent for nonspecific binding. Slides were then incubated overnight with the primary antibody, a rabbit polyclonal antibody against human FLG (1:500; ab24584; Abcam) at 4 °C in a humidified chamber. PBS was used as a negative control replacing the primary antibody.

All immunohistochemistry evaluation was performed in a blinded manner by an attending pathologist. Specimens were graded by staining intensity (0: negative; 1: weak; 2: moderate; 3: strong) and the percentage of tumor cells (0: 0%–5%; 1: 6%–25%; 2: 26%–75%; 3: 76%–100%). The final score was calculated by multiplying these two variables. A score of 1–2 indicated weakly positive (1+), a score of 3–4 indicated moderately positive (2+) and a score > 4 indicated intensely positive (3+).

Statistical analysis

The Chi-square test and Fisher's exact test were used to compare the differences in genotype and allele frequen-

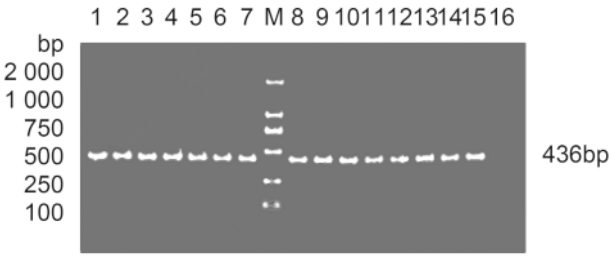

Figure 1. Electrophoresis results of *FLG* gene (rs2065955) PCR products. Lane M: DL 2000 DNA Marker; lane 16: negative control; lanes 1–15: 436 bp *FLG* (rs2065955) PCR products.

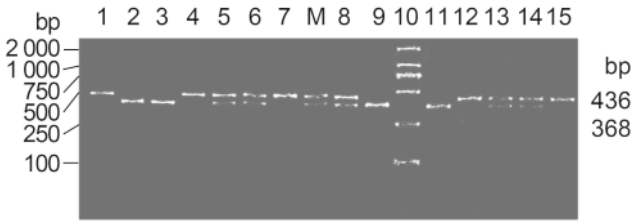

Figure 2. PCR-RFLP analysis of *FLG* gene (rs2065955) genotyping. Lane M: DL 2000 DNA Marker; lanes 1, 4, 7, 12, 15: GG genotype (homozygous wild-type); lanes 5, 6, 8, 9, 13, 14: GC genotype (heterozygous type); lanes 2, 3, 10, 11: CC genotype (homozygous mutated type).

cies of *FLG* rs2065955 in each group. Nonconditional logistic regression was used to estimate the odds ratio (OR) and *P* values to indicate the correlation between genotype and the risk of gastric carcinoma. Results were considered to be statistically significant when $P < 0.05$. Statistical analyses were conducted using SPSS 19.0 statistical software (SPSS, Chicago, IL).

RESULTS

The 436 bp fragments of the *FLG* gene flanking the rs2065955 locus were successfully amplified in 64 EBVaGC, 82 EBVnGC, and 111 normal control samples (Figure 1). After digestion by ApaLI, the homozygous wild-type genotype GG remained as only one 436 bp band. The homozygous mutant genotype CC showed two bands, 368bp and 68bp. The heterozygous genotype GC showed three bands, 368 bp, 68 bp, and 436 bp (Figure 2).

The distributions of *FLG* rs2065955 genotypes and alleles in EBVaGC, EBVnGC, and normal controls are shown in Table 3 and Table 4. The CC genotype was found in 38 of 64 (59.4%) EBVaGC samples, 31 of 82 (37.8%) EBVnGC samples, and 17 of 111 (15.3%) normal controls. The distribution of the CC genotype was significantly different among the three groups (EBVaGC vs normal control: $\chi^2 = 36.566$, $P < 0.01$; EBVnGC vs normal control: $\chi^2 = 12.766$, $P < 0.01$; EBVaGC vs EBVnGC: $\chi^2 = 6.701$, $P = 0.01$). The CC genotype showed higher frequency in EBVaGC and EBVnGC than in normal controls and may be a risk factor for EBVaGC and EBVnGC (EBVaGC vs normal control: OR = 8.081, 95% CI = 3.941–16.571; EBVnGC vs normal control: OR = 3.361, 95% CI = 1.698–6.652; EBVaGC vs EBVnGC: OR = 2.404, 95% CI = 1.231–4.496).

Table 3. Genotype and allele frequencies of *FLG* (rs2065955) in EBVaGCs and EBVnGCs

FLG (rs2065955)	EBVaGC[a] (%)	EBVnGC[b] (%)	OR[c] (95% CI)	*P* value
Genotypic frequencies				
GG	12 (18.8)	13 (15.9)	1.00	
CC	38 (59.4)	31 (37.8)	0.753 (0.301–1.884)	0.544
GC	14 (21.8)	38 (46.3)	2.505 (0.926–6.780)	0.067
Recessive Model				
Others	26 (40.6)	51 (62.2)	1.00	
CC	38 (59.4)	31 (37.8)	2.404 (1.231–4.496)	0.01
Dominant Model				
GG	12 (18.8)	13 (15.9)	1.00	
Others	52 (81.2)	69 (84.1)	1.225 (0.517–2.904)	0.645
Allelic frequencies				
C	90 (70.3)	100 (61)	1.00	
G	38 (29.7)	64 (39)	1.600 (0.973–2.632)	0.097

Note: [a]: EBV associated gastric carcinoma; [b]: EBV negative gastric carcinoma; [c]: odd ratio.

Table 4. Distribution of genotype and allele of FLG in EBVaGC, EBVnGC, and control samples

FLG (rs2065955)	EBVaGC[a] n = 64 (24.9%)	Control n = 111 (43.2%)	EBVnGC[b] n = 82 (31.9%)
Genotype			
GG	12 (18.8)	21 (18.9)	13 (15.9)
GC	14 (21.8)	73 (65.8)	38 (46.3)
OR[c] (95%)	1.198–7.410	0.380–1.862	
P	0.016	0.669	
CC	38 (59.4)	17 (15.3)	31 (37.8)
OR (95%)	0.103–0.636	1.186–7.319	
P	0.003	0.018	
Recessive model			
Others	26 (40.6)	94 (84.7)	51 (62.2)
CC	38 (59.4)	17 (15.3)	31 (37.8)
OR (95%)	8.081 (3.941–16.571)		3.361 (1.698–6.652)
P	< 0.01		< 0.01
Dominant model			
GG	12 (18.8)	21 (18.9)	13 (15.9)
Others	52 (81.2)	91 (81.1)	69 (84.1)
OR (95%)	0.989 (0.450–2.073)		0.807 (0.378–1.726)
P	0.978		0.581
Allele			
C	90 (70.3)	107 (48.2)	100 (61)
G	38 (29.7)	115 (51.8)	64 (39)
OR (95%)	2.687 (1.683–4.289)		1.679 (1.115–2.529)
P	< 0.01		0.013

Note: [a]: EBV associated gastric carcinoma; [b]: EBV negative gastric carcinoma; [c]: odd ratio.

Allele C frequency was 70.3%, 61%, and 48.2% in EBVaGC, EBVnGC, and normal controls, respectively. Allele C exhibited higher frequency in EBVaGC and EBVnGC than in normal controls and may be a risk factor for EBVaGC and EBVnGC (EBVaGC vs normal control: $\chi^2 = 16.137$, $P < 0.01$, OR = 2.687, 95% CI = 1.683–4.289; EBVnGC vs normal control: $\chi^2 = 6.192$, $P = 0.013$, OR = 1.679, 95% CI = 1.698–6.652; EBVaGC vs EBVnGC: $P = 0.097$).

FLG protein expression level in 35 EBVaGC and 51 EBVnGC tissue samples was detected by immunochemical methods (Table 5, Figure 3). The scoring criteria are mentioned in the MATERIALS AND METHODS section. The statistical analysis using Fisher's exact test showed that there was no significant difference in FLG protein expression level between EBVaGC and EBVnGC ($P = 0.079$).

Table 5. FLG protein expression level in EBVnGC and EBVaGC tissues

	EBVaGC n = 35 (%)	EBVnGC n = 51 (%)
–	0	5 (9.80)
1+	30 (85.7)	35 (68.6)
2+	5 (14.3)	11 (21.6)

DISCUSSION

In this study, we found that genotype CC of *FLG* showed higher frequency in gastric carcinoma, especially in EBVaGC, which may be a risk factor for EBVaGC development. To our knowledge, this is the first research describing the association of *FLG* polymorphism with

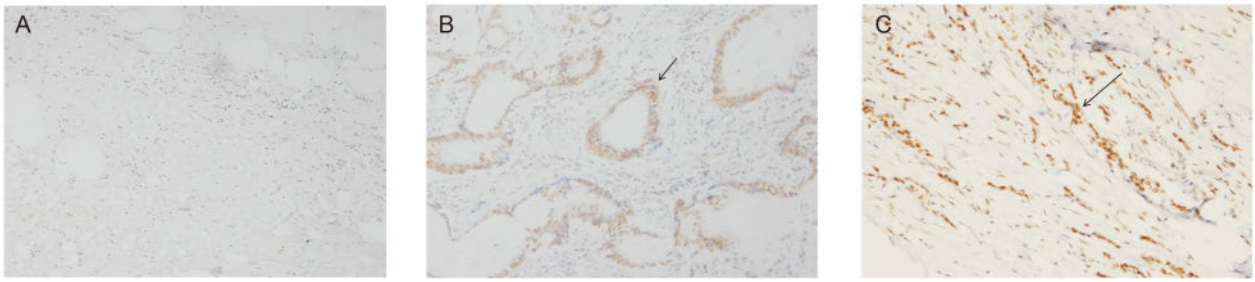

Figure 3. Immunohistochemical analysis of FLG. (A) FLG negative expression in tumor cells. (B) weakly positive (1+). (C) moderately positive (2+). The arrow indicates the specific cytoplasmic expression of FLG in gastric tumor cells. (Magnification: 100 ×)

EBV-associated diseases.

Filaggrin proteins are crucial for the terminal differentiation of the epidermis by aggregating keratin filaments. They play important roles in the barrier function of skin, which may prevent the entry of harmful factors from environmental exposure including microorganisms, allergens, or chemicals (Candi et al., 2005). Loss-of-function mutations in the *FLG* gene reduce epidermal filaggrin levels and may disrupt the skin barrier, and have been shown to be highly associated with ichthyosis vulgaris (Smith et al., 2006) and atopic dermatitis (Palmer et al., 2006). In addition to the skin, filaggrin proteins are also expressed in the oral cavity, cervix, endometrium, and vagina (De benedetto et al., 2008; Irvine et al., 2011). *FLG* loss-of-function mutations also confer a higher risk of rhinitis, asthma, and food allergies in the context of atopic dermatitis (De benedetto et al., 2008). However, the role of filaggrin outside the skin is largely unknown.

A few studies have reported the association of *FLG* mutations with the pathogenesis of certain carcinomas (Chen et al., 2013; Skaaby et al., 2014; Bager et al., 2015). Filaggrin expression was reported to be reduced in cervical lesions infected with HPV in a dose-dependent manner (Cintorino et al., 1988; Mcglennen et al., 1991; Lara et al., 1994). One report declared that *FLG* mutations were not associated with the risk of cervical cancer (Bager et al., 2015). However, Skaaby (2014) reported that *FLG* mutations led to greater susceptibility to infection by HPV and thus may confer a higher risk of HPV-related cancer and pre-cancer.

We detected *FLG* rs2065955 in EBVaGC, EBVnGC, and normal controls. The association of this SNP with disease has not been reported previously. The distribution of the *FLG* gene SNP (rs2065955) was significantly different between the three groups. A higher proportion of genotype CC in gastric carcinoma (EBVaGC and EBVnGC) suggested that this genotype may be a risk factor for development of gastric carcinoma. A higher frequency of genotype CC in EBVaGC than in EBVnGC indicated that genotype CC may be associated with susceptibility to EBV infection and may contribute to the pathogenesis of EBVaGC. Despite significant differences in FLG rs2065955 polymorphism between EBVaGC and EBVnGC samples, there was no significant difference in FLG protein level between the two groups. To date, however, there have been no reports of EBV infection associated with FLG gene expression levels. A nucleotide mutation at one site only may affect the amino acid sequence of the encoded protein, but it does not necessarily cause changes in expression level. However, further work is needed to confirm this phenomenon. First, we have to confirm the expression of *FLG* in mucosa of the nose, oral cavity (the natural entrance of EBV to invade the human body), and stomach, which is the basis of the pathogenic role of *FLG* mutations. Second, a much larger-scale study of gastric carcinomas from different geographical regions should be undertaken to eliminate the possibility of sampling errors. Third, samples from other EBV-associated diseases such as nasopharyngeal carcinoma also should be included. In conclusion, our study found an interesting phenomenon in EBVaGC, which provides a new clue to tumorigenicity of EBV.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that there is no conflict of interest. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

AUTHOR CONTRIBUTIONS

BL and XK designed the experiments. XK, ZZZ, and DZ

carried out the experiments. LS, SL, ZZZ, DZ, and SL analyzed the data. LS, XK, and BL wrote the paper. All authors have read and approved the final manuscript.

Supplementary tables are available on the websites of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

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